Biotechnology and Genetic Engineering Reviews - Vol. 25, 149-164 (2008)

Differentiating Stem Cells into Liver

ALEJANDRO SOTO-GUTIERREZ[1], HESHAM BASMA[2], NALU NAVARRO-ALVAREZ[3], BASAK E. UYGUN[1], MARTIN L. YARMUSH[1], NAOYA KOBAYASHI[3], AND IRA J. FOX[4]*

[1] Center for Engineering in Medicine and Department of Surgery, Massachusetts General Hospital, Harvard Medical School, and the Shriners Hospitals for Children, Boston, MA 02114, USA, [2]Department of Surgery, University of Nebraska Medical Center, Omaha, Nebraska 68198-3285, USA, [3] Department of Surgery, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan, [4]Department of Surgery, Division of Pediatric Transplant Surgery, Children's Hospital of Pittsburgh and McGowan Institute for Regenerative Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA.

Abstract

Research involving differentiated embryonic stem (ES) cells may revolutionize the study of liver disease, improve the drug discovery process, and assist in the development of stem-cell-based clinical therapies. Generation of ES cell-derived hepatic tissue has benefited from an understanding of the cytokines, growth factors and biochemical compounds that are essential in liver development, and this knowledge has been used to mimic some aspects of embryonic development *in vitro*. Although great progress has been made in differentiating human ES cells into liver cells, current protocols have not yet produced cells with the phenotype of a mature hepatocyte. There is a

To whom correspondence should be addressed (foxi@upmc.edu)

Abbreviations: ES cells, embryonic stem cells; CYP, cytochrome P450; AFP, alpha-fetoprotein; alb, albumin; CK18, cytokerain 18; Oct4, Octamer-4; SSEA-4, Stage-Specific Embryonic Antigen-4; Oxt2, orthodenticle homeobox 2; Hesx 1, HESX homeobox 1; Hex, homeobox; Cdx2, caudal-related homeobox 2; EBs, embroyd bodies; GSC, goosecoid; Foxa 2, forkhead box A2; cxcr4, chemokine C-X-C motif receptor 4; Sox17a/b, sex determining region-Y box 17; VEGFR2, vascular endothelial growth factor receptor-2; PDGFRa, platelet-derived growth factor receptor-a; GATA-4, GATA binding protein 4; EpCAM, epithelial cell adhesion molecule; DPPIV, dipeptidyl peptidase IV; TGFß, Tumor growth factor beta; FGFs, Fibroblast growth factors; BMPs, Bone marrow proteins; HNF6, Hepatocyte nuclear factor 6; HGF, Hepatocyte growth factor; C/EBPß, CCAAT/enhancer binding protein beta; Foxm1b, Forkhead Box (Fox) m1b; Xbp1, X-box binding protein 1; Dex, Dexamethasone; CYP, cytochrome p450

significant need to formally establish criteria that would define what constitutes a functional human stem cell-derived hepatocyte. Here, we explore current challenges and future opportunities in development and use of ES cell-derived liver cells. ES-derived hepatocytes could be used to better understand liver biology, begin the process of "personalizing" health care, and to treat some forms of liver disease.

Introduction

Stem cell biology has received much attention in the last several years. Successful production of stem cell-derived neural and pancreatic islet cells could dramatically affect the treatment of Parkinson's disease, Alzheimer's disease, and diabetes (D'Amour *et al*., 2006; Kim *et al*., 2002). Treatment of liver disease through the use of stem cells has received significantly less attention. However, liver failure causes more than 25,000 deaths/year in the United States alone (Lee, 1993; Stravitz *et al*., 2007). Treatment of liver disease has been dramatically improved by organ transplantation, but its more broad application has been limited by the scarcity of organ donors, and the risks and complications associated with the complexity of the surgery (Stravitz *et al*., 2007).

A better understanding of liver regeneration and stem cell biology could potentially be applied clinically to improve the treatment of liver disease. Stem cells, because they retain their capacity to generate new progeny and renew themselves throughout life, play a critical role in the physiological process of cell turnover and regeneration in response to injury. While therapies for liver diseases often aim to reduce damage from infection or other disease processes, it may be possible to reverse damage by replacing lost cells with new ones derived from either tissue-specific stem cells or stem-cells derived from outside the liver. Transplantation of isolated hepatocytes (Fisher and Strom, 2006), a minimally invasive intervention associated with few risks, has been shown to provide temporary support for some patients who have acute and possibly reversible forms of liver failure that may require organ transplantation (Stravitz *et al*., 2007). Recent clinical studies also indicate that hepatocyte transplantation may be effective in treating children with life-threatening liver-based metabolic diseases. Unfortunately, donor availability limits this form of therapy as well. An unlimited supply of stem cell-derived liver cells could dramatically affect the development of cell-based therapies for the treatment of liver disease and could eventually lead to therapies that could improve the lives of other patients with less severe, but debilitating liver-based metabolic disorders. The availability of reliable source of high-quality liver cells would also facilitate the study of liver diseases and revolutionize the early stages of the drug discovery process.

Embryonic stem (ES) cells are an exciting potential source of cells that might be used to produce liver cells. ES cells are pluripotent, self-renewing products of the inner cell mass of the blastocyst. They characteristically express Oct4, SSEA-4, TRA-1-60, and TRA-1-81 and have high telomerase activity levels (Wobus and Boheler, 2005). They proliferate extensively *in vitro*, and can be effortlessly differentiated into derivatives of all three germ layers. Aggregation into spheroid clumps of cells, called embryoid bodies (EBs), leads to spontaneous differentiation of cells with characteristics of ectoderm, mesoderm and endoderm, and a variety of protocols have been generated that lead to production of tissue-specific cells from both rodent and human ES cells (Itskovitz-Eldor *et al*., 2000).

Care must be taken when evaluating studies describing the extent to which mature liver cells have been successfully derived from stem cells. Stem cell-derived "hepatocytes" have been generated using many strategies, and have been shown to secrete albumin and urea, and express cytochrome P450 (CYP) enzyme activity. However, a more detailed analysis of gene expression, metabolic activity, growth potential, and secretory function will be required to determine whether such cells can fully function as primary hepatocytes (Nahmias *et al*., 2007; Runge *et al*., 2000) (*Figure 1*).

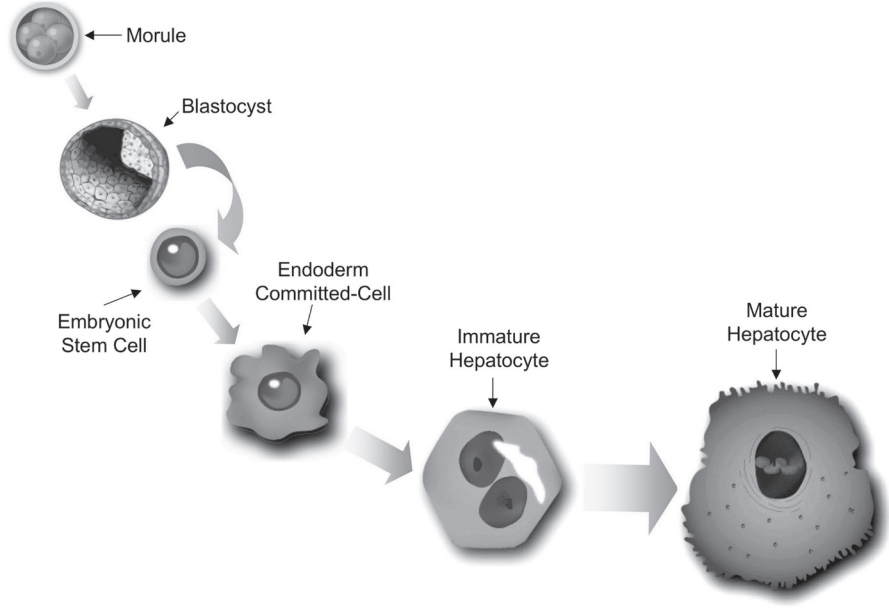

Figure 1. Directed differentiation of hES cells to hepatocyte-like cells by mimicking embryonic development. Key stages of hepatocyte development and stepwise differentiation of ES cells.

Mammalian liver development and its relationship to stem cell differentiation

EARLY GASTRULATION AND FORMATION OF DEFINITIVE ENDODERM

Embryonic stem cells are derived from the undifferentiated cells of the epiblast, which give rise to the three principal germ layers and their differentiated progeny through a process called gastrulation (Wells and Melton, 1999). Of the three germ cell layers, the endoderm gives rise to hepatic, pancreatic, lung, and intestinal tissues in a process that is not well understood. The transcription factors orthodenticle homeobox 2 (Otx2), HESX homeobox 1 (Hesx1), homeobox Hex, and caudal-related homeobox 2 (Cdx2) are associated with formation of definitive endoderm and are expressed prior to activation of organ specific genes (Wells and Melton, 1999). Genes critical for cell-fate determination emerge from the mesendoderm shortly thereafter. Among these are goosecoid (GSC) forkhead box A2 (Foxa 2), chemokine C-X-C motif receptor 4 (CXCR4), sex determining region-Y box 17 (Sox17a/b), brachyury, E-cadherin, vascular endothelial growth factor receptor-2 (VEGFR2), VE-cadherin, platelet-derived growth factor receptor-a (PDGFRa), and GATA binding protein 4, (GATA-4) (Yasu-

naga *et al*., 2005). An appreciation of the genetic patterns and cellular markers that distinguish definitive endoderm from extraembryonic endoderm, such as EpCAM, CD38, and DPPIV, have been critically important in the process of differentiating and isolating tissue-specific cells from stem cells (Sherwood *et al*., 2007).

Definitive endoderm can be generated by a variety of means. Definitive endoderm can be produced through formation of embryoid bodies or by culturing ES cells with collagen-sandwiched hepatocytes (Cho *et al*., 2007). Large numbers of endoderm like-cells can also be produced when mouse ES cells are cultured on fibronectin-coated collagen gels and exposed to follistatin, an Activin binding protein (Parashurama *et al*., 2007). The common transcriptional machinery present in both definitive and visceral endoderm may, however, limit the extent to which differentiation using these techniques can lead to tissue formation. Generation of a pure population of definitive endoderm from ES cells may require selective inhibition of visceral endoderm.

Recently, investigators have facilitated the development of definitive endoderm from mouse and human ES cells using culture in low concentrations of serum in conjunction with growth in Activin A, a TGFβ family member that binds the same receptors as does Nodal. This finding has facilitated generation of endoderm-derived tissues, such as pancreatic beta cells and hepatocytes (D'Amour *et al*., 2005; Kubo *et al*., 2004; Yasunaga *et al*., 2005) and has resolved the problem of forming a mixture of visceral and definitive endoderm during the differentiation process.

HEPATIC INDUCTION

Growth factor signaling from the cardiac mesoderm and septum transversum mesenchyme specifies the underlying endoderm to adopt a hepatic fate such that, by the 6–7 somite stage, hepatic gene expression can be detected in the ventral foregut endoderm (Jung *et al*., 1999; Rossi, 2001). Using a variety of techniques, it has now been shown that fibroblast growth factors (FGFs) can substitute for cardiac mesoderm and bone morphogenic proteins (BMPs) can substitute for the septum transversum mesenchyme to work in concert to induce the ventral endoderm to adopt a hepatic fate (Jung *et al*., 1999). Studies documenting induction of hepatic specification following chick cardiac mesoderm co-culture with ES cells, or following culture in FGF and BMP, illustrate how such developmental principles can assist with stem cell differentiation *in vitro* (Cai *et al*., 2007; Fair *et al*., 2003; Gouon-Evans *et al*., 2006; Soto-Gutierrez *et al*., 2007). Factors identified as proposed targets of FGF and BMP signaling include the Foxa and Gata genes, which regulate the competence of foregut endoderm to respond to hepatic inductive signals (Cirillo *et al*., 2002). In addition, the transcription factor hepatocyte nuclear factor-6 (HNF6) has been shown to play a critical role in the proper morphogenesis of both the intra- and extra-hepatic biliary tree. The mechanism by which HNF6 regulates biliary tree development also appears to involve the related transcription factor, hepatocyte nuclear factor-1 (HNF1) (Clotman *et al*., 2005) (*Figure 2*).

LIVER SPECIFICATION, DEVELOPMENT OF THE LIVER BUD, AND HEPATIC EXPANSION

Hepatocytes and bile duct cells originate from a common precursor, the hepatoblast (Germain *et al*., 1988). Notch signaling promotes hepatoblast differentiation toward the

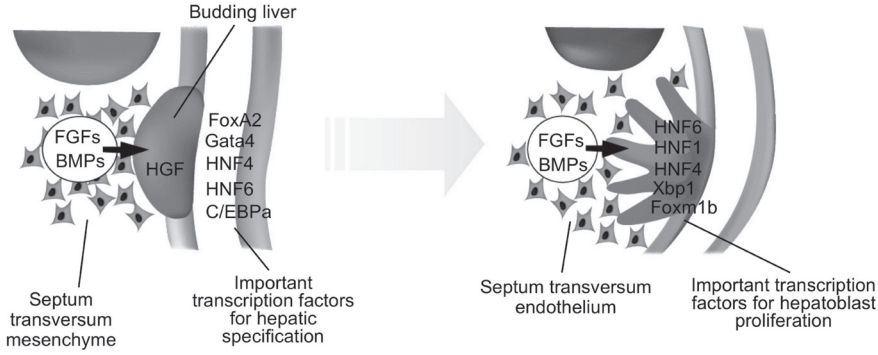

Figure 2. Signaling that induces hepatic genes in the endoderm. Transcription factors and other molecules that influence differentiation of the endoderm into liver. The cardiac mesoderm and prospective septum transversum mesenchymal cells ("mesenchyme") provide signals to the endoderm during this period that promote hepatic induction.

biliary epithelial lineage, while HGF promotes differentiation toward the hepatocyte lineage (Kodama *et al*., 2004). While Notch inhibits differentiation to hepatocytes, HGF induces expression of C/EBPα in albumin-negative fetal liver cells. When C/EBPα activity is blocked, there is no transition from the albumin-negative to the albumin-positive stage. HGF promotes differentiation of albumin-positive cells from albumin-negative precursors, but inhibits further differentiation of albumin-positive cells into biliary cells, suggesting that HGF promotes the establishment of a bipotent state in hepatoblasts (Suzuki *et al*., 2002). Thus, TGFß, HGF, C/EBPα, and HNF6, in combination with Notch, work in concert to form a network of signals that controls bipotency and allows later biliary or hepatocyte differentiation.

The crucial early budding phase of liver development involves hepatic interaction with endothelial cells (Cleaver and Melton, 2003; Matsumoto *et al*., 2001) and results in hepatoblast proliferation. The mesenchymal cells of the liver, derived from the septum transversum mesenchyme, are essential for this proliferation process to take place (Gualdi *et al*., 1996). This requirement for endothelial cells in hepatic endoderm growth has been recapitulated using embryo tissue explants, and appears to persist in the adult liver (Bhatia *et al*., 1999). Hepatocyte growth factor (HGF) signaling also controls proliferation in the fetal liver (Uehara *et al*., 1995). Finally, the transcription factors Foxm1b and Xbp1 are also required for liver bud cell proliferation. Foxm1b knockout mice die in utero with 75% fewer hepatoblasts in their livers, while Xbp1 knockout mice also develop hypoplastic livers (Krupczak-Hollis *et al*., 2004; Reimold *et al*., 2000). (*Figure 2*)

APPLICATION OF PRINCIPLES FROM DEVELOPMENT IN ES CELL DIFFERENTIATION INTO LIVER CELLS

Methods for differentiating stem cells into hepatocytes can be separated into those that involve spontaneous formation of liver-like cells and those involving directed differentiation (Hamazaki *et al*., 2001; Lavon *et al*., 2004; Rambhatla *et al*., 2003).

Spontaneous differentiation involves formation of EBs, plating of ES cells on an adherent matrix as a monolayer, or following transplantation into an hepatic envi-

ronment (Lavon *et al*., 2004; Yamada *et al*., 2002; Yamamoto *et al*., 2003). Directed differentiation usually involves addition of growth factors and cytokines to cells *in vitro* on extracellular matrices (Cai *et al*., 2007; Rambhatla *et al*., 2003; Teratani *et al*., 2005). Combinations of these techniques, involving both formation of EBs with expansion in growth factors and/or co-culture with cells supplying additional factors, have also been successful (Cho *et al*., 2007; Fair *et al*., 2003; Soto-Gutierrez *et al*., 2006; Soto-Gutierrez *et al*., 2007). In addition, culture in sodium butyrate, a histone deacetylase inhibitor, leads to an increase in the number of cells expressing mature hepatocyte-specific genes.

Selection of hepatocyte-like cells based on the use of liver-specific promoters that drive reporter gene expression has been a relatively successful strategy for selecting a homogeneous population of cells with hepatic characteristics (Lavon *et al*., 2004). In the majority of settings, the resultant cells have morphological features similar to those of primary hepatocytes and most of the cells express liver-associated proteins (Rambhatla *et al*., 2003). Whether such cells have the functional characteristics of a mature liver cell will require a more comprehensive analysis.

ES cell differentiation: state of the art

Using a modification of the protocol described earlier using Activin A to enrich for definitive endoderm (D'Amour *et al*., 2005), and using principles from development, Cai *et al*. reported that later addition of FGF, BMP, and HGF to cultured cells induces expression of hepatic fate genes, and that further culture in Oncostatin M (OSM) and dexamethasone (Dex) leads to significant differentiation to cells with a large number of hepatocyte-like characteristics. Transplantation of such differentiated cells into mice with chemically-induced liver injury resulted in engraftment of a small number of ES-derived cells in the liver (Cai *et al*., 2007). Culture of ES cells in Activin A followed by treatment with sodium butyrate and HGF has been also shown to generate cells exhibiting morphologic characteristics of hepatocytes, expression of alpha-fetoprotein and albumin, and cytochrome p450 (CYP) metabolic activity (Hay *et al*., 2007).

ES cells have also been induced to differentiate into "hepatic-like" cells using other approaches (Cai *et al*., 2007; Cho *et al*., 2007; Lavon *et al*., 2004; Rambhatla *et al*., 2003; Soto-Gutierrez *et al*., 2007; Teratani *et al*., 2005). Development of cells that express hepatocyte markers, such as AFP, Alb, and CK18, inducible expression of cytochrome P450, and morphologic characteristics of an epithelial phenotype has been reported widely (Cai *et al*., 2007; Lavon *et al*., 2004; Rambhatla *et al*., 2003). However, useful stem cell-derived hepatocytes will need to not only express the genes found in mature hepatocytes, but the levels of expression will need to be at or near those found in the normal liver. Furthermore, differentiation will need to be lineage specific.

Evidence of protein production will need to be demonstrated to provide verification of gene expression studies, and the metabolic activity of CYP enzymes will be needed to provide definitive evidence of mature hepatic function. While hepatocytes are the only cell that secretes albumin, inferring that any albumin-secreting cell can, by definition, be regarded as a hepatocyte is not justified. Many stem cell-derived "hepatocytes" express albumin but many do not express other genes that would

normally constitute normal hepatocyte function. The same can be said for CYP enzyme activity, as p450 expression is not limited to hepatocytes. Inducible CYP activity has been reported by lung, intestinal epithelial cells, and adipose tissue (Baijal *et al*., 1997; Yoshinari *et al*., 2004; Zhang *et al*., 2003).

To adequately assess the extent to which *in vitro* differentiation of stem cells has been effective, it will be important to clearly demonstrate cellular characteristics and activities that can only be performed by primary hepatocytes.

1. Gene expression by differentiated "hepatocyte-like" cells should be compared to the gene expression profile of human fetal and/or mature liver cells (Hewitt *et al*., 2007; Li *et al*., 1990);
2. Evidence of basal and inducible CYP450 isoform function should be assessed (Kostrubsky *et al*., 1999);
3. Metabolism of xenobiotics or other endogenous substances (hormones and ammonia) should be determined (Duncan *et al*., 1998; Hewitt *et al*., 2007);
4. Synthesis and/or secretion of the following should be performed: albumin, clotting factors, complement, transporter proteins, bile acids, and lipids and lipoproteins. (Hewitt *et al*., 2007; Mita *et al*., 2006);
5. Evidence of restoration of liver function in appropriate animal models, or evidence of repopulation of the liver by derived "hepatocytes" should be examined.

In summary, derived hepatocytes should demonstrate drug metabolism and detoxification activity by both gene expression and function, and they should express the hepatic transport proteins and transcription factors present in mature hepatocytes. They should also, to some degree, secrete albumin and/or produce bile acids, conjugate bilirrubin, metabolize ammonia, and function in animal models of liver dysfunction after transplantation. Finally, differentiated cells should no longer express transcription factors or other genes characteristic of cells of other cell lineages (*Figure 3*).

Application of stem cell derived liver cells

The availability of a reliable and homogeneous source of human hepatocytes would be an invaluable tool for liver tissue engineering, for use in cell-based *in vitro* assays of drug toxicity, for metabolic profiling, for the study of drug–drug interactions, and for use in cell therapy and regenerative medicine (Wobus and Boheler, 2005).

CELL THERAPY

The worldwide shortage of donor organs is likely to increase over the coming decades. As a result, development of alternative methods for treating life-threatening liver disease will become increasingly important. While cell transplantation and other cell-based therapies have great potential for improving the lives of patients with liver failure (Fox *et al*., 1998; Navarro-Alvarez *et al*., 2007), the lack of available donors

for use in such experimental therapies has severely limited their development. (Fisher and Strom, 2006). An unlimited supply of stem cell-derived hepatocytes would be invaluable for development of novel cell therapies.

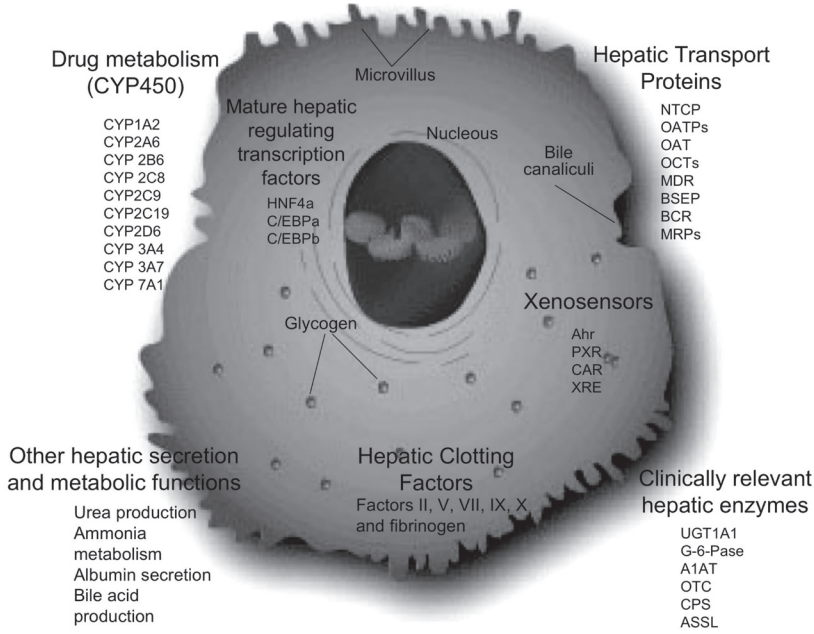

Figure 3. Distinguishing features of mature hepatocytes. Hepatocytes are the chief functional cells of the liver, and make up approximately 80% of the mass of the liver. These cells are involved in protein synthesis, protein storage, and transformation of carbohydrates; synthesis of cholesterol, bile salts and phospholipids; and detoxification, modification and excretion of exogenous and endogenous substances. NTCP, Na+-taurocholate co-transporting polypeptides; OATPs, organic anion-transporting polypeptides; OATs, organic anion transporters; MDR, multi drug resistance proteins; BSEP, bile salt export pump; BCRP, breast cancer resistance protein; MRP's, multi drug resistance associated proteins; UGT1A1, UDP-glucuronosyltransferase; G-6-Pase, Glucose-6-phosphatase; A1AT, Alpha-1-antitrypsin; OTC, Ornithine transcarbamylase; CPS, carbamyl-phosphate synthetase I; ASSL, argininosuccinate synthetase; HNF4α, Hepatocyte nuclear factor 4 alpha; C/EBPα or β, CCAAT-enhancer binding protein alpha or beta; OCTs, Organic cation transporters.

Transplantation of a relatively small number of allogeneic donor hepatocytes can effectively replace several generations of livers in mice. In rodent models of certain liver diseases, significant repopulation of the liver can also be accomplished using human hepatocytes. Remarkable levels of liver repopulation have been attained following transplantation in the severely immune deficient fumarylacetoacetate hydrolase (Fah)-deficient mouse, an animal model of hereditary tyrosinemia. Liver cells recovered from human hepatocyte-transplanted immunodeficient Fah knockout mice were able to partially repopulate the livers of four generations of immunodeficient Fah knockout mice (Azuma *et al*., 2007). While bone marrow-derived cells may be useful as a source of extra-hepatic derived liver stem cells, and mouse bone marrow has been used to repopulate the livers of Fah knockout mice, the degree to which these cells can effectively repair the liver or be efficiently converted into classic hepatocytes is not clear (Navarro-Alvarez *et al*., 2007; Navarro-Alvarez *et al*., 2006). The reason for discrepant results may relate to the fact that numerous animal models have been

employed in such stem cell transplant studies. The form of liver injury or pathology, and the mechanism by which stem cells engraft in the liver may be significantly affected by the form of liver pathology involved.

The risk of tumor development that would be associated with clinical use of differentiated human ES or adult stem cells should not be understated. Embryonic stem cells are known to form teratomas following transplantation (Nussbaum *et al*., 2007). Whether transplantation of early liver progenitor cells can also generate teratomas remains to be determined. Since long-term immune suppression can be somewhat carcinogenic, the extent to which such concerns are valid, and therefore may be prohibitive, will need to be rigorously addressed. It is possible, with development of patient derived, inducible pluripotent stem (IPS) or adult stem cell-derived hepatocytes, it may be possible to circumvent rejection and the need for immune suppressive medications (Wernig *et al*., 2007).

Successful scaling-up of the production of differentiated cells is an area that has yet to be fully examined. The process of expanding ES-derived cells might lead to truncated differentiation or compromise in stem cell integrity. Primary hepatocytes do not divide well *in vitro*, and appear to de-differentiate and lose their hepatic potential after prolonged culture (Nahmias *et al*., 2007; Tanaka *et al*., 2006). Growth in culture may also result in the loss or an alteration in cell homing capacity.

Techniques will also need to be developed to monitor cell function and rejection of engrafted stem cell-derived hepatocytes after transplantion in patients (Fisher and Strom, 2006; Nagata *et al*., 2007). Since cells may not engraft uniformly, percutaneous liver biopsies may not be effective in identifying engrafted cells unless there is selective repopulation of the liver by donor cells. Since there are expected to be few morphological differences between the donor and recipient cells following liver cell transplantation, histologic identification of engrafted donor cells may be challenging (*Figure 4*).

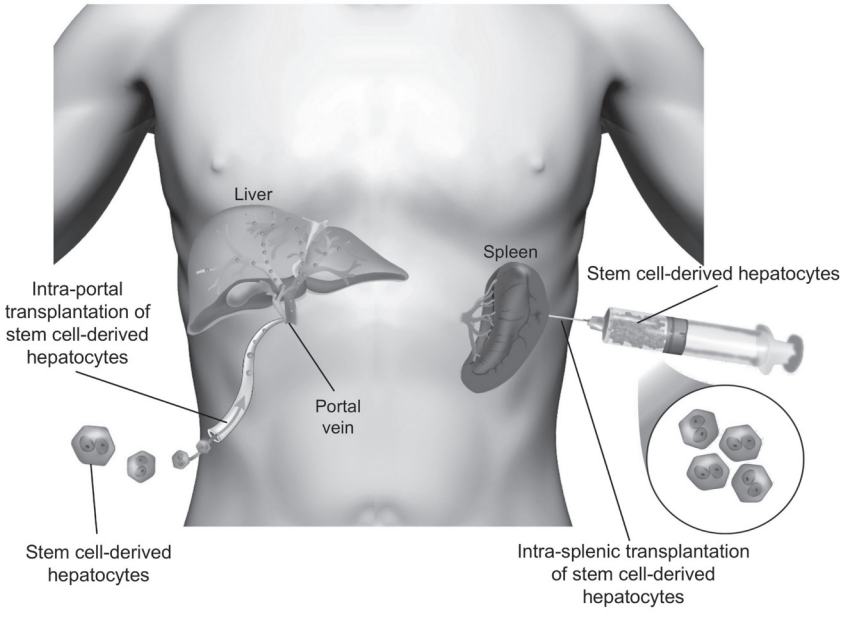

Figure 4. Therapeutic application of stem cell-derived hepatocytes.

Stem-cell technology has the potential to revolutionize the drug discovery process by improving the primary screening process, as well as the metabolic profiling and toxicity evaluation that is required to optimize drug candidate selection (McNeish, 2004; Pouton and Haynes, 2007). Tumor cell lines and cells immortalized by genetic transformation have been used in the past for such studies and for studies on liver tissue engineering, often with limited success. Cell lines usually have an abnormal karyotype and may have a significantly altered extracellular signaling system. (Hewitt *et al*., 2007). Culture and engineering studies involving primary cells should be more informative, however, their use has practical disadvantages. Primary cultures are usually performed in enriched media, which include mitogens. Although the cells may not be cycling at the time of isolation, they are often induced to enter the cell cycle, and generally survive for only a few generations. Their restricted capacity for expansion limits utilization of primary liver cells for culture and, thus, precludes the large scale use of primary cell culture for drug discovery (Hewitt *et al*., 2007).

By contrast, ES cell technology offers the opportunity to develop functional differentiated hepatocytes for *in vitro* analysis. If a functional and homogeneous hepatocyte-like cell population can be produced from ES cells, it will be easier to examine the role of human genetic variation in response to drugs. Also, by deriving cultures from ES cells of known genotypes, it may be possible to examine the mechanisms responsible for genetic predisposition to disease.

The availability of functional adult human hepatocytes derived from ES cells may also affect improvement in bioartificial liver devices, whose development has also been hindered by the limited availability of adult human hepatocytes. Using tissue engineering techniques, researchers have studied the microstructure of the liver in order to improve *in vitro* culture techniques that allow maintenance of signals similar to the intact hepatocyte microenvironment. Successful modifications have involved manipulation of the extracellular matrix environment (Griffith and Swartz, 2006), alterations in the composition of culture media (Nahmias *et al*., 2007), and other techniques that promote cell-cell interactions and signalling (Bhatia *et al*., 1999). In recent years, there has also been an immense growth in knowledge about hepatocyte survival, differentiation, and function both *in vivo* and *in vitro*. Advances in microfabrication and bioimaging technologies have allowed microscale control and evaluation of the cellular microenvironment *in vitro* (Tilles *et al*., 2002).

These advances have provided a better understanding of hepatic differentiation, and have led to creation of hepatic tissue engineering models that more closely mimic the *in vivo* physiology and pathology of the liver. A combination of techniques from cellular and molecular biology, liver development, tissue engineering, and microelectromechanical systems will produce new designs of systems that should facilitate the creation of liver-on-chip devices that could be used to screen hepatotoxic effects of drugs or environmental toxins, screen for potential growth factors or provide programmed release of specific growth factors to enhance hepatic differentiation of stem cells (Wieder *et al*., 2005).

Conclusions and prospects

Whether stem cell-derived liver cells will soon be available to treat liver disease is not known. Although there are many promising laboratory studies, only a handful

of disease models have been examined concerning whether stem cells can correct liver disease. It is a bit premature to conclude that hepatocytes can be generated from non-hepatic cells in culture that will be clinically useful. Standard criteria will need to be developed to assess the extent to which human stem cell-derived hepatocytes have been produced.

References

Azuma, H., Paulk, N., Ranade, A., Dorrell, C., Al-Dhalimy, M., Ellis, E., Strom, S., Kay, M. A., Finegold, M., and Grompe, M. (2007): Robust expansion of human hepatocytes in Fah(-/-)/Rag2(-/-)/Il2rg(-/-) mice. *Nat Biotechnol* **25**, 903-10.

Baijal, P. K., Fitzpatrick, D. W., and Bird, R. P. (1997): Phenobarbital and 3-methylcholanthrene treatment alters phase I and II enzymes and the sensitivity of the rat colon to the carcinogenic activity of azoxymethane. *Food Chem Toxicol* **35**, 789-98.

Bhatia, S. N., Balis, U. J., Yarmush, M. L., and Toner, M. (1999): Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *Faseb J* **13**, 1883-900.

Cai, J., Zhao, Y., Liu, Y., Ye, F., Song, Z., Qin, H., Meng, S., Chen, Y., Zhou, R., Song, X., Guo, Y., Ding, M., and Deng, H. (2007): Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* **45**, 1229-39.

Cho, C. H., Parashurama, N., Park, E. Y., Suganuma, K., Nahmias, Y., Park, J., Tilles, A. W., Berthiaume, F., and Yarmush, M. L. (2007): Homogeneous differentiation of hepatocyte-like cells from embryonic stem cells: applications for the treatment of liver failure. *Faseb J*.

Cirillo, L. A., Lin, F. R., Cuesta, I., Friedman, D., Jarnik, M., and Zaret, K. S. (2002): Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* **9**, 279-89.

Cleaver, O., and Melton, D. A. (2003): Endothelial signaling during development. *Nat Med* **9**, 661-8.

Clotman, F., Jacquemin, P., Plumb-Rudewiez, N., Pierreux, C. E., Van der Smissen, P., Dietz, H. C., Courtoy, P. J., Rousseau, G. G., and Lemaigre, F. P. (2005): Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. *Genes Dev* **19**, 1849-54.

D'Amour, K. A., Agulnick, A. D., Eliazer, S., Kelly, O. G., Kroon, E., and Baetge, E. E. (2005): Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* **23**, 1534-41.

D'Amour, K. A., Bang, A. G., Eliazer, S., Kelly, O. G., Agulnick, A. D., Smart, N. G., Moorman, M. A., Kroon, E., Carpenter, M. K., and Baetge, E. E. (2006): Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* **24**, 1392-401.

Duncan, S. A., Navas, M. A., Dufort, D., Rossant, J., and Stoffel, M. (1998): Regulation of a transcription factor network required for differentiation and metabolism. *Science* **281**, 692-5.

Fair, J. H., Cairns, B. A., Lapaglia, M., Wang, J., Meyer, A. A., Kim, H., Hatada, S., Smithies, O., and Pevny, L. (2003): Induction of hepatic differentiation in embryonic stem cells by co-culture with embryonic cardiac mesoderm. *Surgery* **134**, 189-96.

Fisher, R. A., and Strom, S. C. (2006): Human hepatocyte transplantation: worldwide results. *Transplantation* **82**, 441-9.

Fox, I. J., Chowdhury, J. R., Kaufman, S. S., Goertzen, T. C., Chowdhury, N. R., Warkentin, P. I., Dorko, K., Sauter, B. V., and Strom, S. C. (1998): Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* **338**, 1422-6.

Germain, L., Blouin, M. J., and Marceau, N. (1988): Biliary epithelial and hepatocytic cell lineage relationships in embryonic rat liver as determined by the differential expression of cytokeratins, alpha-fetoprotein, albumin, and cell surface-exposed components. *Cancer Res* **48**, 4909-18.

Gouon-Evans, V., Boussemart, L., Gadue, P., Nierhoff, D., Koehler, C. I., Kubo, A., Shafritz, D. A., and Keller, G. (2006): BMP-4 is required for hepatic specification of mouse embryonic stem cell-derived definitive endoderm. *Nat Biotechnol* **24**, 1402-11.

Griffith, L. G., and Swartz, M. A. (2006): Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol* **7**, 211-24.

Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J. R., and Zaret, K. S. (1996): Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev* **10**, 1670-82.

Hamazaki, T., Iiboshi, Y., Oka, M., Papst, P. J., Meacham, A. M., Zon, L. I., and Terada, N. (2001): Hepatic maturation in differentiating embryonic stem cells in vitro. *FEBS Lett* **497**, 15-9.

Hay, D. C., Zhao, D., Ross, A., Mandalam, R., Lebkowski, J., and Cui, W. (2007): Direct differentiation of human embryonic stem cells to hepatocyte-like cells exhibiting functional activities. *Cloning Stem Cells* **9**, 51-62.

Hewitt, N. J., Lechon, M. J., Houston, J. B., Hallifax, D., Brown, H. S., Maurel, P., Kenna, J. G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A. P., LeCluyse, E., Groothuis, G. M., and Hengstler, J. G. (2007): Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* **39**, 159-234.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. (2000): Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* **6**, 88-95.

Jung, J., Zheng, M., Goldfarb, M., and Zaret, K. S. (1999): Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* **284**, 1998-2003.

Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., and McKay, R. (2002): Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* **418**, 50-6.

Kodama, Y., Hijikata, M., Kageyama, R., Shimotohno, K., and Chiba, T. (2004): The role of notch signaling in the development of intrahepatic bile ducts. *Gastroenterology* **127**, 1775-86.

Kostrubsky, V. E., Ramachandran, V., Venkataramanan, R., Dorko, K., Esplen, J. E., Zhang, S., Sinclair, J. F., Wrighton, S. A., and Strom, S. C. (1999): The use

of human hepatocyte cultures to study the induction of cytochrome P-450. *Drug Metab Dispos* **27**, 887-94.

Krupczak-Hollis, K., Wang, X., Kalinichenko, V. V., Gusarova, G. A., Wang, I. C., Dennewitz, M. B., Yoder, H. M., Kiyokawa, H., Kaestner, K. H., and Costa, R. H. (2004): The mouse Forkhead Box m1 transcription factor is essential for hepatoblast mitosis and development of intrahepatic bile ducts and vessels during liver morphogenesis. *Dev Biol* **276**, 74-88.

Kubo, A., Shinozaki, K., Shannon, J. M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H. J., and Keller, G. (2004): Development of definitive endoderm from embryonic stem cells in culture. *Development* **131**, 1651-62.

Lavon, N., Yanuka, O., and Benvenisty, N. (2004): Differentiation and isolation of hepatic-like cells from human embryonic stem cells. *Differentiation* **72**, 230-8.

Lee, W. M. (1993): Acute liver failure. *N Engl J Med* **329**, 1862-72.

Li, Y. C., Wang, D. P., and Chiang, J. Y. (1990): Regulation of cholesterol 7 alpha-hydroxylase in the liver. Cloning, sequencing, and regulation of cholesterol 7 alpha-hydroxylase mRNA. *J Biol Chem* **265**, 12012-9.

Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K. S. (2001): Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**, 559-63.

McNeish, J. (2004): Embryonic stem cells in drug discovery. *Nat Rev Drug Discov* **3**, 70-80.

Mita, S., Suzuki, H., Akita, H., Hayashi, H., Onuki, R., Hofmann, A. F., and Sugiyama, Y. (2006): Inhibition of bile acid transport across Na+/taurocholate cotransporting polypeptide (SLC10A1) and bile salt export pump (ABCB 11)-coexpressing LLC-PK1 cells by cholestasis-inducing drugs. *Drug Metab Dispos* **34**, 1575-81.

Nagata, H., Nishitai, R., Shirota, C., Zhang, J. L., Koch, C. A., Cai, J., Awwad, M., Schuurman, H. J., Christians, U., Abe, M., Baranowska-Kortylewicz, J., Platt, J. L., and Fox, I. J. (2007): Prolonged survival of porcine hepatocytes in cynomolgus monkeys. *Gastroenterology* **132**, 321-9.

Nahmias, Y., Berthiaume, F., and Yarmush, M. L. (2007): Integration of technologies for hepatic tissue engineering. *Adv Biochem Eng Biotechnol* **103**, 309-29.

Navarro-Alvarez, N., Soto-Gutierrez, A., and Kobayashi, N. (2007): Hepatocyte transplantation: a step forward. *Curr Opin Organ Transplant* **12**, 652-658.

Navarro-Alvarez, N., Soto-Gutierrez, A., Rivas-Carrillo, J. D., Fox, I. J., and Kobayashi, N. (2006): Stem cell-derived hepatocytes. *Curr Opin Organ Transplant* **11**, 659–664.

Nussbaum, J., Minami, E., Laflamme, M. A., Virag, J. A., Ware, C. B., Masino, A., Muskheli, V., Pabon, L., Reinecke, H., and Murry, C. E. (2007): Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *Faseb J* **21**, 1345-57.

Parashurama, N., Nahmias, Y., Cho, C. H., van Poll, D., Tilles, A. W., Berthiaume, F., and Yarmush, M. L. (2007): Activin Alters the Kinetics of Endoderm Induction in Embryonic Stem Cells Cultured on Collagen Gels. *Stem Cells*.

Pouton, C. W., and Haynes, J. M. (2007): Embryonic stem cells as a source of models for drug discovery. *Nat Rev Drug Discov* **6**, 605-16.

Rambhatla, L., Chiu, C. P., Kundu, P., Peng, Y., and Carpenter, M. K. (2003): Generation of hepatocyte-like cells from human embryonic stem cells. *Cell Transplant* **12**, 1-11.

Reimold, A. M., Etkin, A., Clauss, I., Perkins, A., Friend, D. S., Zhang, J., Horton, H. F., Scott, A., Orkin, S. H., Byrne, M. C., Grusby, M. J., and Glimcher, L. H. (2000): An essential role in liver development for transcription factor XBP-1. *Genes Dev* **14**, 152-7.

Rossi, J. D., NR. Hogan, BL. Zaret, KS. (2001): Distinct mesodermal signals, including BMPs from the septum transversum mesenquyme, are require in combination for hepatogenesis from the endoderm. *Genes Dev* **15**, 1998-2001.

Runge, D., Runge, D. M., Jager, D., Lubecki, K. A., Beer Stolz, D., Karathanasis, S., Kietzmann, T., Strom, S. C., Jungermann, K., Fleig, W. E., and Michalopoulos, G. K. (2000): Serum-free, long-term cultures of human hepatocytes: maintenance of cell morphology, transcription factors, and liver-specific functions. *Biochem Biophys Res Commun* **269**, 46-53.

Sherwood, R. I., Jitianu, C., Cleaver, O., Shaywitz, D. A., Lamenzo, J. O., Chen, A. E., Golub, T. R., and Melton, D. A. (2007): Prospective isolation and global gene expression analysis of definitive and visceral endoderm. *Dev Biol* **304**, 541-55.

Soto-Gutierrez, A., Navarro-Alvarez, N., Rivas-Carrillo, J. D., Chen, Y., Yamatsuji, T., Tanaka, N., and Kobayashi, N. (2006): Differentiation of human embryonic stem cells to hepatocytes using deleted variant of HGF and poly-amino-urethane-coated nonwoven polytetrafluoroethylene fabric. *Cell Transplant* **15**, 335-41.

Soto-Gutierrez, A., Navarro-Alvarez, N., Zhao, D., Rivas-Carrillo, J. D., Lebkowski, J., Tanaka, N., Fox, I. J., and Kobayashi, N. (2007): Differentiation of mouse embryonic stem cells to hepatocyte-like cells by co-culture with human liver nonparenchymal cell lines. *Nat Protoc* **2**, 347-56.

Stravitz, R. T., Kramer, A. H., Davern, T., Shaikh, A. O., Caldwell, S. H., Mehta, R. L., Blei, A. T., Fontana, R. J., McGuire, B. M., Rossaro, L., Smith, A. D., and Lee, W. M. (2007): Intensive care of patients with acute liver failure: recommendations of the U.S. Acute Liver Failure Study Group. *Crit Care Med* **35**, 2498-508.

Suzuki, A., Zheng, Y. W., Kaneko, S., Onodera, M., Fukao, K., Nakauchi, H., and Taniguchi, H. (2002): Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. *J Cell Biol* **156**, 173-84.

Tanaka, K., Soto-Gutierrez, A., Navarro-Alvarez, N., Rivas-Carrillo, J. D., Jun, H. S., and Kobayashi, N. (2006): Functional hepatocyte culture and its application to cell therapies. *Cell Transplant* **15**, 855-64.

Teratani, T., Yamamoto, H., Aoyagi, K., Sasaki, H., Asari, A., Quinn, G., Sasaki, H., Terada, M., and Ochiya, T. (2005): Direct hepatic fate specification from mouse embryonic stem cells. *Hepatology* **41**, 836-46.

Tilles, A. W., Berthiaume, F., Yarmush, M. L., Tompkins, R. G., and Toner, M. (2002): Bioengineering of liver assist devices. *J Hepatobiliary Pancreat Surg* **9**, 686-96.

Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, N. (1995): Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373**, 702-5.

Wells, J. M., and Melton, D. A. (1999): Vertebrate endoderm development. *Annu Rev Cell Dev Biol* **15**, 393-410.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E., and Jaenisch, R. (2007): In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**, 318-24.

Wieder, K. J., King, K. R., Thompson, D. M., Zia, C., Yarmush, M. L., and Jayaraman, A. (2005): Optimization of reporter cells for expression profiling in a microfluidic device. *Biomed Microdevices* **7**, 213-22.

Wobus, A. M., and Boheler, K. R. (2005): Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* **85**, 635-78.

Yamada, T., Yoshikawa, M., Kanda, S., Kato, Y., Nakajima, Y., Ishizaka, S., and Tsunoda, Y. (2002): In vitro differentiation of embryonic stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green. *Stem Cells* **20**, 146-54.

Yamamoto, H., Quinn, G., Asari, A., Yamanokuchi, H., Teratani, T., Terada, M., and Ochiya, T. (2003): Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. *Hepatology* **37**, 983-93.

Yasunaga, M., Tada, S., Torikai-Nishikawa, S., Nakano, Y., Okada, M., Jakt, L. M., Nishikawa, S., Chiba, T., Era, T., and Nishikawa, S. (2005): Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat Biotechnol* **23**, 1542-50.

Yoshinari, K., Sato, T., Okino, N., Sugatani, J., and Miwa, M. (2004): Expression and induction of cytochromes p450 in rat white adipose tissue. *J Pharmacol Exp Ther* **311**, 147-54.

Zhang, Q. Y., Dunbar, D., and Kaminsky, L. S. (2003): Characterization of mouse small intestinal cytochrome P450 expression. *Drug Metab Dispos* **31**, 1346-51.