Journal of
Stem Cell Research & Therapy

Han, J Stem Cell Res Ther 2012, S10
DOI: 10.4172/2157-7633.S10-008

Review Article Open Access

Generation of Functional Hepatic Cells from Pluripotent Stem Cells

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Abstract

Liver diseases affect millions of people worldwide, especially in developing countries. According to the American Liver Foundation, nearly 1 in every 10 Americans suffers from some form of liver disease. Even though, the liver has great ability to self-repair, in end-stage liver diseases including fibrosis, cirrhosis, and liver cancer induced by viral hepatitis and drugs, the liver regenerative capacity is exhausted. The only successful treatment for chronic liver failure is the whole liver transplantation. More recently, some clinical trials using hepatocyte transplantation have shown some clinical improvement for metabolic liver diseases and acute liver failure. However, the shortage of donor livers remains a life-threatening challenge in liver disease patients. To overcome the scarcity of donor livers, hepatocytes generated from embryonic stem cell or induced pluripotent stem cell differentiation cultures could provide an unlimited supply of such cells for transplantation. This review provides an updated summary of hepatic differentiation protocols published so far, with a characterization of the hepatic cells generated *in vitro* and their ability to regenerate damaged livers *in vivo* following transplantation in pre-clinical liver deficient mouse models.

Keywords: Hepatic cells; Pluripotent stem cells; Embryonic stem cells

Introduction

The liver is a remarkable regenerative organ that can restore normal mass and function after an injury or partial hepatectomy. After mild injury, this ability is supported by the proliferation of fully mature hepatocytes, biliary epithelial cells [1] as well as the proliferation and help of surrounding cells including stellate cells, endothelial sinusoidal cells [2] and macrophages [3]. After severe injury or chronic disorder, hepatic progenitors or "oval cells-ductular hepatocytes" are recruited to ensure regeneration of the hepatocytic and biliary cell mass [1]. However, in end-stage liver diseases, such as fibrosis, cirrhosis, or liver cancer induced by viral hepatitis or drugs, the regenerative capacity of the liver is exhausted. Consequently, liver diseases are becoming one of the most common causes of mortality all over the world, especially in developing countries. Until now, liver transplantation is the most successful treatment for patient with chronic liver failure [4,5]. Alternatively to the whole organ transplantation, hepatocyte transplantation has been recently used in clinical trials for the treatment of acute failure and life-threatening metabolic liver diseases [6-9]. Unfortunately, these two options face the major concern of shortage of human donor livers [10].

To overcome the scarcity of donor livers, hepatocytes generated from pluripotent stem cell (PSC) differentiation cultures could provide an unlimited supply of such cells for transplantation in liver diseases. Embryonic Stem Cells (ESC) established from the inner cell mass of the blastocyst from early embryos or induced pluripotent stem cells (iPSC) from somatic adult cells are pluripotent, and proliferate indefinitely in an undifferentiated state *in vitro* [11,12]. Since the first establishment of mouse iPSC from embryonic fibroblasts by Takahashi and Yamanaka using viral vectors of Oct3/4, Sox2, Klf4 and c-Myc transcription factors, this technology has been widely and successfully applied to different species including human, established from different terminally differentiated adult cell types using various gene delivery systems (Recently discussed by Yamanaka, [13]).

Numerous studies summarized in this manuscript have shown that functional hepatocyte-like cells could be derived from ESCs (ESC-Hep) or induced pluripotent stem cells (iPSC-Hep), which express transcription factors and markers for mature hepatocytes as well as possess the metabolism and secretion functions both *in vitro* and *in vivo* (Table 1 and Table 2). Therefore, with their pluripotency and self-renewal abilities, ESCs and iPSCs have been proposed as a very valuable and unlimited transplantable hepatic cell source for patients with end-stage liver diseases [14]. The patient-specific iPSC-Hep have the advantage over the ESC-Hep to be immunologically compatible with the host for cell therapy approaches, and also to provide *in vitro* liver disease models [15]. As a proof of principle that iPSC can be a valuable source of functional hepatic cells, Espejel et al. [16] have injected iPSC into the blastocysts of the liver deficient Fah-/- mice, and found that by 70 days after birth, 100% of the hepatocytes in adult livers derived from the iPSC. These liver-chimeric mice display very healthy liver functions, demonstrating the definite ability of iPSC to generate functional hepatocytes *in vivo* avoiding the limitations of current *in vitro* differentiation protocols. Similar experiments were performed by Duncan's group by producing embryos by tetraploid complementation from mouse iPSC [17]. All embryos including their livers were derived from the iPSC except the extra embryonic tissues that were derived from the donor tetraploid embryos. The authors demonstrated that fetal mouse livers derived from iPSCs were indistinguishable from wild-type livers based on histological and gene expression assays, thereby supporting the ability of iPSCs to generate functional hepatocytes as ESCs do.

This review summarizes the results of various protocols using pluripotent stem cells published so far, and compares their efficiency in generating functional hepatic cells and their therapeutic relevance

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Received July 06, 2012; **Accepted** August 13, 2012; **Published** August 15, 2012

Citation: Han S, Bourdon A, Hamou W, Dziedzic N, Goldman O, et al. (2012) Generation of Functional Hepatic Cells from Pluripotent Stem Cells. J Stem Cell Res Ther S10:008. doi:10.4172/2157-7633.S10-008

References	Species	Protocol specificity	EB monolayer	Endoderm induction		Hepatic specification and maturation		In vitro functional assays	In vivo assay
				Inducers	Efficiency & marker	Inducers	Efficiency & marker		
Cytokines only									
Gouon-Evans et al. [50]	mESC		EB	Act	55% Foxa2/ckit/ Cxcr4	BMP4, bFGF, HGF, Dex, TGFa, EGF, VEGF	70% Afp, 60% Alb	Alb, Glycogen	yes
Cai et al. [25]	hESC		monolayer	Act, ITS	80% Foxa2/Sox17	FGF4 BMP2, HGF, OSM, Dex	70% Alb	Alb, Glycogen, ICG, LDL, p450	yes
Hay et al. [26]	hESC		monolayer	Act, Wnt3a		Serum, Insulin, HGF, OSM	up to 90% alb	Urea, Gluconeogenesis, Afp	yes
Agarwal et al. [40]	hESC		monolayer	Act, low serum	72% Cxcr4	FGF4, HGF	70%		yes
Basma et al. [115]	hESC		EB monolayer	Act, bFGF		FGF, DMSO, Dex	26% Asgr-1	Alb, Urea, AAT, p450	yes
Si-Tayeb et al. [17]	hiPSC		monolayer	Act	80%	BMP4, FGF2, HGF	80%	Glycogen, LDL, Oil red O storage, ICG, Urea	yes
Touboul et al. [27]	hESC		monolayer	Act, FGF2, BMP4, LY294002	80% CXCR4	FGF10, RA, SB431542, FGF4, HGF, EGF	50-60% Hnf4α, Afp or Ck19, 35% Asgr-1	Glycogen, p450, ICG, LDL	yes
Ghodsizadeh et al. [127]	hiPSC		EB	Act		DMSO, HGF, Dex		p450, Alb, Afp, Urea, LDL. ICG, Glycogen, Oil red O storage	
Mfopou et al. [35]	hESC		monolayer	Act, Wnt3a, low serum	60-80% Cxcr4/ Foxa2	FGF10, BMP, Cyclo, DAPT, Exendin-4, IGF1, HGF	40-60% Afp	Urea, Glycogen	
Sullivan et al. [34]	hiPSC		monolayer	Act, Wnt3a		β-ME, DMSO, Insuli, HGF, OSM	70-90% Alb	p450,Fibrinogen, Fibronectin, Transthyretin, Afp secretion	
Rashid et al. [31]	hiPSC		monolayer	Act, FGF2, BMP4, LY294002		Act, HGF, OSM	83% Alb	Alb, p450, Glycogen, LDL	
Liu et al. [41]	hiPSC		monolayer	Act, low serum	90% Cxcr4	FGF4, HGF, OSM, Dex	90% Afp	Alb, p450	yes
Li et al. [119]	miPSC		monolayer	Act, ITS		FGF4, HGF, OSM, Dex	~ 90% Afp, ~ 90% Alb	LDL, Glycogen, p450	yes
Woo et al. [39]	hESC/ hiPSC		EB	LiCL, GSK-3 inhibitor (BIO), Wnt3a	35% Foxa2/Sox17	HGF, OSM, Dex	~69% Alb/Ck18	Urea, Alb, Glycogen	yes
Pauwelyn et al. [37]	mESC		monolayer	Act, Wnt3a, low serum		FGF2, BMP4,FGF8b, FGF1, FGF4, Follistatin		Alb, Urea, Glycogen, p450	
Sancho-Bru et al. [36]	miPSC		monolayer	Act, Wnt3a, low serum	70% Foxa2	BMP4,FGF2, FGF1, FGF4, FGF8, HGF, Follistatin	30% Hnf4α	Alb, Glycogen, Urea, p450	
He et al. [113]	mESC		EB monolayer	Act	75% Cxcr4/ckit (monolayer)	BMP4, bFGF, EGF, TGFα, VEGF, HGF, Dex	31% Alb (monolayer)	Alb, Ammonia, Glycogen, LDL, ICG	yes
Chen et al. [38]	hiPSC		monolayer	Act, Wnt3a, HGF	61-64%	OSM, Dex, ITS		p450, Urea, LDL, Glycogen	yes
Genetic modification									
Kubo et al. [71]	mESC	Hex expression	EB	Act		Hex, BMP4		Alb, Transferrin	
Inamura et al. [69]	hESC / hiPSC	Hex expression	monolayer	Act, bFGF, Hex expression	46%	BMP4, FGF4, HGF, OSM, Dex		p450	
Takayama et al. [70]	hESC / hiPSC	Sox17 and Hex expression	monolayer	Act, Sox17 and Hex expression	67% Cxcr4/ckit, 57% Hex	BMP4, FGF4, HGF, OSM, Dex	50% p450	LDL, p450	

Takayama et al. [56]	hESC / hiPSC	Sox17 and Hex expression	monolayer	Act, Sox17 and Hex expression		BMP4, FGF4, HGF, OSM, Dex	80% p450, 80% Asgr1, c-met or Alb	LDL, p450, ICG, Glycogen, Metabolism-mediated toxicity	
Epigenetic modification									
Hay et al. [79]	hESC	NaBu	monolayer	Act, NaBu	70% Cxcr4	DMSO, HGF, OSM, 8.3% serum	71% Alb, 65% Hepar-1	Glycogen, generation and secretion of plasma proteins, p450	
Li et al. [78]	mESC	NaBu	monolayer	Act, NaBu	100% Cxcr4, 65% ckit, 63% Epcam	BMP4, FGF2, HGF, EGF, TGFα, Dex	51% Afp, 62% Alb	ICG, Glycogen	yes
Duan et al. [57]	hESC	NaBu	monolayer	Act, NaBu	85% Cxcr4, Sox17 and Foxa2	HGF, BMP2, FGF4, BMP4, DMSO, serum, OSM, Dex	90% Alb	ICG, Drug matabolism	
Ren et al. [74]	mESC	NaBu	EB	NaBu		HGF, Dex	50%	Glycogen	
Scaffold									
Fang et al. [100]	mESC	scaffold-alginate microbeads	EB	FGFα		HGF, OSM, Dex, Insulin, Transferrin, Selenium	49% Alb, 50% Ck18	Alb, Urea	
Li et al. [95]	mESC	scaffold-polyacrylamide substrate	monolayer	DMSO		NaBu, cells placed on polyacrylamide substrate	70% functional hepatocyte-like cells	Alb, Urea	
Mizumoto et al. [97]	mESC, monkey ESC	scaffold-hollow fibers	fibers			NaBu		Ammonia, Alb	
Matsumoto et al. [93]	mESC	3d scaffold-Polyurethane Foam	EB			αFGF, HGF, OSM, Dex, ITS		Glycogen, Ammonia, Alb	
Lee et al. [94]	mESC	Fibronectin / collagen1, collagen 4 / laminin	monolayer						
Farzaneh et al. [99]	hESC	scaffold-ultraweb nanofiber	monolayer	Act	86% Foxa2, 94% Sox17, 92% Cxcr4	FGF4, HGF,OSM, Dex	66% Afp	Afp, Alb, Urea, LDL, Glycogen, ICG, PROD activity	
Shiraki et al. [128]	miPSC/ hESC	scaffold	monolayer	Act, bFGF	40% Cxcr4 / E-cad (mouse)	RA, Act, bFGF, ITS-DMEM, Dex, HGF, DMSO, NA, AsP, Akt inhibitor treated cells	45% Alb		
Miki et al. [59]	hESC	scaffold-hydrophilic hollow fiber microfiltration membranes	monolayer	Act		FGF4, BMP2, HGF, OSM, Dex	30% Asgpr	Alb, Ammonia, Glycogen, p450	
Haque et al. [101]	mESC	E-cadherin substratum	monolayer	Act, bFGF	~ 55%	HGF, OSM, Dex	92% Alb	Glycogen	
Amimoto et al. [96]	mESC / miPSC	cellulose triacetate hollow fibers	fibers			NaBu, Dex, OSM, ITS		Ammonium, Alb	
Co-culture or conditional medium									
Cho et al. [84]	mESC	Co-culture with rat hepatocytes	monolayer					Urea	
Zhao et al. [82]	hESC, primate ESC	Co-culture with STO feeder cells	monolayer	Act		FGF4, BMP2, HGF, OSM, Dex	90% Afp	Alb, Glycogen, ICG, LDL, PROD activity	
Fukumitsu et al. [88]	mESC	Co-culture with murine fetal liver stromal cell line	monolayer	Trans retinoic acid		HGF, bFGF, Nicotinamide, l-ascorbic acid phosphate, ITS, Dex, OSM		Glycogen, Ammonia, Alb	
Huang et al. [83]	primate ESC	Co-culture with human ESC-derived fibroblast-like cells	monolayer					Haptoglobin, Urea, Alb, EROD, Glycogen, HBV infection	
Tuleuova et al. [89]	mESC, hESC	Culture with protein microarray matrices	monolayer						
Ishii et al. [87]	mESC, hESC	Co-culture with murine fetal liver stromal cells and mesenchymal cells	monolayer	Act		HGF		Glycogen, Ammonia, p450	

Yu et al. [81]	hESC	Co-culture with mitomycin treated 3T3-J2 feeder cells	EB	Act					
Nishiofuku et al. [90]	mESC, ratESC	Co-culture with rat hepatic stellate cells	EB						
Han et al. [86]	mESC	Co-culture with D4T endothelial cells	EB monolayer	Act	~ 50% Foxa2/ Foxa3	EGF, TGFα, Dex, VEGF, bFGF, HGF, BMP4	60% Afp, Alb		
Pal et al. [80]	hESC	Conditioned medium from HepG2 cells	EB	bFGF				Glycogen, Afp, SGOT, SGPT, GGT	

Table 1: Summary of PSC-Hep differentiation protocols in serum free media.

PSC: pluripotent stem cell; Act: Activin A; NaBu: sodium butyrate; Afp: afp secretion; Alb: albumin secretion; LDL: low-density lipoprotein uptake; ICG: indocyanine green uptake; p450: cytochrome p450 activity; ammonia: ammonia metabolism; Urea: urea secretion and production; glycogen: glycogen storage (PAS staining); ASGPR: asiaglycoprotein receptor 1; ITS: Insulin, transferin, selenium.

in pre-clinical animal models of liver disease. The use of PSC-Hep for drug screening and liver disease models will not be discussed here.

Hepatic Cell Generation from Pluripotent Stem Cells (PSC-Hep): The Embryonic Stem Cell-derived Hepatic Cells (ESC-Hep) and the Induced Pluripotent Stem Cell-derived Hepatic Cells (iPSC-Hep)

In the last decade, numerous protocols have been established to generate ESC-Hep and iPSC-Hep. The most efficient and reproducible hepatic differentiation approaches are those that recapitulate in ESC and iPSC cultures the appropriate signalling pathways uncovered in embryo studies, thereby subsequently mimicking endoderm development, endoderm hepatic specification followed by hepatic cell maturation.

Cytokine-based hepatic differentiation protocols

The use of a specific cocktail of cytokines in a serum-free media became a pre-requisite to control in a timed manner the successive steps of liver organogenesis in differentiation cultures. Studies in *Xenopus* have demonstrated that different concentrations of the TGF β (Transforming growth factor beta) family member, activin-A used as a surrogate for nodal, induce mesodermal and endodermal fates in animal cap cells in culture [18-23]. Consequently, since its first use in the mouse ESC cultures [24], high doses of Activin-A are now widely utilized for endoderm induction in ESC and iPSC cultures from human and mouse lines [25-31]. Associated with Activin-A, activation of other pathways has also been shown to promote endoderm development including FGF (Fibroblast growth factor) and Wnt signalings [26,27,29,32-39]. In some protocols, low doses of serum have been reported to be necessary for Activin-A to induce an efficient endoderm program [30,40,41]. The presence of PI3kinase inhibitor in the serum was suspected to be responsible for promoting Activin-A-induced endoderm development [42].

Originally, most of the ESC differentiations were performed using embryoid bodies (EBs), the three-dimensional structure that was meant to mimic the blastocyst and epiblast architecture [43,44]. Few years ago, D'Amour et al. [30] demonstrated efficient endoderm induction from monolayers of hESC that was subsequently reproduced by many groups. The monolayer induction is thought to better synchronize the endodermal cell fate by exposing the cells evenly to the endodermal inducer, Activin-A.

Regardless of the induction system used (EBs or monolayers), heterogeneity of endoderm cultures remains inherent to pluripotent stem cell differentiation cultures. To overcome this issue, Activin-A-induced endoderm generated from pluripotent stem cell differentiation cultures have been enriched using the cell surface receptors CXCR4 [45], cKit [46], ENDM-1 [47], E-cadherin and PDGF receptor-α or through the selection of reporter molecules targeted to the Brachyury, Foxa2, Foxa3, goosecoid, sox17 and Hex loci [30,32,33,47-50]. Interestingly, endoderm induction appears to be always more effective in human cultures than in murine cultures, with the human endoderm fraction reaching ~90% of the total population versus ~60% at the most in murine cultures. The discrepancy of efficiency between both species may be due to the quicker endoderm induction in the mouse embryo compared to the human embryo, thereby restricting the time flexibility to modulate signaling to generate homogenous endoderm cell population in mPSC differentiation cultures. Overall, pluripotent stem cells have been successfully and reproducibly differentiated in serum-free media to endoderm with the help of Activin-A (Table 1).

To recapitulate the liver specification signaling based on developmental studies in the mouse and the *Xenopus* models [51-55], many groups including ours have used the combination of BMPs (BMP2, BMP4) and FGFs (FGF1, FGF2, FGF4, FGF8, FGF10) to specify endoderm cells generated in pluripotent stem cell cultures [17,25,27,28,35,36,40,50,56-59]. Shh inhibition found in *Xenopus* to be required for hepatic and pancreas fate decision, has been shown to improve the generation of mESC-Hep with the use of cyclopamine [35]. In the same study, inhibition of Notch by DAPT also helped mESC-Hep development supposedly by favoring hepatocyte fate over the cholangiocyte fate [35]. Hepatocyte growth factor (HGF) is known to promote hepatoblast proliferation, migration and survival through its tyrosine kinase receptor c-Met [60,61], and is therefore always used in PSC hepatic differentiation protocols. Following hepatic specification, combination of FGF10, retinoic acid and inhibition of Activin-A in the presence of SB431542 has been also reported to improve hepatic endoderm maturation [27]. Maturation of hepatoblasts in the mouse fetal liver requires Oncostatin M (OSM) secreted by hematopoietic cells [62]. Consequently, OSM has been also widely used in PSC hepatic cultures (Table 1).

Genetic modification-based hepatic differentiation protocols

The transcriptional machinery of the specified hepatic endoderm is very well documented including the homeodomain protein Mixer/Mix.3 [63], the Sry-related HMG-box transcription factor Sox17 [64], the zinc-finger transcription factors Gata5, Gata4 and Gata6 [65,66], Hepatocyte nuclear factors HNF4α [67] and Hex [68]. Some studies have used this information to improve efficiency of hPSC-hep

Reference	Animal model	Type and number of transplanted cells	Percentage of repopulation	Improved liver functions	Mouse survival after transplantation
Teratani et al. [118]	129X1/SvJ and BALB/c nude mice (DMN, cirrhosis)	mESC-Hep 5 e6/mouse (IV)	ND	suppression of onset of fibrosis/cirrhosis plasma fibrinogen Alb	~40% after 12 weeks
Heo et al. [116]	MUP-uPA/SCID + CCL4	mESC-Hep 1 e6/mouse (IS)	1.94% +/- 5.81 after 82 days	ND	ND
Gouon-Evans et al. [50]	Dpp4-/-, Rag2-/- (CCl4 and retrorsine) and Fah-/-	mESC-Hep 0.25 - 1.5 e6/mouse (IS)	scattered endothelial and hepatocitic clusters	ND	ND
Agarwal et al. [40]	NOD/SCID (CCl4 and retrorsine, acute liver failure)	hESC-Hep 1 e6/mouse (PV)	sporadic hepatocytic clusters	ND	ND
Hay et al. [26]	NOD/SCID	hESC-Hep 1 e6/mouse (IS)	clusters of CK18/CK19 and Alb cells after 3 days in the spleen	Alb	ND
Cai et al. [25]	SCID (CCl4 acute liver failure)	hESC-Hep 1 e6/mouse (IS)	scattered AAT human cells in livers	ND	ND
Duan et al. [121]	NOD/SCID	hESC-Hep 0.5 e6/site (IH)	ND	Alb	ND
Haridass et al. [114]	Alb uPA, Rag2-/-, IL2Rg-/-	ES-Hep 0.5-1 e6/mouse (IS)	few cells scattered and teratoma formation	ND	ND
Basma et al. [115]	NOD/SCID (retrorsine and 50% hepatectomy) Alb-uPA SCID	hES-Hep 0.1-0.2 e6/mouse (IS) ASGPR+ cells	few clusters of albumin+ cells after 28 days	Alb, AAT	ND
Li et al. [78]	Fah-/-	mES-Hep 1 e6/mouse (IS) ckit-Epcam+ day13	24%+-15%	ND	at least 10 weeks
Touboul et al. [27]	Alb uPA, Rag2-/-, IL2Rg-/-	hES-Hep 0.5 e6/mouse (IH)	small and large clusters throughout the liver 8 weeks after injection.	Alb, AAT	ND
Si-Tayeb et al. [17]	Fah-/-	hiPSC-Hep 0.3 e6/mouse (IH)	ND	ND	ND
Huang et al. [102]	Fah-/-, Rag2-/-	m iHep from fibroblasts 0.833 e6/mouse (IS)	5%-80%	ALT, AST, Tyrosine, Phenylalanine, Bilirubin	5/12 mice survived after 8 weeks
Sekiya and Susuki [103]	Fah-/-	m iHep from fibroblasts (IS)	large clusters	Bilirubin, ALT, ALP, Alb	40% after 10 weeks
Li et al. [119]	BALB/c nude (TAA, liver fibrosis)	m iPSC-Hep ~0.25 e6/mouse (IV)	scattered cells in the liver after 24 hours	ALT, AST, Bilirubin, Ammonia, decreased ROS levels and necrosis	~80% after 14 days
Chang et al. [117]	BALBc nude (CCl4, acute liver failure)	m iPSC-Hep 0.2-5 e6/mouse (IP)	Necrosis : 70% control, 20% transplant	ALT, AST, Bilirubin	90% after 14 days
Woo et al. [39]	BALBc nude (CCl4, acute liver failure)	hES-Hep / hiPSC-Hep 2 e6/mouse (IS) ICG+ cells	~20% at day 3 versus ~10% after 35 days	Alb	ND
Chen et al. [38]	NOD/SCID (CCl4, acute liver failure)	hiPS -Hep (IS)	ND	rescue of hepatic necrosis; glutamyl oxaloacetic aminotransferase; glutamyl pyruvic aminotransferase; Bilirubin; lactate dehydrogenase; HepPar1; Alb	5/7 (71%) after 21 days
Liu et al. [41]	NSG (DMN, cirrhosis)	iPSC-Hep 0.1-2 e6/mouse (IV)	2~17%	Alb, CYP2E1	90% after 8 weeks
He et al. [113]	Fah-/-	mESC-Hep (IS)	0.001%-12.5% after 8-10 weeks	ALT, AST, Bilirubin, Alb, succinylacetone, tyrosine and phenylalanine secretion, 30% of wt FAH activity in recipients	8/20 survived after 2nd transplantation
Bandi et al. [120]	CD17.NOD/SCID (Rif + Phen + MCT, acute liver failure)	hESC-hep 4-6 e6/mouse (IP with microcarriers)	Detection of transplanted cells in the peritoneal cavity	Endogeneous hepatocytes proliferation Phenobarbital metabolism Ammonium chloride	11/11 after 14 days (MCT 125, mild injury) 38% after 2 weeks (MCT 160, strong injury)

Table 2: Summary of in vivo functions of PSC-Hep in mice.

PSC: pluripotent stem cell, IV: intra venous injection; IS: intra splenic injection; IH: intra hepatic injection; IP: intra peritoneal injection; hESC-MEC: hESC meso-endodermal cells. Detection in serum of Alb (Albumin), AAT (alpha 1 anti-trypsin), ALT (alanine aminotransferase), AST (aspartate aminotransferase), bilirubin, AP (Alkaline phosphatase) and CYP2E1 activity.

generation by overexpression in a timely manner either Sox17, Hex and HNF4α [56,69,70]. Sox17 and Hex were transduced into the hESCs and hiPSCs differentiation cultures at day 3 and day 6 of differentiation respectively to induce hepatic commitment. Adenovirus vector-mediated overexpression of HNF4α in later cultures could further promote the hepatic maturation of the hPSC-Hep. Similarly, using a Hex inducible mESC line, Kubo et al. [71], have shown that forced expression of Hex in endoderm cells dramatically improved mESC-Hep hepatic specification and maturation synergistically with BMP-4.

Epigenetic modification-based hepatic differentiation protocols

In addition to genetic modulation, epigenetic modifications have been shown to improve hepatic differentiation protocols. For instance, sodium butyrate, a well-known specific HDACs inhibitor was used in ESCs and iPSC differentiation a long time ago [72]. In addition to decreasing cell death and promoting viability of ESCs, sodium butyrate has been reported to induce ESC differentiation toward different cell lineages including the neural cells [73], cardiac cells [29], pancreatic cells [73,74], and hepatic cells [74-79]. Ren et al. [74], showed specifically that the cell fate induced by sodium butyrate depends on the concentration and time of the treatment. For instance, a low concentration of sodium butyrate with shorter exposure time induce generation of pancreatic progenitors over the hepatic progenitors, while higher concentrations with longer exposure favor the hepatic lineage fate

Role of supportive cells and matrices in hepatic differentiation protocols

To recapitulate the inductive signals established in liver embryogenesis in PSC hepatic differentiation cultures, some studies have proposed to combine the use of cytokines or chemicals with co-cultures with supportive cells or cultures on specific matrices found in the developing liver. For instance, Pal et al. used conditioned media derived from the HepG2 liver carcinoma cell line during hESC differentiation, and obtained a high yield population of mature hESC-Hep that served as a functional *in vitro* hepatic cell model to study the effects of ethanol toxicity [80]. Fibroblast cells from different sources (STO feeder cells, 3T3 cells or ESC-derived fibroblast like cells) were utilized in several studies as supportive cells to improve hepatic differentiation of hESC as well as primate ESC and iPSC [81-83]. Huang et al. identified FGF2 and Activin A, as two factors secreted by the ESC-derived fibroblast important for endoderm induction. Additional source of hepatocytes harvested from rats in co-culture with mESC-derived cell cultures have been reported to improve mESC-Hep maturation [84]. Endothelial cells, mesenchymal cells, kupffer cells and stellate cells constitute the microenvironment of the developing hepatocytes, and were also reported to provide support for PSC-hep specification and maturation [85-90]. Our lab used an immortalized endothelial cell line derived from mESC cultures to improve mESC-Hep generation [86]. We demonstrated that endothelial cells are not only required for hepatic endoderm outgrowth as demonstrated previously by Zaret's group [91], but are also essential earlier to induce hepatic specification of endoderm through dual repression of Wnt and Notch signaling in endoderm cells [86]. This was one example of few studies showing that PSC differentiation cultures are not only a source of hepatic cells for future cell therapy, but also an *in vitro* culture system to understand liver organogenesis as much as embryology studies in animal models help improving PSC differentiation protocols.

Another alternative to produce better PSC-Hep *in vitro* is to use specific matrices or scaffold that would mimic the proper architecture of the *in vivo* microenvironment of the developing liver. Using an extracellular matrix microarray platform for the differentiation of mESC toward an early hepatic fate, Flaim et al. [92] have established combinations of extracellular matrices that synergistically impact both hepatic ESC differentiation and mESC-Hep hepatic functions. Specific studies have identified a wide variety of artificial materials and natural matrices to improve both human and mouse PSC-Hep generation such as collagen type I, vitrogen, matrigel, polyurethane foam [93], fibronectin, laminin [94], polyacrylamide [95], hollow fibers [59,96,97], poly-l-lactic acid plus polyglycolic acid [98], ultraweb nanofibers [99], alginate microbeads [100] and also recombinant E-cadherin substratum [101]. Compared to the 2D culture, the 3D scaffold system provides the physical support to enable spontaneous spheroid formation and mass cultivation of PSC-derived cells. With the support of the scaffold and cytokines, hepatocyte-like cells could be generated with the purity of up to 98% without cell isolation [101].

Hepatic cell generation from direct reprogramming of fibroblasts

Since the discovery of iPSC by Yamanaka in 2006, many groups have successfully reprogramed somatic cells directly to specialized cells bypassing the pluripotent stem cell stage. Last year, two studies have shown that fibroblasts can be reprogrammed directly to hepatic cells with the help of several transcription factors essential for liver development. Huang et al. [102] have demonstrated that the transduction of mouse fibroblasts from p19arf-/- mice with GATA4, FoxA3 and Hnf1 leads to the generation of hepatic cells that express hepatic markers, and restore liver functions following transplantation in the Fah liver deficient deficient mouse model. The second study from Sekiya et al. [103] used a different set of transcription factors, HNF4α, FoxA1 and FoxA2 or FoxA3 to reprogram mouse fibroblasts into hepatic cells, and demonstrated the *in vivo* ability of the cells to improve 40% of survival 10 weeks after cell transplantation in Fah deficient mice [103].

In vitro characterization of the PSC-Hep

To characterize the PSC-Hep *in vitro*, most of the above studies analyzed hepatic markers that are expressed in the early (alpha-fetoprotein, transthyretin) and later stages of liver maturation (albumin, cytokeratin 19 (CK19), CK7, CK18, cytochrome p450 enzymes (CyP), α1-antitrypsin (ATT), tyrosine aminotransferase (TAT), γ-glutamyltranspeptidase (GGT), glutathione S transferase (GST), tryptophan 2,3-dioxygenase (TDO), asialoglycoprotein receptor 1 (ASGPR), phosphoenolpyruvate carboxykinase (PPC), glucose-6-phosphatase (G6P), hepar-1 apolipoprotein F, fibrinogen, fibronectin, constitutive androstane receptor (Table 1). In addition to marker expression analyses supporting the differentiation stages, functional *in vitro* assays are also performed and usually compared to those from primary hepatocytes. Since liver exerts functions related to metabolism, protein synthesis, urea production and detoxification, the functional assays usually include albumin secretion, glycogen storage, low-density lipoprotein uptake, indocyanine green uptake and release, cytochrome P450 enzyme metabolism, urea production and metabolism-mediated toxicity. Results of these *in vitro* assays for each study are summarized in Table 1.

Pre-clinical *In vivo* Relevance of PSC-Hep

The definitive functional assay for PSC-Hep is their ability to

regenerate diseased livers from animal models and ultimately to increase the animal survival rate. Chronologic studies of repopulation assays (summarized in Table 2) clearly indicate an improvement over the last years of pre-clinical relevance of the PSC-Hep. The differences between pre-clinical outcomes are the result of the combination of the intrinsic functionality of the PSC-Hep generated *in vitro* discussed above and the liver-deficiency animals used for each study.

To study the liver regenerative ability of the PSC-Hep *in vivo*, cells are transplanted into animal models in which the liver is injured to provide space for the transplanted cells and a proliferative stimulus for the regeneration to occur. Some of these models provide specific growth advantage for the transplanted cells. The liver injury models used in these studies include two genetic models, some chemical models and partial hepatectomy reviewed recently by Shafritz and Oertel [104].

The first genetic mouse model was established in 1991 by Sandgren et al. [105] by overexpressing the transgene urokinase type plasminogen activator under the albumin promoter (Alb-uPA mice). In this mouse model, uPA is expressed strictly in hepatocytes and appeared to be highly cytotoxic for these cells. Most of the uPA is secreted in serum, however a small amount remains in the liver resulting to intense liver damage. Consequently, most of the newborns die from hemorrhaging between 4 and 6 week-of-age, the rest of the newborns survive from extensive liver toxicity due to clonal growth of revertant hepatocytes that lost the transgene. Rhim et al. have subsequently tested the ability of the Alb-uPA mice to host transplanted normal hepatocytes [106]. They demonstrated that Alb-uPA mice provide a growth advantage to transplanted cells over the dying endogenous uPA expressing hepatocytes, and hence offer a permissive environment for survival, expansion and function of transplanted hepatocytes. Alternatively, Sandgren's group has developed another transgenic mouse model expressing uPA under the major urinary protein (MUP) promoter in which uPA expression was initiated in hepatocytes only from 2 to 4 week-of-age, thereby preventing the uPA-mediated neonatal lethality [107].

The second genetic mouse model, the fumarylacetoacetate hydrolase deficient mice (Fah-/-mice), was created by Grompe et al. [108] in 1995 to recapitulate the hereditary tyrosinemia type 1 (HT1) in human characterized by mutation in the Fah gene. The lack of the Fah enzyme, which is involved in the tyrosine catabolism pathway, induces an accumulation in hepatocytes of toxic metabolites including fumarylacetoacetate (FAA), leading to chronic liver damage. Accumulation of FAA can be prevented by adding in drinking water the chemical agent NTBC (2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione) that is used in clinical settings to treat HT1 patients [108]. NTBC stops the tyrosine catabolism pathway upstream of the Fah enzyme, blocking the accumulation of FAA, thus preventing liver cell toxicity.

Therefore, the liver injury in Fah deficient mice can be easily controlled by the NTBC intake and removal. Overturf et al. [109] have shown that transplanted wild type hepatocytes have a growth advantage, and hence are able to repopulate mutant livers and restore liver functions. More recently, both the Fah deficient mice and Alb-uPA transgenic mice have been crossed to the immunosuppressed mouse models Rag2-/-, Il2Rγ-/- mice or the SCID mice (for Alb-uPA) to allow liver repopulation by human hepatocytes and mouse hepatocytes harboring any genetic background [110-112].

These 2 genetic deficient liver mouse models provide not only liver injury but also selective growth advantage for transplanted human and mouse hepatocytes generated from pluripotent stem cells (27,50,78,113-116). Among those studies, Li et al. [78] demonstrated extensive liver repopulation capacity (24% +/- 15%) of purified mESC-Hep based on EpCAM expression (and negative for cKit) 10 weeks following transplantation in Fah-/- mice. Percentage of repopulation from PSC-Hep varied from 1.94%+/- 5.81 in MUP-uPA treated with CCl4 at 82 days [116], or 0.001%-12.5% in Fah-/- mice at 8 weeks [113], to detection of few clusters of transplanted cells in either Fah deficient mouse model [50] or uPA transgenic mouse model [27,114,115]. Two interesting studies describing the direct conversion of mouse fibroblasts by transducing a specific set of hepatic transcription factors demonstrated a remarkable ability of these cells to repair the damaged livers from Fah-/- mice [102,103].

The chemical liver injuries used to test the *in vivo* functionality of PSC-Hep include the carbon tetrachloride (CCl4)-induced hepatic centrilobular necrosis (38,39,117), the dimethylnitrosamine (DMN)-induced chronic cirrhotic injury [41,118] and the thioacetamide (TAA)-induced hepatic fibrosis [119]. The acute liver injury induced by CCl4 has recently been shown to be a successful mouse model to allow high liver repopulation ability of mouse [117] and human [38,39] PSC-Hep. Transplantation of miPSC-Hep or hPSC-Hep in the CCl4-treated BALBc-nude mice reduced hepatic necrotic area, oxidative stress, and improved hepatic functions and the survival rate [117], and in a different study, regenerate about 10% of the liver mass 35 days after transplantation [39]. Similarly, Chen et al. [38] have successfully rescued the acute liver injury induced by CCl4 in NOD/SCID mice following transplantation of hiPSC-Hep. Another acute liver injury was developed by Bandi et al. [120] using a combination of the hepatotoxic drugs rifampicin and phenytoin that inhibit host hepatocyte proliferation followed by injection of monocrotaline, a pyrrolizidine alkaloid agent which induces sinusoidal endothelial cell injury to facilitate transplanted cell engraftment. This regiment produced 50-70% liver necrosis in NOD-SCID mice with 90-100% mortality over 2 weeks. Transplantation of hESC-derived meso-endodermal cells (hESC-MEC) intraperitoneally in this acute liver failure mouse model rescued the survival rate 2 weeks following surgery although further hepatic maturation of hESC-MEC was not successful [120].

Another variable parameter to consider when investigating the regenerative ability of PSC-Hep is the mode of cell delivery. Most of the published studies use the intra-splenic (IS) route to deliver PSC-Hep to the liver through the portal vein. These studies showed variable repopulation abilities of the transplanted cells, and in some cases partial rescue of the liver damage (38,39,50,78,113-116). Injections of cells through the portal vein deliver cells directly to the liver bypassing the spleen. Agarwal et al. [40] transplanted hESC-Hep through the portal vein of the CCl_4 treated NOD/SCID, and showed some human cells integrated into the host liver. Cells can also be directly and locally targeted to the liver parenchyma or under the liver capsule. This mode of delivery is appropriate to newborn injection as the liver is visible through the skin. Few studies showed liver function improvement using this route [17,27,121]. The least invasive routes of delivery are intra-peritoneal (IP) and intra-venous (IV) injections.

Mostly, IP transplanted cells remain in the intra-peritoneal cavity [120] where they can still exert hepatic functions, even though one study has shown integration of the IP transplanted cells into the liver [117]. In the first study, cells were delivered IP with microcarriers that eventually revascularized in the peritoneal cavity and secreted proteins in blood circulation improving hepatic functions, and rescuing 100%

of animal survival 2 weeks after a mild injury and 38% after a stronger injury [120]. IV injections of PSC-Hep allowed integration of cells into the host livers and have shown some liver function improvement [41,118,119].

Conclusions

In summary, the liver field of pluripotent stem cells has tremendously advanced the last decade with the generation *in vitro* of cells harboring hepatocytic functions and capable of improving some liver functions *in vivo* of mouse models with liver deficiency. Even though the hepatic differentiation protocols are presently very efficient and reproducible among different groups, the variability of hepatic differentiation efficiency between PSC lines remains unclear.

A recent publication from Yamanaka's group has shown that the variation in hepatic differentiation efficiency of hiPSCs was mostly due to donor differences [122] rather than the tissue from which the iPSCs were derived as indicated by other studies using mouse [123-125] or human [126] tissues, or the iPSC derivation methods. This finding may impact our current vision of personalized medicine using hiPSC-Hep for liver functions rescue. In addition to differences in the propensity for hepatic differentiation among PSC lines, the consistency of the regenerative ability of PSC-Hep is still a challenge. Before the use of the PSC-Hep in clinical settings, many issues need to be resolved including namely the purity of the PSC-Hep cultures, the risk of teratoma development and the choice of delivery mode based on the liver damage. In addition to these technical limitations, there is a need to understand the interactions between the transplanted cells and the damaged microenvironment to improve PSC-Hep integration into the liver and their proliferation. One can suspect that some of these crosstalks are universal to all liver injuries, while some are specific to particular damages. Consequently, it is conceivable that understanding the response of the damaged microenvironment will help the generation of customized PSC-Hep to specific liver injuries.

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This article was originally published in a special issue, **Embryonic and Induced Pluripotent Stem Cells** handled by Editor(s). Dr. Jianlong Wang, Mount Sinai School of Medicine, United States