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REVIEW

Differentiation of human embryonic stem cells along a hepatocyte lineage and its application in liver regeneration

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Hepatocyte transplantation and bioartificial liver (BAL) as alternatives to liver transplantation offer the possibility of effective treatment for many inherited and acquired hepatic disorders. Unfortunately, the limited availability of donated livers and the variability of their derived hepatocytes make it difficult to obtain enough viable human hepatocytes for the hepatocyte-based therapies. Embryonic stem cells (ESCs), which could be isolated directly from the blastocyst inner cell mass, have permanent self-renewal capability and developmental pluripotency and therefore might be an ideal cell source in the treatment of hepatic discords. However, differentiation of hESCs into hepatocytes with significant numbers remains a challenge. This review updates our current understanding of differentiation of ESCs into hepatic lineage cells, their future therapeutic uses and problems in liver regeneration.

embryonic stem cell, hepatocyte, differentiation, liver regeneration

CELL BIOLOGY

Liver is the largest organ in mammals and it exerts a variety of important functions, including metabolizing diverse dietary molecules, storing glycogen, detoxifying compounds and swallowing for defense. This organ also has hematopoietic function in embryonic/fetal stage. Since liver has so many pivotal roles, patients with acute hepatic failure or end-stage liver diseases are in great danger. Orthotopic liver transplantation remains the most successful treatment for many cases of end-stage liver disease. However, the efficacy of liver transplantation is limited by the shortage of available donor organs, risk of rejection, infections, and other complications caused by the lifelong immunosupression. Liver cell therapies, including hepatocyte transplantation and bioartificial liver (BAL), are considered promising new approaches to treatment of patients with end-stage liver disease. However, the usage of mature hepatocytes is also hampered by limited tissue source and inability to proliferate and maintain the function for a long term *in vitro*. Embryonic stem cells (ESCs) might serve as an ideal source for cell therapy because of their immortalization and unique ability to give rise to all somatic cell lineages. ESCs were first isolated from mouse embryos in 1981[1], and the establishment of the first human ESC line in 1998[2] undoubtedly further expanded the potential of ESCs not only as an important tool for basic research but also as a promising source for cell therapy.

1 Predominance and underlying problem of ESCs

ESCs, derived from the inner cell mass of preimplantation blastocyst stage embryos, are primitive and highly undifferentiated cells. ESCs have several advantages over adult stem cells: (1) High self-renewal ability. ESCs can be propagated indefinitely in prolonged culture under special conditions as undifferentiated cells. (2) Devel-

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opmental potential to form derivatives of all three embryonic germ layers. Actually, ESCs can produce teratomas composed of derivatives of all three embryonic germ layers after being injected into severely combined immunodeficient (SCID) mice. (3) Genetic manipulation. After genetic manipulation, ESCs can be induced into desired types, and immunologic rejection can be removed through therapeutic cloning. These characteristics of ESCs offer a new hope for therapies against diseases due to cell loss, such as liver disorders, blood diseases, diabetes, Parkinson' s disease and miocardial infarction.

Although ESCs can be differentiated into all kinds of cell types, the process is "un-located". At present, we cannot make ESCs differentiate into desired cells in specific sites, which may result in teratomas theoretically. Therefore, it is urgent to carry out comprehensive investigations to induce ESCs into functional mature cells prior to the clinical applications.

2 Liver development and differentiation

2.1 Molecular mechanisms of liver development

Molecular events that occur during normal liver development *in vivo* are likely to provide clues for understanding the differentiation of pluripotent stem cells, such as ESCs, into hepatic cells *in vitro*. Liver develops from the ventral endoderm of the foregut at the 14—20 somite stage[3]. A hepatic bud is formed first, and then expands and proliferates into the septum transversum to form the embryonic liver. It has been shown that the ventral endoderm must express Gata-4 and Foxa-2 (previously known as Hnf-3β) and undergo hepatic differentiation[4]. An outgrowing bud of proliferating endodermal cells present in the ventral floor of the foregut requires signaling from fibroblast growth factors (FGFs) that are secreted from the adjacent cardiac mesodermal cells. Then, the endodermal cells start to proliferate and bud into the stromal environment of the septum transversum mesenchyme derived from the lateral plate mesoderm, from which bone morphogenetic proteins (BMPs) are secreted to stimulate hepatogenesis[5] (Figure 1[6]). In the absence of these signals, the endoderm assumes a pancreatic fate. In the process of proliferation and outgrowth of the liver cells, other signals, including hepatocyte growth factor (HGF) derived from mesenchymal cells, Oncostatin M (OSM) secreted from hematopoietic cells, glucocorticoid, Endothelial cells, cMET, c-jun, SEK1 and gp130, are also critical. Several transcription factors, such as HNF1a, β; the FoxA family; HNF4a, β; HNF6 and the c/EBP family, are implicated in the differentiation of hepatoblast to hepatocyte[5,7]. In addition, the composition of extracellular matrix and cell-cell interactions play important roles during the liver development[8].

2.2 Markers in hepatic differentiation

Hepatic cells are short of specific markers, only having some high expressed genes during liver development. At present, different liver-relative genes and proteins have been identified, which laid a foundation for defining differentiated hepatic cells.

(i) Embryonic markers. The earliest genes expressed in murine liver development are alpha-fetoprotein (AFP) and albumin (ALB), which precede changes of morphology. Another relative-specific marker[9] is Ankyrin Repeat Domain 17 (Ankrd17).

AFP AFP is a globulin, which was first detected in serum of patient suffering from liver cancer by Bergstyandh and Czar in 1956; it is also a marker of embryonic

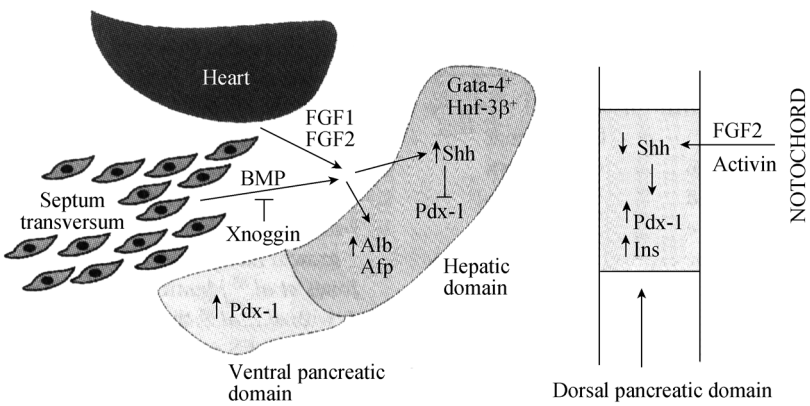

Figure 1 Signals involved in the specification of the liver and pancreas from the ventral endoderm.

liver and differentiation of definitive endoderm[10,11]. This protein is highly expanded in the embryonic liver and is down-regulated shortly after birth[12]. However, AFP is also expressed in the yolk sac[9,10]. The function of this 70-kD protein is not yet fully understood, but it has been thought to have a role in growth control, steroid binding, immune modulation, and osmotic pressure maintenance.

ALB ALB is a soluble monomeric protein, which makes up about half of all serum protein in an adult. ALB functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones, and plays a role in stabilizing extracellular fluid volumes by contributing to the colloid osmotic pressure of the blood[13]. Shortly after the presumptive hepatic endoderm starts to produce AFP, ALB mRNA is synthesized, which occurs in the 7—8 somite stage in the mouse (embryonic day 8.5)[14]. Although ALB has a more restricted pattern of expression than AFP, it is expressed in yolk sac as well as liver[15,16].

Ankyrin Repeat Domain 17 Ankrd 17 is first expressed in embryonic day 8.5 mouse embryos in the cells of ventral endoderm that have been specified to a hepatic fate. It therefore provides the earliest specific marker of a hepatic phenotype. However, its expression is down-regulated in hepatocytes as development proceeds, and it is not expressed in most adult hepatocytes, which limits its use as a hepatic marker.

(ii) Mature hepatocyte markers. As hepatocytes get mature, they begin to express several other proteins, such as a-1 antitrypsin (Aat), glutamine (Gs), and transthyretin (Ttr). Although these proteins are enriched in liver, none of them exhibit liver-specific expression. However, the combined expression of these genes in a single cell can be useful in defining the hepatic phenotype. The gluconeogenic enzyme glucose-6-phosphatase (G6P) is expressed in embryonic liver and kidney[17]. Tyrosine aminotransferase (Tat) is expressed in hepatocytes but not until the neonatal period[18]. These two enzymes are often used as markers of hepatic maturation as they are not expressed early in hepatic development.

3 Hepatic differentiation of ESCs

3.1 Hepatic differentiation of mESCs

During the past 30 years, not only the pluripotency of mESCs was testified *in vitro* and *in vivo*, but also huge progress in differentiation studies has been achieved.

(i) Spontaneous differentiation. Using ESCs carrying a gene trap vector insertion (I.114) into an ankyrin repeat-containing gene (Gtar) Jones et al.[19] obtained an exclusive beta-galactosidase marker for the early differentiation of hepatocytes *in vivo*. Beta-Galactosidase-positive cells were differentiated from I.114 ESCs *in vitro*. Yamada et al.[20] examined the cellular uptake of ICG to identify differentiated hepatocyte within developing embryoid bodies (EBs). ICG-stained cells appeared around 14 d of EBs culture and posess characteristics of hepatocytes identified by electron microscope, immunocytochemistry and RT-PCR. Because ICG staining was detected almost exclusively near areas of beating cells, cardiomyocytes in the differentiating cultures may contribute to hepatocyte development. Miyashita et al.[21] observed ESCs differentiation through formation of EBs and attached them on gelatin. Quantitative PCR analysis revealed that hepatic gene expression related to early and late-stage (G6P) liver development were enhanced through *in vitro* differentiation of ESCs. The presence of albumin-producing cells in the peripheral region of attached EBs was confirmed by immunocytochemical analysis. All this showed that the formation of EBs can re-create the inductive microenvironment required for liver organogenesis and initiate differention along a hepatocyte lineage. Considering that the efficacy of ESCs spontaneous differentiation was low and their derivatives were miscellaneous, reseachers focused on the induction and separation of ESCs derived hepatocyte-like cells.

(ii) Induced differentiation. Hamazaki et al.[22] investigated the potential of mESCs to differentiate into hepatocytes *in vitro*, and indicated that the *in vitro* ES differentiation system had a potential to generate mature hepatocytes. Differentiating ESCs expressed endodermal-specific genes, such as *afp*, *alb*, and *ttr* when cultured without additional growth factors and late differential markers of hepatic development, such as TAT and G6P, when cultured in the presence of growth factors (aFGF, HGF, OSM) critical for late embryonic liver development. However, Chinzei et al.[23] had a different result that ESCs can be differentiated into hepatocytes when cultured without additional growth factors both *in vitro* and *in vivo*. The difference might be relative with concentration, timing, ES cell lines and maturation of EB. Kuai et al.[24] examined the differentiating potential of mouse ESCs into hepatocytes in the presence of retinoic acid (RA), HGF and beta-nerve growth factor

(beta-NGF). The results showed that, in the presence of HGF and beta-NGF, mESCs could differentiate into functioning hepatocytes. Hu et al.[25] observed that the cell growth factors such as transform growth factor (TGF), bFGF and HGF could induce the differentiation of EBs into hepatocytes. The result suggested that the mRNA of AFP, ALB, G6P and TAT expression could be detected at days 3, 9, 11 and 13. Hepatic proteins such as AFP, ALB, CK8, CK18 expression in cytoplasm aslo could be detected at days 7, 9, 9 and 11. At day12, the concentration of Urea in culture medium was 8.3 μmol/L. In an effort to promote ESCs commitment to the hepatocyte lineage, Novik et al.[26] evaluated the effects of four culture conditions on ALB and gene expression in differentiating ESCs. Quantitative *in situ* immunofluorescence and cDNA microarray analyses suggested that spontaneous and collagen-mediated differentiation induced cells with the highest levels of ALB expression, while mature liver specific genes were only expressed in the spontaneous condition, which indicated that two distinct mechanisms may govern spontaneous and collagen-mediated differentiation. Teratani et al.[27] identified growth factors that allowed direct hepatic fate-specification from ESCs by using simple adherent monolayer culture conditions. ES cell-derived hepatocytes showed liver-specific characteristics, including several metabolic activities, suggesting that ESCs can differentiate into functional hepatocytes without the requirement for EB formation. Most importantly, transplantation of ES cell-derived hepatocytes in mice with cirrhosis showed significant therapeutic effects, and no teratoma formation was observed in the transplant recipients.

In addition to these classic induction methods, more and more ways to induce ESCs into hepatocytes are being tried.

Zhou et al.[28] differentiated mouse ESCs into hepatic progenitor cells after the withdrawal of sodium butyrate, which was characterized by scant cytoplasm, ovoid nuclei, the ability of rapid proliferation, expression of a series of hepatic progenitor cell markers, and the potential of differentiation into hepatocytes and bile duct-like cells.

Because cell-cell interactions between embryonic cardiac mesoderm and definitive endoderm are essential for liver development *in vivo*, Fair et al.[29] co-cultured murine ESCs with chick embryonic cardiac mesoderm and found that FGFs derived from cardiac mesoderm were very importance for development of fetal liver. Soto-Gutierrez A et al.[30] induced mESCs differentiate into hepatocytes by co-culture of human liver nonparenchymal cell lines with combination of FGF2, human activin A and HGF. ESC-derived hepatocytes expressed liver-specific genes, secreted ALB and metabolized ammonia, lidocaine and diazepam. Treatment of 90% hepatectomized mice with a subcutaneously implanted BAL seeded with ESC-derived hepatocytes or primary hepatocytes improved liver function and prolonged survival.

Ogawa et al.[31] succeeded in establishing EBs prepared from the ESCs that differentiated into not only hepatocytes but also into at least two mesodermal lineages: cardiomyocytes that supported liver development and endothelial cells corresponding to sinusoids. This allowed the development of an *in vitro* system using mESCs that approximated the events of liver development *in vivo*. Gouon-Evans et al.[32] found when differentiated in the presence of activin A in serum-free conditions, mESCs efficiently generated an endoderm progenitor population defined by the co-expression of either Brachyury, Foxa2 and c-Kit, or c-Kit and Cxcr4. Specification of these progenitors with BMP-4 in combination with bFGF and activin A resulted in the development of hepatic populations highly enriched (45%—70%) cells that expressed the AFP and ALB proteins. These cells also expressed transcripts of Afp, Alb, Tat, Cps1, Cyp7a1 and Cyp3a11; they secreted albumin, stored glycogen, showed ultrastructural characteristics of mature hepatocytes.

Imamura et al.[33] reported that EBs derived from ESCs could be induced into hepatocyte-like cells by a collagen scaffold three-dimensional culture system and could be stimulated with exogenous growth factors and hormones *in vitro* and *in vivo*. Maguire et al.[34] developed an mESCs alginate poly-l-lysine microencapsulation hepatocyte differentiation system. Their results indicated that the alginate microenvironment was conducive to differentiation of ESCs and maintenance of differentiated cellular function.

(iii) Genetic Marker of Hepatocytes. Recently, with the development of genetic manipulation *in vitro*, the study on hepatic differentiation has made much headway. Kanda et al.[35] investigated the ability of a genetically altered ESC line to promote endodermal differentiation toward hepatocytes *in vitro* by transfecting the HNF-3β

gene. By adding FGF2 after mESCs transfection, expression of *afp*, *alb* and *ttr* were detected in all of transfected mESCs. To obtain a uniform population of differentiated cells, Yin et al.[36] found that liver progenitor cells could be purified from differentiated ESCs using AFP as a marker. Hamazaki et al.[37] purified liver progenitor cells with a similar method. By adding FGF1, HGF and OSM, expression of AFP, ALB, CK19 and CYP1A1 were detected in green fluorescent protein (GFP)-positive cells, and proteins expression levels of AFP and ALB were high. To drive expression of the enhanced green fluorescent protein (EGFP) transgene in hepatic cells, Yamamoto et al.[38] constructed pALB-EGFP plasmid and established stable transfectant. After transplantation into the carbon tetrachloride-injured mouse liver, ES-derived GFP-positive cells were incorporated into liver tissue and rescued mice from hepatic injury and no teratoma formation was observed in the transplant recipients in three months. To address whether hepatocytes can be differentiated into developing EBs *in vitro*, Asahina et al.[39] identified Cyp7a1 encoding CYP7A1 (cholesterol 7α-hydroxylase) as a gene expressed in liver but not in the yolk sac, and found that its expression could be induced in developing EBs *in vitro*. Furthermore, they generated an ES cell line expressing GFP under the control of the Cyp7a1 enhancer/promoter, and visualized GFP-positive epithelial-like cells in the developing EBs. These results demonstrated that ESCs can be differentiated into hepatocytes of definitive endoderm *in vitro*. Using mESCs transfected with the GFP reporter gene regulated by ALB enhancer/promoter, Heo et al.[40] found that highly enriched populations of early-stage hepatocyte precursors could be isolated from EB outgrowths by fluorescence-activated cell sorting (FACS) using GFP expression driven by a lineage-specific (ALB) promoter. Furthermore, they demonstrated that in a mouse model of liver injury, ES-derived GFP-positive cells gave rise to functional hepatocytes and bile duct-like cells that integrated into and replace diseased parenchyma without forming teratomas following transplantation.

3.2 Hepatic differentiation of rat embryonic stem cells (rESCs)

At present, the isolation, establishment and application of ESCs derived from animals, except mouse, were still not well establised. Ruhnke et al.[41] first reported the establishment of rat embryonic stem cell-like cell line and identified their differentiating ability. rESCs were directly grown on martrigel and treated with FGF-4. The result of RT-PCR showed that hepatocyte-specific genes (*afp*, *ttr*) were strongly upregulated. However, it should be mentioned that some late markers of hepatocyte differentiation (CK18, albumin, HNF-1α, and CYP2B1) were expressed but not upregulated.

3.3 Hepatic differentiation of hESCs

Actually, the research on mESCs made a contribution to the hESCs isolated several years ago. Rambhatla et al.[42] showed that hESCs differentiated into hepatocyte-like cells after treating cultures with sodium butyrate. This not only induced hepatic differentiation but also resulted in significant cell death. The remaining cells displayed some morphologic characteristics of hepatocytes and expressed hepatocyte-specific proteins (ALB, AAT, cytokeratin 8 and 18). Furthermore, the induced cells executed a range of hepatocyte functions such as glycogen deposits and inducible cytochrome P450 activity. However, these cells did not express AFP. Levenberg et al.[43] utilized biodegradable polymer scaffolds to promote hESCs growth and differentiation. Addition of activin A and insulin-like growth factor1 (IGF) induced the formation of structures with biochemical features found in developing liver. Shirahashi et al.[44] investigated culture conditions for differentiating h/mESCs into hepatocyte-like cells *in vitro*. Various combinations of culture media, growth and differentiation factors, and substratum were evaluated, and it was found that a combination of Iscove's modified Dulbecco's medium with 20% fetal bovine serm, human insulin, dexamethasone, and collagen type I precoating was optimal for directing ESCs along a hepatocyte lineage. Schwartz et al.[45] examined specific cytokines and extracellular matrix (ECM) proteins that supported differentiation of hESCs to hepatocytes. Tests of several different conditions showed that addition of FGF-4 and HGF in completely serum-free cultures of hESC-derived EBs subsequently allowed to attach to type I collagen-coated dishes led to maximal differentiation into cells. Lavon et al.[46] had characterized the expression level of liver-enriched genes in hESCs upon differentiation into EBs by cDNA microarrays. In order to isolate the hepatic-like cells, they introduced a reporter gene regulated by a hepatocyte-specific promoter into hESCs and detected GFP-positive clusters of hepatic-like cells in 20-d-old EBs. Result of immunostaining showed that most of

these cells expressed ALB, while some cells still expressed the earlier expressed protein AFP.

4 Promise and problems for ESCs in liver regeneration

An overall understanding of the differentiation of ESCs into hepatocytes is important not only for elucidation of molecular events that govern normal liver development *in vivo*, but also for clinical application of ESC-based therapies against liver failure due to hepatic cirrhosis and hepatonecrosis. All of these are of great importance to China, a country with a high morbidity in liver disorders.

In spite of pivotal progress in animal studies, many issues are still to be addressed before the ESC-derived hepatocytes can be used in clinical transplantations. (1) At present, the feeder layers supporting hESC growth are almost derived from mouse, which would inevitably cause contamination of xenogeneic proteins. The best measure to avoid this is establishing human-derived feeder layer or feeder-free/serum-free culture system, and the related investigation has been initiated in our lab now. (2) How to get purified functional hepatocytes. It is difficult to separate hESCs derived hepatocyte-like cells from other differentiated cell types for short of reliable liver-specific markers. In our laboratory, we have made great efforts to induce various kinds of stem cells into hepatocytes[47−52]. Currently, cytokines are combined with ECM to induce the differentiation of hESCs into hepatocytes through EBs. The hepatocyte-specific ultra-structures are confirmed by a transmission electron microscope (TEM), including plentiful round or elliptical mitochondria, endoplasmic reticulum, glycogenic granules, and microvillus on the surface of the cells. Moreover, these cells also express hepatocyte-specific genes (AFP, ALB, CK18, CK19, CYP1B1, etc.), and demonstrate multiple functions, i.e. ICG uptake/excretion, glycogen deposition, albumin production and ammonium metabolism. Meanwhile, hESCs are labeled with GFP under the control of an ALB promoter, ensuring the direct visualization and sorting of GFP-positive hepatocyte-like cells during the course of differentiation. (3) How to generate significant numbers of the desired cell type? Given that a large number of cells, such as 10^7—10^{10} cells in BAL, are needed in transplantation, higher efficiency of hepatic differentiation is critical. By developing three-dimensional culture system via bioreactors, our lab is trying to generate enough cells for cell therapies. (4) How to refrain from immunological rejection after cell transplantation. The study on therapeutic cloning and banking on hESCs must be performed at a rapid pace[53]. (5) It remains to be determined whether the transplanted cells are home to the liver——and if not, what is the effect of engraftment in nonhepatic tissues. Answers to these questions will facilitate clinical application of ESC-based therapies.

Taken together, the study on differentiation of ESCs is still preliminary and many key questions are unsolved. Thus there is still a long way to go before the clinical application of ESC-based therapies. It is our hope that this goal will be accomplished soon.

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