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Study of Hepatocyte Differentiation Using Embryonic Stem Cells

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Abstract The liver has many crucial functions including metabolizing dietary molecules, detoxifying compounds, and storing glycogen. The hepatocytes, comprising most of the liver organ, progressively modify their gene expression profile during the fetal development according to their roles in the different phases of development. Embryonic stem (ES) cells serve as a major tool in understanding liver development. These cells may also serve as a source of hepatic cells for cellular therapy. In this review, we aim to summarize the research that has been performed in the field of hepatocyte differentiation from mouse and human ES cells. We discuss the various methodologies for the differentiation of ES cells towards hepatic cells using either spontaneous or directed differentiation protocols. Although many protocols for differentiating ES cells to hepatic cells have been developed, the analysis of their status is not trivial and can lead to various conclusions. Hence, we discuss the issues of analyzing hepatocytes by means of the specificity of the markers for hepatocytes and the status of the cells as fetal or adult hepatocytes. J. Cell. Biochem. 96: 1193–1202, 2005.

Key words: embryonic stem cells; hepatocytes; endoderm; liver; development; genetic manipulation

The liver is the largest organ in mammals and it serves a variety of important functions. Hepatocytes, the primary cells of the liver, perform various functions, including metabolizing diverse dietary molecules, detoxifying compounds, and storing glycogen. The organ has also endocrine and exocrine functions. Through the basal surface, the hepatocytes condition the venous blood coming into the liver by the secretion of serum factors. Through the apical surface, the hepatocytes secrete bile into canaliculi that join the bile ducts. The remaining cells of the liver consist largely of cholangiocytes, Kupffer cells, stellate cells, and a variety of endothelial cells.

The developmental processes leading to the final complex structure and function of the liver have been studied for years using mainly rodent embryos. In the last 20 years, mouse embryonic stem (ES) cells have served as a major biological tool for studying early embryonic development. These pluripotent cells, isolated from the blastocyst stage embryos, have been shown to differentiate into derivatives of the three embryonic germ layers. Human ES cells, isolated on 1998, now enable the study of human development in culture. The human ES cells may also serve as a source of cells for cellular therapy of liver pathologies. Here we aim to review the recent findings in the field of hepatocyte development that has been provided from the study of mouse and human ES cells.

STRUCTURE AND DEVELOPMENT OF THE LIVER IN RODENTS AND HUMANS

The adult human liver is composed of two different sized lobes, each subdivided into lobules [Braunwald et al., 2004]. The lobules are tiny hexagonal or pentagonal cylinders about 2 mm high and 1 mm in diameter. A small branch of the hepatic vein extends through the center of each lobule. Around this central vein, in plates or irregular walls radiating outward, are arranged the hepatic cells. Around the periphery of each lobule, several sets of three tubes of vessels exist: (a) branches of the hepatic artery; (b) the portal vein (interlobular veins); (c) the hepatic duct (interlobular bile ducts). The hepatocytes comprise approximately 80% of the total liver mass and the rest of the hepatic parenchyma is mainly

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biliary epithelial cells and Kupffer cells. The unique structure along with the enzymes expressed in the liver enables the organ to perform the processes related to macromolecular synthesis, energy generation and storage, catabolism, and disposal of toxic substances, and waste products of intermediary metabolism.

During mouse development, the induction of hepatic genes occurs in a segment of the definitive endoderm at about 8.5 days of gestation (E8.25, 7–8 somites) [Lemaigre and Zaret, 2004]. The induction requires signaling from two different mesodermal cell types: fibroblast growth factors (FGFs) are secreted from the adjacent cardiac mesodermal cells [Jung et al., 1999] and bone morphogenetic proteins 2 & 4 (BMP2 & BMP4) are secreted from the septum transversum mesenchyme cells [Rossi et al., 2001]. Then, the endodermal cells start to proliferate and bud into the stromal environment of the septum transversum mesenchyme (E9, 14–20 somites) [Douarin, 1975; Medlock and Haar, 1983]. The proliferating cells interact with endothelial cells, that are also mesodermal derivatives, and this interaction is crucial for this early budding phase [Matsumoto et al., 2001]. When the hepatic endoderm is specified and the liver bud begins to grow, the cells are referred to as hepatoblasts (E9) [Lemaigre and Zaret, 2004]. They seem to be bipotential, capable of differentiating into hepatocytes or bile duct cells (cholangiocytes), even though they already express some genes specific to mature hepatocytes such as albumin [Shiojiri et al., 2001]. The hepatoblasts migrate in cord-like structures and the endothelial cells associate with them to form capillary-like structures between them [Duncan, 2003]. Hematopoietic cells migrate into the liver bud during E12 and further proliferate there, apparently emitting a growth signal for the liver [Kamiya et al., 1999]. Numerous signal transduction molecules and transcription factors are than needed for the continued growth of the fetal liver and to prevent apoptosis [Zaret, 2002; Duncan, 2003]. The polarization of the hepatic epithelium allows the liver to form its final architecture and it becomes ready to perform its aforementioned tasks.

The differentiation of hepatoblasts into hepatocytes is a gradual process, taking several days during rodent embryonic development. Prior to day 12 of gestation in the mouse, the hepatoblasts remain in a morphologically undifferentiated state [Medlock and Haar, 1983]. The onset of synthesis of secreted proteins is around rat embryonic day 12, while deposition of glycogen rosettes is identifiable after embryonic day 18 in the rat [Duncan, 2003]. It was also shown that the fetal rat liver tissue gets the polygonal characteristic adult structure just prior to birth [Vassy et al., 1988]. This data suggests that hepatocytes in different developmental phases are functionally diverse and we may discriminate between them.

The mouse is the best-studied animal model for embryonic development; however, there are some differences between mouse and human development that we should consider when we study human development using mouse models. Although mouse and human genome sizes are very similar, there are many genomic differences, for example, 15% of the genes in one species have a homologue but lack an orthologue in the other species. Moreover, 1% of human genes are absent in the mouse genome [Waterston et al., 2002]. These differences lead to altered biochemical pathways and different histological and pathological phenotypes. Mouse and human possess the expected range of intra-abdominal organs that, in developmental terms, are formed as outgrowths from the primitive gut, such as the liver and pancreas, but their gross morphology is dissimilar [Strachan et al., 1997]. A major difference in the time scale of development and in the morphologic features of mouse and human are expressed by size, growth, and anatomy [Strachan et al., 1997].

LIVER PATHOLOGIES AND THERAPIES

Since the liver has so many pivotal roles, patients with acute hepatic failure or end-stage liver disease must be treated by liver transplantation. Liver transplantation has become an accepted treatment for liver failure, but the scarcity of organ donors limits its potential. Transplantation of hepatocytes has been proposed as an aid and as an alternative to whole organ transplantation (for review [Horslen and Fox, 2004]). Hepatocytes are transplanted into the liver or ectopic sites as the spleen and can support liver function in time of hepatic insufficiency. Although clinically used, the source for the hepatocytes is also limited. Discarded livers, which cannot be used as a source for organ transplantation, are a limited source for hepatocytes.

Until other sources for hepatocytes are found, a wider use of the treatment will not be possible.

Three major sources for hepatocytes, other than liver donations, are hepatocytic cell lines, xenogenic hepatocytes, and hepatocytes derived from stem cells. Primary hepatocytes have a very limited proliferating potential thus hepatic cell lines were established. In order to increase the amount of the human hepatocytes available for transplantation, the cells should be immortalized. SV40 T-antigen immortalized hepatocytes have been demonstrated to restore hepatic function when transplanted into animal models but carry a potential for increased malignant transformation [Nakamura et al., 1997; Tada et al., 1998; Cai et al., 2002]. In a recent publication, transduction of fetal human hepatocytes with the catalytic subunit of telomerase reverse transcriptase enabled them to divide for a longer period as compared to control cells [Wege et al., 2003]. The cells expressed functions normally associated with liver cells and upon transplantation to immunocompetent mice integrated and expressed RNA of hepatic genes. The telomerase immortalization is advantageous over other methods since the cells are less amenable to neoplastic transformation. The cells maintained contact inhibition and did not display characteristics of transformed cells in culture and upon transplantation in suitable recipients [Wege et al., 2003]. Although this work presents the creation of hepatocyte cell lines, the differentiation status of the cells is not clear. The immortalized cells exhibited functions normally associated with fetal hepatic cells and not to the adult hepatocytes. Since there are characteristics of adult hepatocytes that develop during late phases of development, the fetal hepatocytes may not be able to acquire them upon transplantation to adult liver. Only transplantation into a metabolic model will demonstrate the functionality of the engrafted cells.

Xenogeneic cells have been shown to be effective in many animal models [Horslen and Fox, 2004]. In a latest report, xenotransplants of porcine hepatocytes into a rat model of terminal cirrhosis displayed improvement in liver functions [Nagata et al., 2003]. Animals that received hepatocyte transplants showed significant improvement in their encephalopathy scores, plasma bilirubin and albumin, and ammonia levels compared with untreated controls. Porcine hepatocytes were as effective as syngeneic rat hepatocytes in the correction of the animal's liver functions. The survival of the animals was significantly improved and was equivalent in the animals treated with xenotransplant and those treated with syngeneic rat hepatocytes [Nagata et al., 2003]. The importance of this study is the fact that xenotransplants can be as effective as cells from the same species. However concerns of zoonoses and possible pathogens transferred from animal to human dramatically limit the usefulness of xenotransplantation.

The third possibility to derive hepatocytes for transplantation is the use of stem cells. Adult stem and ES cells have been shown to differentiate into hepatocytes. Oval and hepatic stem cells were identified in liver repopulation studies in animals and were shown to differentiate into mature hepatocytes [Fausto and Campbell, 2003]. Stem cells from non-endodermal origin, such as bone marrow, were also reported as a source for hepatocytes [Jiang et al., 2002; Austin and Lagasse, 2003]. This process of transdifferentiation of mesodermal stem cells into differentiated endodermal hepatocytes received a great deal of attention. This process is very controversial since the dogma of development is based on the commitment of cells to specific fate and it is not clear how the cells expand their potential and change their fates. In later reports, it was shown that this process of transdifferentiation may be misinterpreted because of cell fusion between the transplanted cells and the native hepatocytes [Vassilopoulos et al., 2003; Wang et al., 2003]. ES cells are self-renewing pluripotent cells capable of differentiating into derivatives of the three germ layers. Mouse and human ES cells were shown to differentiate into hepatocytes using various different protocols. In this review, we will focus on the topic of ES cells as a source for hepatocytes.

EMBRYONIC STEM CELLS AND HEPATOCYTES

ES cells were first isolated from mouse embryos [Evans and Kaufman, 1981; Martin, 1981]. The pluripotency of ES cells is evident both in vivo and in vitro. The most prominent proof for pluripotency is that mouse ES cells injected into the blastocyst cavity contributes to all cell types, including germ cells, in the resultant embryo. The germ cells derived from the ES

cells are a source for the future generation of embryos carrying the ES genotype [Capecchi, 1989; Rossant and Joyner, 1989].

The isolation of human ES cells several years ago [Thomson et al., 1998; Reubinoff et al., 2000] expanded the potential of ES cells not only as a source of cells for developmental studies but also as a source of cells for cell therapy. The pluripotency of human ES cells was proven in vivo and in vitro. In vivo, injection of the human ES cells into immune deficient mice generates teratomas harboring derivatives of all three embryonic germ layers [Thomson et al., 1998; Reubinoff et al., 2000]. In vitro, ES cells grown in suspension aggregate to form spheroid clumps of cells called embryoid bodies (EBs) [Itskovitz-Eldor et al., 2000]. The cells within the EBs were shown to express molecular markers specific for the three embryonic germ layers. The EBs mature by the process of spontaneous differentiation and cavitations, and the cells acquire molecular markers for differentiated cell types. Dissociation of EBs and plating the differentiated cells as a monolayer revealed many cell lineages [Itskovitz-Eldor et al., 2000]. The ability to induce specific differentiation was demonstrated by the addition of various growth factors to differentiating human ES cells, followed by analysis of cell morphology and expression of a large number of cell specific markers [Schuldiner et al., 2000]. Specific protocols were developed in order to enrich various cell types during the differentiation of ES cells. The production of neuronal, cardiac, hematopoietic, endothelial, and hepatic cells was subsequently demonstrated [Schuldiner and Benvenisty, 2003]. Figure 1 schematically illustrates the various ways for differentiating ES cells and methods for selecting specific subsets of cells.

Although stem cells were shown to differentiate into most cell types we should bear in mind two major limitations in the study of ES cells. Since the differentiation of ES cells in culture is heterogeneous, in order to study the differentiation towards specific cell types there is a need to define markers that are cell specific. Thus, for example, in order to follow up on the differentiation of hepatic cells, genes that are predominantly expressed in the liver tissue and not in other tissues, such as yolk sac or pancreas, should be identified. The second limitation is the differentiation status of the cells. Since the process of differentiation is gradual, there is a need to discriminate between the genes expressed in each phase of development. Various tissues change their gene expression profile during the embryonic development. For example, during the development of the hematopoietic system, there is a change in the expression of

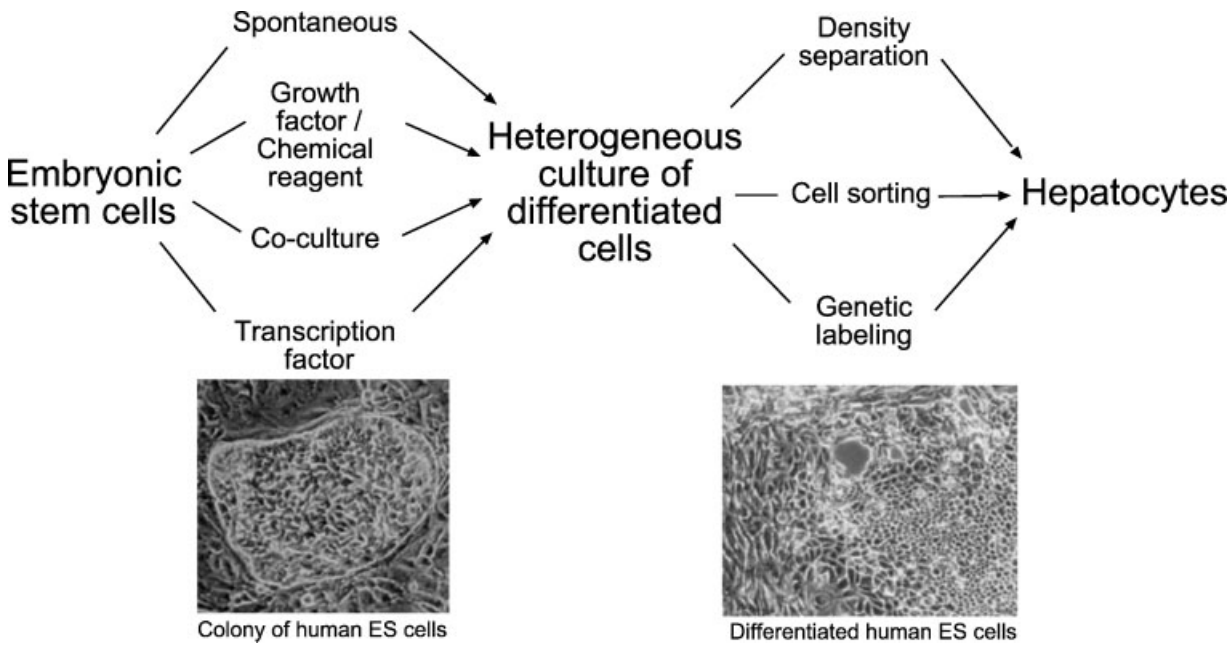

Fig. 1. Methodologies for the differentiation of hepatic cells from human embryonic stem cells.

the globins [Weatherall, 1986]. Hence, genes expressed during the early stages of development cannot represent adult cells.

Since the first reports on differentiation of mouse ES cells into endodermal cells [Abe et al., 1996; Levinson-Dushnik and Benvenisty, 1997], many studies demonstrated differentiation of ES cells into hepatic-like cells. In Table I, we summarize the studies on differentiation of mouse and human ES cells into hepatic-like cells. The various studies are detailed by means of the differentiation protocol, molecular markers tested, functional assay, histological assay, and in vivo assay. In general, we can divide the methods of differentiation into spontaneous and directed differentiation. In the protocol of spontaneous differentiation, the cells are grown as EBs for a few days and then usually they are plated on an adherent matrix as a monolayer either as dissociated cells or as clumps of cells. In the directed differentiation, the protocol involves elements that promote the differentiation of the ES cells into the endodermal lineage. The enrichment of a specific differentiated cell type is due to an induction of the endoderm lineages, inhibition of other lineages or both. We can subdivide the directed differentiation into three categories according to the major element in the protocol of differentiation compared to the spontaneous one (Table I). Thus, the directed differentiation is due to addition of growth factors and hormones, limited exposure to serum and constitutive expression of hepatic transcription factors.

Spontaneous differentiation of mouse ES cells occurs when the cells are grown in suspension and form EBs [Abe et al., 1996; Chinzei et al., 2002; Jones et al., 2002; Miyashita et al., 2002; Yamada et al., 2002; Asahina et al., 2004; Kumashiro et al., 2005]. In order to direct the differentiation towards the endoderm lineage, various growth factors were used. In most of the protocols the cells were grown as EBs for several days and then plated on adherent matrix and treated with several growth factors [Hamazaki et al., 2001; Kuai et al., 2003; Hu et al., 2004; Imamura et al., 2004; Jochheim et al., 2004; Kania et al., 2004; Shirahashi et al., 2004; Teratani et al., 2005]. Usually, collagen was chosen as the matrix for growing the cells since the liver bud proliferates and migrate into the septum transversum mesenchyme that is composed of loose connective tissue containing collagen. The most abundant factors that were used are aFGF, hepatocyte growth factor (HGF), oncostatin M (OSM), and dexamethasone. aFGF was added since it is secreted by the mesoderm and is the first factor (along with bFGF) to commit the foregut endoderm to forming the liver primordium. HGF supports fetal hepatocytes during mid-stage hepatogenesis and it was shown that mice lacking HGF had reduced sized embryonic liver [Maina et al., 1996]. OSM is produced by the hematopoietic cells and induces the maturation of fetal hepatocytes [Kamiya et al., 1999]. Dexamethasone is a synthetic glucocorticoid hormone active in induction of enzymes concerned in gluconeogenesis in the liver such as phosphoenolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT), and others and thus it was usually added last to the culture media [McGrane et al., 1990]. Kubo et al. [2004], enriched the endodermal population of cells by a limited exposure to serum and the addition of activin A alone or with dexamethasone. Some protocols used a constitutive expression of a hepatic transcription factor in order to direct the differentiation towards the endoderm lineage. The nuclear factors used were the forkhead box (FOX) transcription factors, FOXA1 or FOXA2 [Levinson-Dushnik and Benvenisty, 1997; Ishizaka et al., 2002; Kanda et al., 2003]. The FOXA transcription factors, previously named hepatocyte nuclear factors 3 (HNF3), were shown to be involved in endoderm differentiation [Kaestner, 2000]. They bind specifically to sequences required for hepatocyte-specific expression of genes and sequentially activated during the differentiation of definitive endoderm [Duncan et al., 1998].

The characterization of the differentiated mouse hepatic cells was carried out by analyzing their endodermal gene expression (RNA and protein), functional metabolic assays, morphological assays, and in vivo assays. Marker genes for endodermal differentiation were tested for the presence of RNA transcripts by RT-PCR and in situ hybridization. The markers included hepatic transcription factors such as transcription factor 1 & 2 (TCF1 & 2), FOXA1, FOXA2, HNF4, and HNF6, the carrier proteins alpha-fetoprotein (AFP) and albumin, and enzymes such as tryptophan-2,3-dioxygenase (TDO), TAT, and the cytochrome P450 (CYP) enzymes. AFP and albumin were usually also tested on the protein level by immunostaining or Western blot analysis. The functional assays

TABLE I. Differentiation Potential of ES Cells into Hepatic Cells

Differentiation protocol	RNA markers	Protein markers	Functional assay/ morphology	In vivo assay	Ref
Mouse					
Spontaneous (through EBs)	AFP, ALB, TTR, TCF1, TCF2, HNF4, FOXA2				Abe et al. [1996]
	AFP, ALB, HNF4	ALB, AFP, GTAR, Transferrin	E.M.		Jones et al. [2002]
	AFP, ALB, TTR, AAT, TDO2, CPSI, PEPCK, LST1, FOXA2	ALB	ICG uptake/E.M.	Incorporation Immuno.—ALB	Yamada et al. [2002]
	AFP, ALB, TTR, AAT, G6P, GST, ASGR1, FOXA1,A2,A3	ALB			Miyashita et al. [2002]
	AFP, ALB, TAT	ALB	Urea synthesis	Incorporation Immuno.—ALB	Chinzei et al. [2002]
	AFP, ALB, TAT, TTR, APCS, FGB, TDO2, CYP1A2, CYP7A1, MAT1A, STH2	ALB			Asahina et al. [2004]
	ALB, TAT, CYP7A1	ALB, CYP1A2	Urea synthesis E.M., PAS	Incorporation Immuno.—ALB PT, T-Bil	Kumashiro et al. [2005]
Directed by growth factors					
aFGF, HGF, OSM, Dex., ITS	ALB, G6P, TAT				Hamazaki et al. [2001]
HGF & beta-NGF	ALB, G6P, TTR, HNF4, SEK1	AFP, AAT	Epithelial shape		Kuai et al. [2003]
aFGF, TGF, AFP, HGF, OSM, Dex.	AFP, ALB, TTR, TAT, G6P, FOXA2	ALB, AFP, CK8, CK18	ALB & urea synthesis		Hu et al. [2004]
Monothioglycerol on collagen type I & HCM	AFP, ALB, TTR, AAT, TAT, CYP2B9, CYP2B13, NESTIN, FOXA2, HNF4, HNF6, CEBPB	ALB, AFP, NESTIN, CK18, CK19, AAT, AMY, cMET, DPPIV	ALB secretion epithelial-like shape, multi nucleated, PAS		Kania et al. [2004]
HCM on collagen type I	AFP, ALB, TCF1, TCF2, FOXA1, FOXA2, HNF4, HNF6, CEBPA, CEBPB	ALB, AFP			Jochheim et al. [2004]
RA, HGF, aFGF,FGF4, OSM/ Gel.-Col./ESM-HCM (monolayer only)	ALB, TTR, TAT, G6P, TDO2, CK8, LST1, CPS1, PEPCK, CYP1A1, FOXA2, HNF4	CK18, TTR, ALB-GFP	E.M.	Incorporation, survival, fibrin & ALB levels	Teratani et al. [2005]
Insulin, Dex., collagen type I	AFP, ALB, CK19, G6P, GGT	ALB	Urea synthesis		Shirahashi et al. [2004]
AFGF, HGF, OSM, Dex., ITS & 3D scaffold	AFP, ALB, TAT, TTR, G6P	ALB	E.M.	Immuno.—ALB, CK18	Imamura et al. [2004]
Directed by serum limitation & GFs					
Limited exposure to serum & Dex.	AFP, ALB, TTR, AAT, TAT, CPS1				Kubo et al. [2004]
Act.A, Dex. & limited exposure to Serum	AFP, ALB			Teratomas (gut) Immuno.—FOXA2	
Directed by FOX TFs					
FOXA1 or FOXA2	ALB, TTR, TCF2, FOXA1, FOXA2				Levinson-Dushnik and Benvenisty [1997]
FOXA2 & bFGF	AFP, ALB, TTR, AAT, TDO2, PEPCK, FOXA2, HNF4	ALB	ICG uptake/polygonal shape		Kanda et al. [2003]
FOXA2 & bFGF, Dex., Asc., Nicotinamide	ALB, C3, CYP1A1, PEPCK, PXMP1-L	ALB, CK18	Urea &lipid synthesis E.M., PAS		Ishizaka et al. [2002]

Human				
Spontaneous (through EBs)	AFP, ALB, APOA4, APOB, APOH, FGA, FGG, FGB,	ALB, AFP		Lavon et al. [2004]
Directed by				
Insulin, Dex., collagen type I (through EBs)	ALB, AAT	ALB	Urea synthesis	Shirahashi et al. [2004]
Na Butyrate & DMSO (EBs/monolayer + HCM)	ALB, AAT, AGRP, GATA4, HNF4, TTR, CEBPA, CEBPB	ALB, AAT, CK8, CK18, CK19	ALB synthesis, CYP1A2 activity, PAS	Rambhatla et al. [2003]

Differentiation: Ebs, embryoid bodies; GFs, growth factors; FOX, forkhead box; TFs, transcription factors; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; OSM, oncostatin M; Dex., dexamethasone; ITS, insulin transferrin and selenious acid; NGF, nerve growth factor; TGF, transforming growth factor; AFP, alpha fetoprotein; HCM, hepatocyte culture medium; RA, retinoic acid; Gel., gelatin; Col., collagen; ESM, embryonic stem cell medium; 3D, 3 dimensional; Act.A, activin A; Asc.-L-ascorbis-2-phosphate; DMSO, dimethyl sulfoxide.
Molecular markers: ALB, albumin; TTR, transthyretin; TCF, transcription factor; HNF, hepatocyte nuclear factor; AAT, alpha-1-antitrypsin; TDO2, tryptophan-2,3-dioxygenase; CPSI, carbamyl phosphate synthetase 1; PEPCK, phosphoenolpyruvate carboxykinase; LST1, liver specific organic anion transporter 1; G6P, glucose-6-phosphatase; GST, glutathione-s-transferase; ASGR1, asialoglycoprotein receptor; TAT, tyrosine aminotransferase; APCS, serum amyloid *p* component; FG, fibrinogen; CYP, cytochrome P450; MAT, methionine adenosyltransferase; STH, hydroxysteroid sulfotransferase; SEK1, SAPK/ERK kinase-1; CEBP-CCAAT/enhancer binding protein; CK, cytokeratin; GGT, gamma-glutamyl transpeptidase; C3, complement C3; PXMP1-L-peroxisomal membrane protein 1-like protein; APO, apolipoprotein; AGRP-DGAT, Diacylglycerol *O*-acyltransferase; GTAR, ankyrin repeat-containing gene; AMY, amylase; cMET-HGF receptor; DPP, dipeptidyl peptidase; GFP, green fluorescent protein.
Histology: E.M., electron microscopy; ICG, indocyanine green; PAS, periodic acid shiff staining for glycogen; H&E, hematoxylin and eosin staining; Immuno., immunostaining; PT, prothrombin time; T-Bil, total bilirubin.

for hepatocytes were mainly urea and albumin synthesis. Using electron microscopy, the morphological features of the cells were tested and periodic acid shiff staining was used for tracing glycogen granules. In the few studies that used an in vivo assay in order to characterize the differentiated cells, the incorporation of the transplanted cells into the liver of the mice and the production of albumin by those cells was tested.

Human ES cells were shown to differentiate into derivatives of the three germ layers, among them endodermal cells expressing AFP and albumin [Itskovitz-Eldor et al., 2000; Schuldiner et al., 2000]. This first clue of endodermal differentiation led us to analyze the potential of human ES cells to differentiate into hepatic cells and the spontaneous differentiation of human ES cells into hepatic-like cells was demonstrated [Lavon et al., 2004]. Human hepatic-like cells were isolated and characterized for their phenotype. Through gene manipulation hepatic cells were labeled and for the first time, a homogenous population of differentiated cell types was demonstrated. In addition, the hepatic-like cells were suggested to develop in a niche next to cardiac mesodermal cells and that aFGF may play a role in this differentiation. The differentiation towards hepatic-like cells were also demonstrated by using other factors [Rambhatla et al., 2003; Shirahashi et al., 2004]. Shirahashi et al. [2004], added insulin and dexamethasone to EBs cultured on collagen type I and showed that the cells express various endodermal genes. Rambhatla et al. [2003], added sodium butyrate to the culture media inducing hepatic differentiation as well as significant cell death. The resultant cells had morphological features similar to that of primary hepatocytes and most of the cells expressed liver associated proteins.

As we described above, there are various ways to cause ES cells to differentiate into hepatic-like cells. However, the nature of the hepatic-like cells should be analyzed very carefully under several constrictions. (a) Most of the genes that are expressed in the liver tissue and also expressed in the extra embryonic yolk sac tissue. AFP, transthyretin (TTR) and FOXA2 for example, are expressed in both tissues and were widely used to characterize the differentiated cells as hepatocyte. In order to discriminate between these two populations of cells, we should identify discrete markers for

hepatocytes and yolk sac tissues. In mouse, it was shown that CYP7A1 is expressed in the liver and not expressed in the yolk sac tissue, and thus it can be a good marker for hepatocytes [Asahina et al., 2004]. The synthesis of urea is also performed only by the hepatocytes and not by the yolk sac and should fit as a test for hepatocytes only. (b) Some genes that are expressed form hepatocytes are also expressed in other somatic cells such as lung, intestine, pancreas, and kidney. For example, metabolic enzymes such as TAT and PEPCK are not solely expressed by the liver and thus do not fit as hepatocyte markers. Furthermore, many groups used endodermal transcription factors such as FOXA2 and HNF4 in order to prove endodermal differentiation, but we should bear in mind that they are also expressed in mesodermal and ectodermal tissues. (c) Embryonic, fetal, and adult hepatocytes are different by means of their gene expression. When a cell is characterized as a hepatocyte we should also state its developmental stage. Embryonic hepatocytes transplanted into the liver cannot replace adult hepatocyte since they lack many functions. Some genes such as albumin and TTR are first expressed in early embryos and further on in fetal and adult hepatocytes and thus their expression cannot tell the state of the differentiated cell. AFP on the other hand is expressed very early in embryonic development and later on in the fetus but is turned off during adult life hence, a hepatocyte that had stopped to express AFP can be considered as adult hepatocyte. Many of the metabolic and detoxifying enzymes, but not all, are functional only upon birth although they are expressed during fetal stages. Functional assays for enzymes should be carried on in order to tell the differentiation state of the cells.

FUTURE STUDIES

In this review, we have summarized the studies on differentiation of mouse and human ES cells into hepatic-like cells. Different groups have used various protocols to yield hepatic-like cells and have characterized them. Since the characterization of hepatocytes is problematic, as we described above, we believe that in order to further characterize the hepatic-like cells, more assays should be performed. In vitro, since the differentiated population is heterogeneous there is a need to specifically characterize the hepatic cells. This can be performed by analyzing the expression of several genes within the same cell and by identifying the gene expression profile of isolated homogenous populations of hepatic-like cells. In-situ hybridization and immunostaining allow analyzing the expression of few genes within the same cell in the level of RNA and protein, respectively. In order to isolate a specific population of cells, usually the cells are sorted out from the rest by tagging a specific surface molecule that is expressed only in these cells. Unfortunately hepatocytes do not exhibit this kind of molecule and thus should be genetically manipulated in order to sort the cell out from heterogeneous population. The cells can be labeled by a fluorescent reporter gene under hepatic-specific promoter and sorted out using fluorescent activated cell sorter or manipulated to express a selection marker under hepatic specific promoter and thus under selection pressure only the hepatic-like cells will survive. Extracting the RNA from the homogenous population of hepatic-like cells and analyzing their gene expression profile by microarray analysis would provide valuable information about the nature of these cells. In order to prove in vitro the functionality of the hepatic-like cells, functional assays for metabolic enzymes and detoxifying enzymes are also necessary. But the ultimate proof that the hepatic-like cells are functional hepatocytes should be in vivo. Improved survival of animals serving as models for hepatic diseases, after transplantation with hepatic-like cells, is necessary in order to finally confirm their hepatocytic nature.

PROMISE

The ability of ES cells to differentiate into hepatocytes combined with their unlimited proliferating potential are of major importance from several reasons. ES cells can serve as a tool to study the early developmental processes during the embryonic development of the liver. Understanding of these developmental processes may help in diagnosis and treatment of liver associated congenital pathologies. Hepatic-like cells derived from ES cells may be also used for toxicity tests of new chemical compounds instead of the rat hepatocytes being used, currently. They are more suitable for mimicking the human reactions to the toxic compounds and save the need of using many

animals as a source of cells. Hepatocytes derived from human ES cells may also serve as a source of cells for transplantation medicine. Transplantation of hepatocytes has proved to be efficient, but the sources for human hepatocytes are limited and ES cells may be an excellent alternative source.

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