Progress and future challenges in stem cell-derived liver technologies

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Dalgetty DM, Medine CN, Iredale JP, Hay DC. Progress and future challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol 297: G241–G248, 2009. First published June 11, 2009; doi:10.1152/ajpgi.00138.2009.—The emergence of regenerative medicine has led to significant advances in the identification and understanding of human stem cells and adult progenitor cells. Both cell populations exhibit plasticity and theoretically offer a potential source of somatic cells in large numbers. Such a resource has an important role to play in the understanding of human development, in modeling human disease and drug toxicity, and in the generation of somatic cells in large numbers for cell-based therapies. Presently, liver transplantation is the only effective treatment for end-stage liver disease. Although this procedure can be carried out with high levels of success, the routine transplant of livers is severely limited by organ donor availability. As a result, attention has focused on the ability to restore liver mass and function by alternative approaches ranging from the bioartificial device to transplantation of human hepatocytes. In this review we will focus on the generation of human hepatic endoderm from different stem/progenitor cell populations with a view to its utility in regenerative medicine.

THE HUMAN LIVER HAS A REMARKABLE capacity to regenerate after physical or toxic injury (17, 54, 71, 80). However, upon repeated insult, the liver becomes progressively fibrosed and eventually loses most of its function. This requires patients to be put on effective liver support systems using various artificial devices since liver transplantation is often not possible or delayed. Existing treatments for liver failure and disease are reliant on liver cell or organ transplantation. At present orthotopic liver transplantation is the only long-term treatment for end-stage liver disease. Although successful, the procedure suffers from a number of drawbacks, such as the shortage of donor organs, operative damage, and the risk of immune rejection. Therefore, recent attention has been focused on the ability to use cellular resources to bridge patients until transplantation or to restore liver mass and function.

There have been significant advances in the identification of stem/progenitor cells within the body that will ultimately improve our understanding of cellular differentiation and growth and diseases and present an opportunity to generate cells and tissues for cell-based therapies. The use of ex vivo adult human hepatocytes is a desirable option for cellular therapies. However, these cells are scarce, have limited proliferation potential, and lose function and viability upon isolation. It has therefore been necessary to find a readily available source of hepatocytes that can be grown for longer in culture. In recent years, there have been great advances in liver stem cell biology, and a number of studies have proposed the use of fetal liver stem cells (hepatoblasts) and adult liver stem cells (oval cells) to generate primary hepatocytes. Both these hepatic progenitors have the potential to give rise to hepatocytes or cholangiocytes (38, 49, 64). Therefore, an attractive strategy would be to expand and transplant patient hepatic progenitor cells. However, it was subsequently shown that these hepatic progenitor cells are low in numbers within tissue, making their isolation, purification, and expansion consequently unfavorable to large-scale expansion (18). In an attempt to bypass the limitations associated with human liver stem cells and terminally differentiated hepatocytes, several groups have attempted to immortalize hepatocytes by introducing telomerase (hTert) constructs (85), SV40 T antigen (11), and viral transfection (4, 11). Regardless of the strategy the cells established using these methods exhibited phenotypic changes, poor liver function, and karyotypic abnormalities over prolonged culture (20).

Therefore, in recent years there has been a focus on deriving human hepatocytes from other sources, in particular human embryonic stem cells (hESCs). hESCs are derived from the inner cell mass of blastocyst stage embryos and are highly primitive cells that demonstrate self-renewal and pluripotency (62, 79). Therefore, hESCs are scalable and have the potential to provide an unlimited supply of replacement somatic cells, which are significant advantages over their adult stem cell counterparts. Initial models for deriving hepatocyte-like cells (HLCs) from hESCs employed direct differentiation or multicellular aggregate strategies. Direct differentiation approaches use a two-dimensional tissue culture approach employing extracellular matrixes, growth factors, cytokines, and hormones. The aggregation of hESCs results in the formation of three-dimensional structures termed embryoid bodies (EBs). The culture of EBs on adherent matrixes in the presence of growth factors, cytokines, and hormone cocktails gave rise to varying levels of HLCs. In recent years, hESC differentiation to HLCs has been modified with efficient and functional hepatocyte differen-
tiation demonstrated by several groups (1, 7, 12, 22, 24, 34–36). Although there has been major progress in the field, there is still the requirement to select HLCs from other contaminating cell types and undifferentiated stem cells in final cell preparations. Recent reports offer significantly improved yields of HLCs and the prospect for their homogenous isolation.

Although hESC derived HLCs are not immediately applicable to cell transplantation therapies or extracorporeal device construction. HLCs are being applied in the modeling of human liver development, disease, transplantation, and drug toxicology (Fig. 1), which is integral to developing safe and efficient cell-based therapies. Future studies in the field will build on those strong foundations and increase our knowledge of human liver biology. In this review we will focus on key stem cell populations capable of hepatocyte differentiation and discuss their merits.

**Human Liver Progenitor Cells**

During development the liver is formed from pluripotent stem cells of the inner cell mass of the blastocyst. Following blastocyst implantation, cells derived from specific regions of the epiblast migrate through a structure called the primitive streak (PS) and give rise to both mesoderm and endoderm (51, 79). The liver develops from the ventral foregut endoderm (53, 94) patterned by adjacent tissues, the cardiac mesoderm and septum transversum, to form the liver bud (32, 55). The liver and intrahepatic biliary tree arise from the anterior portion of the liver bud, whereas the posterior portion forms the gall bladder and extrahepatic bile ducts (102).

During human liver development cellular differentiation is coordinated through a cascade of cell signaling events. In the context of embryonic development, hepatic progenitor cells found in the early fetal liver bud are termed hepatoblasts and comprise ~0.1% of first trimester fetal liver mass (66). Hepatoblasts are characterized by their expression of α-fetoprotein (AFP), albumin, CK19, delta-like kinase (Dlk), and Epcam (75, 76, 93) and their bipotentiality, giving rise to both hepatocytes and cholangiocytes (66, 69). Some groups have described the isolation and expansion in culture of hepatoblasts from fetal livers of several species (32, 67, 76) and determined the signals required for hepatoblast differentiation to both lineages. Activin A, TGF-β, and the transcription factors HNF-6 and OC-2 are all shown to play critical roles in the differentiation of hepatoblasts to hepatocytes and cholangiocytes (15, 16). A gradient of activin A/TGF-β signaling has been shown to control hepatoblast differentiation to biliary epithelium (15). Furthermore, the transcription factors HNF-6 and OC-2 have been shown to inhibit activin A/TGF-β signaling from promoting hepatocytic differentiation (15, 16).
Another stem cell population found in the developing liver are side population (SP) cells and represent another potential source of liver progenitor cells. By virtue of their ability to actively efflux Hoechst dye, SP cells have been identified and characterized in the developing human liver (13, 28, 56, 78). It is suggested that these SP cells are present in the early gestational phase during liver organogenesis and contribute to hematopoietic and epithelial lineage generation (78). Both marker expression and functional similarities demonstrate the possibility that SP cells may share a relationship with oval/progenitor cells in the developing liver, responsible for liver regeneration after hepatic injury. The expression of the hematopoietic progenitor cell markers CD34, Sca-1, and Thy-1 combined with the ability of developing liver SP cells to adopt a hematopoietic and epithelial fate (78) support the possibility that SP cells represent an oval/progenitor and/or hematopoietic precursor population.

In the adult liver, hepatic oval cells account for ~0.3–0.7% of liver mass (66). When adult liver injury is so severe that normal hepatocyte proliferation is blocked, liver oval cells emerge from the canals of Hering located in the periportal region of the liver and have been shown to be directly involved in liver regeneration (3). Oval cells are phenotypically similar to fetal hepatoblasts and exhibit bipotentiality (2, 25, 84). Furthermore, research groups have documented the expression of liver stem cell and hematopoietic markers, including Ov-6, Thy-1, CD34, c-kit, and Sca-1 in oval progenitor cells (58, 61, 77). Because of their similarity to hepatoblasts, oval cells are considered to have valuable properties for therapeutic liver repopulation. In support of a role in liver regeneration, oval cell activation has been detected in chronic liver injury caused by inflammation, chronic hepatic necrosis, chronic alcoholism-induced cirrhosis, and hepatitis models (37, 61). Although the full complements of signals required for oval cell activation are still unknown, both continuous metabolic stress and chemical hepatotoxic substances have been implicated as potential oval cell activators when hepatocyte proliferation is inhibited (37, 61). A recent study reported the production of a chemokine known as stromal derived factor-1 alpha (SDF-1α) in the liver following tissue damage (33). Another chemokine, stem cell factor (SCF), has also been shown to have a fundamental role in the proliferation and migration of stem cells to damaged liver sites (26). It may therefore be possible that chemokines in combination with other growth factor/receptor systems may be directly involved in the early activation of the oval cell compartment and their subsequent cellular differentiation during liver regeneration. Of note, levels of the growth factors HGF, TGF-α, and TGF-β are elevated during oval cell proliferation (10, 26, 68, 81), and oval cells have been shown to express the receptors of these growth factors (2). Additionally, HGF in combination with SCF has been shown to expand hepatic stemlike cells obtained from fetal livers (55). Therefore, these growth factors appear to play an important role in hepatic regeneration via the activation of the stem cell niche.

The fetal and the adult liver provide ideal models with which to study stem cell populations. However, the hepatic stem cell populations discussed are low in number, making their isolation, purification, and growth unfavorable to large scale expansion (18). Although they are unlikely to be used as a cellular resource, the translation of these studies to in vitro stem cell-derived models will contribute to the generation of more physiological models with which to study human development and treatment of disease states (Fig. 1).

**Directed and Spontaneous hESC Hepatic Endoderm Differentiation**

Over the last decade huge progress has been made in the differentiation of hESCs to HLCs. A variety of approaches have been utilized for the differentiation of hESCs into functional HLCs. Current methods involve both spontaneous differentiation via the formation of EBs and directed differentiation by using inducing factors to hESC-derived hepatic endoderm. In the first instance, mouse and human ESCs were differentiated in suspension cultures in ESC aggregation, resulting in the formation of EBs, which exhibit regional differentiation into embryonically specific cell types of all three germ layers. EBs plated onto adherent matrix substrates allowed outgrowths of HLCs in the presence of growth factors, cytokines, and hormones (6, 14, 70). Although the formation of EBs creates a permissive microenvironment required for hepatocyte differentiation, it also possesses several drawbacks. The differentiation process through EB formation is spontaneous and stochastic and results in the formation of mixed cell types originating from all three germ layers (43), and until recently the purity of HLCs was low. In an elegant set of experiments Gadue et al. (27) addressed the problem of heterogeneity by engineering a genetically modified embryonic stem (ES) cell line that allowed cell populations representative of the PS to be purified. Using this model they were able to demonstrate that Wnt and TGF-β/nodal/activin signaling were required simultaneously for the generation of PS-like cells in mouse ES cell differentiation. Subsequently, those cells could be differentiated to HLCs as determined by marker expression (27). More recently, impressive experiments by Basma et al. (7) generated human HLCs through EBs and purified those populations by use of fluorescent activated cell sorting (FACS) for the asialglycoprotein receptor, ASGPR. The resulting cell population derived in a three-dimensional manner displayed hepatocellular function in many ways comparable to primary hepatocytes (7).

In addition to genetic modification and FACS, several groups have employed direct differentiation of hESCs to HLCs to avoid multicellular aggregation and permit scalability. Using this approach, efficient levels of hepatic endoderm (1, 7, 12, 22, 34–36) have been generated. Directed differentiation utilizes intracellular and extracellular signaling during development by employing extracellular signals to mimic human development and drive hESC differentiation toward a specific lineage. More recently, an in vitro direct differentiation model employing growth factors known to induce definitive endoderm and hepatic endoderm has been developed (34). These studies exemplified the need for a physiological component to the differentiation model and as a consequence improved both efficiency and function. Hay et al. (34) demonstrated that Wnt3 is expressed at critical stages of human liver development in vivo, highlighting the essential role of Wnt3 signaling in development. The exposure of hESCs to Wnt3a signaling, in a manner designed to mimic events within the developing embryo, resulted in the production of relatively homogenous hepatocellular differentiation (~90%) and improved functionality both in vitro and in vivo (34).
With the improvements in the ability to derive, purify, and scale hESC-derived hepatocytes in vitro, HLCs can be now produced in sufficient quantities for both in vitro and in vivo experimental applications essential to clinical translation. That being said, the production of hESC-derived HLCs exhibiting all major liver functions remains a challenge. Future work should address the way in which hESCs are differentiated to hepatocenderm with a focus on developmental physiology and cell culture microenvironments. Both approaches are essential step in improving the technology before clinical deployment.

Human iPSCs: Tailor-Made Health Care?

The generation of human induced pluripotent stem (iPS) cells (iPSCs) from human somatic cells has revolutionized the stem cell field (90). Reprogramming of somatic cells is achieved with the introduction of a defined set of transcription factors. This was first demonstrated by Shinya Yamanaka’s (74) group in both mouse and human fibroblasts by retroviral insertion of four transcription factors: Oct 3/4, Sox2, c-Myc, and Klf4. This was the first demonstration of direct reprogramming of cells with defined lineage back to a pluripotent state and exhibiting ES-like characteristics. The aforementioned group of transcription factors were obtained from an initial screen of 24 predetermined target factors in the mouse (74) and were subsequently shown to be essential in human cell reprogramming (73). The advent of iP technology has given rise to the possibility of patient-specific cell therapy (90) in which the use of genetically identical cells would prevent immune rejection. The technology is still in the early stages and a number of hurdles need to be overcome before iPSC-derived therapy can become a reality.

Inducing pluripotency in somatic cells has since been employed by a number of groups with different types of starting material, for example liver, stomach, and neural stem cells (51, 60, 73, 91). It has also recently been established that different combinations of transcription factors can be used to induce pluripotency. Studies have demonstrated that the transcription factors c-Myc and Klf4 can be substituted with other factors and combinations, including Nanog and Lin28 (8, 23, 41, 44, 57, 91). More recently it has been reported that only two factors (Oct3/4 and Klf4) are required for the generation of iPSCs from neural stem cells, which have high endogenous expression of Sox2 (60). Additionally, previous observations showed that certain chemical compounds increased the efficiency of somatic cell nuclear transfer (9, 45) and prompted investigators to try those molecules in somatic cell reprogramming to iPSCs. The translation of these studies has had an immediate impact on the iPSC field. Mouse embryonic fibroblasts reprogrammed with the four transcription factors Oct3/4, Sox2, Klf4, c-Myc and treated with an inhibitor of methyltransferase (5’-azacytidine) showed a 10-fold increase in reprogramming efficiency (40). To date there are three known such inhibitors; the other two are namely suberoylanilide hydroxamic acid and valproic acid (VPA), with the latter proving the most effective at increasing the efficiency of reprogramming (40). In the presence of VPA and two reprogramming factors (Oct3/4 and Sox2), iPSCs were obtained from primary human fibroblasts with greater efficiency than those reprogrammed with three factors (Oct3/4, Sox2, and Klf4) in the absence of VPA (40), suggesting that the rate-limiting step in the process of reprogramming is related to chromatin accessibility. Although promising, these strategies still require retrovirus transduction and integration. Although these strategies are acceptable for in vitro and in vivo modeling, they are not appropriate for clinical translation. In an attempt to overcome these issues, two groups have focused on and successfully reprogrammed fibroblasts using a strategy that allows the DNA insertion of all four transcription factors in one transgene (Oct4, Sox2, Klf4, and c-Myc) without viral transduction. Following the reprogramming process, the transgene was removed enzymatically through the utilization of a cre/ transposase-based method of excision (42, 88), providing a solution to the problems associated with the use of retrovirus/foreign DNA insertion in iP generation.

A variety of functional cell types have been derived from iPSCs, including neurons (21, 87), hematopoietic cells (31), and cardiomyocytes (52, 92). It has also been shown that differentiated iPSCs can be used to rescue a diseased phenotype. Hanna et al. (31) used a mouse model for sickle cell disease by applying gene targeting coupled with direct reprogramming to correct the defect (31). It has also been reported that dopaminergic neurons differentiated from iPSCs alleviated the symptoms of Parkinson’s disease in the rat (87). Additionally, the phenotype for hemophilia A could be corrected by iPSCs in a murine model of the disease (89). Together, these studies suggest that human iPSCs could be used in future regenerative medicine for patient-specific therapy. Although cell therapy is promising, a more immediate use for this technology is likely to be in vitro cell-based assays that model disease and drug toxicology and as such are likely to prove invaluable (Fig. 1). This would provide a greater understanding of the disease mechanisms and pathogenesis, thereby enabling an improved opportunity for therapy. The added value would lie in having a human experimental platform as opposed to an animal model. The creation of disease-specific iPSCs allows experiments on the disease phenotype and importantly an opportunity to repair the relevant gene defect. The iPSC system is therefore a powerful stem cell modeling tool with which to derive patient-specific or disease-model cell types. Additionally, with translation of hESC stem cell models to the iPSC systems it may be possible to generate the desired cell types in sufficient quantity and scale for downstream application.

Stem Cell-Derived Hepatocyte Utility in Drug Screening and Disease Modeling

The cost of drug development is heavily influenced by compound attrition rate. For every drug that reaches the market, ~5,000–10,000 compounds have been tested preclinically. These figures demonstrate the requirement for developing more accurate predictive toxicity models. The availability of hESC and iPSCs offers exciting opportunities for reliable high throughput drug screening. The ability to use human cell types in toxicology studies has the potential to increase the efficiency of novel human drug development, while reducing drug attrition in the final stages of development and therefore costs. Additionally, the use of iPSCs would also enable the study of a number of single nucleotide polymorphisms that influence the ability of an individual to effectively metabolize and clear drugs and toxins. The existence of such variations between individuals and in different ethnic populations gives rise to a number of adverse drug reactions or ineffective drug
dosing regimes. One such example of variable drug metabolism is that of warfarin, in which polymorphisms in cytochrome P-450 2C9 create problems with obtaining an appropriate pharmatherapeutic range. The use of iPSC technology as a research tool in the investigation of human disease could prove invaluable in modeling drug metabolism and the provision of tailor-made health care (39). Additionally, this would provide a greater understanding of disease mechanisms and pathogenesis, thereby enabling an improved opportunity for therapy.

**Stem Cell-Derived Hepatocytes in Human Bioartificial Liver Construction**

Since the 1950s many supportive modalities have been added to standard therapy to bridge patients awaiting organ transplant. These therapies range from drug treatment to liver perfusion and liver support devices. Liver support devices can be grossly divided into biological and nonbiological systems, with the latter exhibiting limited success (19). In contrast, biological support systems have shown great promise and demonstrate the importance of not only restoration of failing detoxification, but also restoration of liver metabolic function. Phase I trials employing a bioartificial liver (BAL) device improved both the patients' neurological state and hemodynamics (19). Although these studies provided great hope the BAL devices were “fueled” with xenogeneic material (porcine hepatocytes) because of cost and availability, and as a direct result BAL use was prohibited in many European countries. Therefore, the need for a humanized BAL device still remains and ideally BALs would be fueled with human hepatocytes. At present there are two main sources of human hepatocytes: freshly isolated adult hepatocytes or hepatocyte cell lines. Unfortunately, both sources of hepatocytes suffer major drawbacks such as scarcity and diminishing function and proliferation in vitro. Immortalization strategies have therefore been applied to primary hepatocytes in an attempt to overcome these problems. However, those attempts proved unsuccessful because both proliferation and hepatocellular function are mutually exclusive (83). Recently a human hepatoma cell line, HepaRG, has been characterized and demonstrated to be a useful research tool in liver metabolism and toxicity studies (82). Although the HepaRG cell lines provide some of the attributes of primary hepatocytes, they remain a cancer cell line and therefore may be restricted in functions essential to in vitro modeling of human drug toxicity and their incorporation in extracorporeal support devices. Therefore, the development of a humanized BAL has to date been limited by our inability to generate efficient numbers of metabolically competent primary human hepatocytes. With recent developments in deriving HLCs from hESCs (7, 34) and the translation of those models to iPSCs, it is tempting to speculate that stem cell-derived HLCs hold great promise in the provision of functional “humanized” extracorporeal support.

**Stem Cell-Derived Hepatocellular Therapy**

Hepatocytes are the main functional cells of the liver and perform a variety of important endocrine and exocrine functions. Research employing human hepatocytes for cellular transplantation has been constrained by the difficulties in sourcing and maintaining viable hepatocytes. A number of innovative cell-based and animal model studies of human liver disorders have highlighted the remarkable regenerative capacity of hepatocytes in vivo, indicating the feasibility of hepatocyte transplantation as a means of replacing lost or diseased hepatic tissue. In urokinase plasminogen activator (uPA) mice, transplanted hepatocytes underwent greater than 12 cell divisions on average, with plasma uPA levels returning to normal within 2 mo and replacement of the entire host liver (65). In transgenic mice with a defect in hepatic growth potential and function, the transplanted hepatocyte population replaced up to 80% of the diseased host liver (63). Furthermore, in other studies of fumarylacetoacetate hydrolase-deficient mice, transplanted hepatocytes underwent at least 18 cell divisions (59) or greater than 69 cell divisions on average (30). Together these findings demonstrate the enormous proliferative potential of adult hepatocytes within in vivo transplantation. However, tumor risk still remains an issue when hESCs are transplanted into immunodeficient animal models and must be addressed. In a recent study by Basma et al. (7), stem cell-derived HLCs were purified by FACS before transplantation. Recipient animals did not develop teratomas or tumors in the liver; however, the peritoneum contained tumors histologically consistent with well-differentiated adenocarcinomas (7). Although these studies are encouraging, there is a clear need to improve the stem cell-derived resource so that tumor formation is not observed in vivo. Nevertheless the experiments by Basma et al. have provided great hope for cell therapy.

**Defined Culture Conditions Are Essential for Stem Cell Clinical Translation**

As stem cells move closer to diagnostic or clinical application, there is an imperative for standardization of cell culture systems and routine tissue culture models. Therefore further efforts are necessary to establish defined culture conditions permitting the expansion and differentiation of stem cells. At present, hESCs and iPSCs are maintained on extracellular matrixes in medium containing animal products that pose a number or problems. The presence of xenogeneic animal products in stem cell culture increases the risk of contamination via animal pathogens, may evoke immunological rejection upon transplantation, and the presence of variable and unidentiﬁed factors greatly complicates developmental studies. The cell culture industry is making the shift away from animal-based materials and now offers a range of media and reagents for the growth and differentiation of stem cells as it moves toward clinical applications. To eliminate serum-related problems, several groups have optimized serum-free culture conditions for hESC lines (5, 46, 53). As this drive progresses, it is vital for research groups to agree on the stem cell policies that extend from the derivation/reprogramming, culture, and cell banking. Furthermore, the poor repopulation efficiencies must also be addressed before hESC-derived hepatocytes can be used effectively in the clinic.

**Conclusion**

Recent progress in the production of stem cell-derived hepatocytes has provided an enormous therapeutic opportunity from in vitro models to in vivo transplantation. Although a number of successful techniques have been developed, stem cell-derived hepatocyte function is still not as broad as that of
primary hepatocytes. Therefore the elucidation of other key developmental factors and tissue culture environments, coupled with iPSC technology, are essential if the true potential of stem cell-derived hepatic endoderm is to be realized.

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