Pluripotent stem cells to hepatocytes, the journey so far (Review)

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Received December 5, 2016; Accepted February 15, 2017

DOI: 10.3892/br.2017.867

Abstract. Over the past several years, there has been substantial progress in the field of regenerative medicine, which has enabled new possibilities for research and clinical application. For example, there are ongoing efforts directed at generating functional hepatocytes from adult-derived pluripotent cells for toxicity screening, generating disease models or, in the longer term, for the treatment of liver failure. In the present review, the authors summarise recent developments in regenerative medicine and pluripotent stem cells, the methods and tissues used for reprogramming and the differentiation of induced pluripotent stem cells (iPSCs) into hepatocyte-like cells. In addition, the hepatic disease models developed using iPSC technologies are discussed, as well as the potential for gene editing.

Contents

1. Introduction

Although modern medicine has made great progress during the last century, there are still few effective ways of treating the root cause of many congenital conditions, diseases and injuries. In many cases, clinicians can only manage the symptoms exhibited by a patient, and there is a requirement for better and safer drugs for treating such conditions. Methods such as organ transplantation are increasingly unable to meet the needs of patients, due to the lack of sufficient donor organs and issues related to immunocompatability. Regenerative medicine has the potential to revolutionise drug discovery and clinical therapy using stem cells, thereby providing hope for patients with conditions that are currently beyond repair.

Pluripotent stem cells are cells that are capable of generating daughter cells belonging to any of the three embryonic germ layers: The endoderm, mesoderm and ectoderm. The discovery of such cells dates back to the 1960s and 1970s, when researchers identified that the cells of the inner cell mass of the mouse blastocyst were pluripotent in nature (1,2). Further progress followed when Evans and Kaufman (3) and Martin (4) isolated mouse embryonic stem cells (ESCs), and this was further developed by Thomson *et al* (5) in 1998, who demonstrated a culture of human ESCs isolated from human blastocysts that offered limitless possibilities for regenerative therapy. However, the use of human embryos to generate human ESC lines sparked widespread controversy, restricting the research surrounding the creation of new human ESC lines. Human ESC lines that were already established were later approved for research, yet they were limited in number and lacked the diversity necessary to address many scientific questions. In addition, most of the human ESC lines represented generic genotypes and were not matched to a particular disease or patient (6). The immunological issue of tissue compatibility and the possibility of tumour formation severely restricted their suitability for use in therapy.

In order to capitalise on the potential of these cells, researchers tried to develop personalised pluripotent stem cells by somatic nuclear transfer without fertilisation. Nuclear transfer-generated pluripotent lines would have the complete genome of patients and could be differentiated to specific cell types for cell therapy (7). However, the issues of efficiency, anxiety over artificially-created embryos and the potential use of such cells for human cloning led to criticism, and research in this direction was severely curtailed. In 2006, Takahashi and Yamanaka (8) illustrated how to restore pluripotency in somatic cells through the ectopic co-expression of reprogramming factors, rewriting the fundamental idea on the stability of cellular identity, thereby providing a strategy to create induced pluripotent stem cells (iPSCs). It was a momentous contribution that provided a new direction for research into disease modelling, *in vitro* differentiation and transdifferentiation, and allowed new hope for the development of personalised therapies.

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Key words: pluripotent cells, hepatocytes, differentiation

2. Induced pluripotent stem cells

The last decade has seen rapid development in the field of stem cells with the advent of human ESCs and, more recently, human induced pluripotent stem cells (hiPSCs). Human ESCs and hiPSCs can undergo unlimited self-renewal, retaining their potential to differentiate into any type of somatic cell. This is a significant achievement, as these iPSCs can provide an inexhaustible human cell source (9).

A major advantage of iPSC technology is the ability to generate patient- or disease-specific pluripotent stem cells that can be grown indefinitely *in vitro*. This is an invaluable resource for medical research, which was previously largely dependent on tumour cell lines or transformed derivatives of adult tissues. hiPSCs from patients with a variety of genetic diseases, such as trisomy 21 syndrome, Parkinson's disease, Huntington disease and type 1 diabetes mellitus have been generated (10). These disease-specific stem cells offer a unique opportunity to study the diseased cells of a tissue *in vitro* and may aid the development of new drugs for therapeutic intervention.

Advancements in reprogramming technology during the last decade have led to the derivation of many hiPSC lines that can propagate indefinitely. In order to induce pluripotency in somatic cells multiple transcription factors, the most widely used being Oct4, Sox2, Klf4 and c-Myc, require to be transfected into somatic cells. This combination of factors was initially identified by Yamanaka's group through the screening of 24 pre-selected factors in a murine system (11), and later, the same combination of factors was demonstrated to be sufficient for reprogramming human cells (11,12). During reprogramming, DNA methylation and histone modifications occur which induce epigenetic changes in the chromatin structure thereby mimicking the embryonic stage. However, the exact role of transcription factors and their mode of action during reprogramming remain elusive and puzzling. Following Yamanaka's reports (9,10), different groups have now successfully produced hiPSC cells using a variety of starting cell types, different combinations of transcription factors and different delivery techniques of these factors into the cells.

The potential to use of autologous hiPSCs as therapeutic medicines holds tremendous promise. Initially, retroviruses were used to introduce transcription factor genes into the nucleus of somatic cells. Retroviral methods require proliferating cells and have poor transfection efficiency. The major disadvantage of the retroviral technique is the potential to simultaneously introduce insertional mutations into the genome and, thereby, promote tumour formation. This drawback shifted the attention of researchers to explore the use of other techniques for the insertion of genes, such as lentiviral and adenoviral vectors. Lentiviruses can infect non-proliferating cells, and can integrate into the genome of the target cell, without expressing viral genes (13,14). However, the limited insertion size, difficulty in storage/quality control and safety concerns of using virus is the primary drawbacks of using this system. Conversely, adenoviral vectors allow transient, high-level expression of exogenous genes without integrating into the host genome (15). Adenoviruses have been used to generate iPSCs from mouse fibroblasts and liver cells (16). Liver cells are highly permissive for adenoviral infections and, therefore, less adenovirus is required for the efficient reprogramming of liver cells compared with fibroblasts. However, the continuous expression of transgenes in iPSCs can limit their differentiation potential (17). Many studies have explored the removal of viral vectors after reprogramming. These techniques include plasmid transfection and piggyback transposition system, in which the inserted transgene can be subsequently excised to remove the risk of residual expression and re-activation of reprogramming factors (18,19). The risk of insertional mutagenesis still remains, as the sequences beyond the Lox site cannot be fully excised.

Recently, there have been many studies to identify alternatives to viral vectors for reprogramming somatic cells. Among them, the technologies that do not require genome integration appear more promising. These technologies include the use of small chemical molecules (20), episomal vectors (21), recombinant proteins (22), microRNAs (23) and synthetic modified mRNAs (24). Owing to its high reproducibility and simplicity, the transient transfection technique seems promising and may find widespread application.

In addition, different tissue sources have been considered for generating iPSCs. Due to the ease of isolation and maintenance for reprogramming, the most popular source is fibroblasts. Other cell types, including keratinocytes (25), mesenchymal stem cells (26), adipose stem cells (27), hair follicular cells (28), neural stem cells (29) and urinary cells (30), have also been used successfully. A more desirable source of starting material is human peripheral blood, which can be readily obtained through non-invasive routine clinical procedures. However, the non-adherent nature of most blood cells complicates the process of reprogramming. Recently, a robust method for reprogramming peripheral blood mononuclear cells using a non-viral, feeder cell-free methodology has been developed (21).

3. iPS and ES cells, how similar are they?

Even though iPSCs possess phenotypic and behavioural similarity to ESCs, recent critical analysis of their genetics and epigenetics demonstrates that iPSCs can carry residual DNA methylation patterns from their source tissue (31,32). Transcribed genes and mutational load have demonstrated small distinctive dissimilarities between iPS and ES cells (33), and this epigenetic memory can favour the differentiation of iPS cells towards the parental cell type. However, this phenomenon appears to be transitory in many lines and, in some instances, can be erased by additional reprogramming, by chromatin-modifying drugs, or with extended passaging of the cells (34). Differences in endogenous signalling activity may also contribute to the heterogeneity of iPSC lines generated from the same tissue sample (35). A recent study on variation between pluripotent cell lines indicates that a difference in genetic background is the major factor for variation, rather than aberrations arising during viral transfection (36). In the authors' experience, extended passaging of iPSC lines did not promote differences in hepatic differentiation, irrespective of the parental tissue of origin. However, previous work of the University of Edinburgh has observed that iPSCs generated using episomal vectors are able to produce healthy hepatocyte-like cells comparable to those produced with lentiviral or Sendai vector systems (21).

Table I. Protocols used to generate hepatic differentiation (3 stage methods).

	Signalling molecules	Duration (days)	Markers	Refs.
Definitive endoderm differentiation	100 ng/ml Activin A, 10 μM LY294002 (inhibit insulin, PI3K/AKT pathway) 2 μM bromo-indirubin-3'-oxime (mimic Wnt signalling)	6	FOXA2, SOX17	(53)
	100 ng/ml Activin A, 50 ng/ml Wnt3A	3	FOXA2, SOX17	(46,53,54)
	3 μM CHIR99021(mimic Wnt signalling)	1	FOXA2, SOX17,	(55)
	followed by medium alone	1	HHEX, GATA4	
	100 ng/ml Activin A, 100 ng/ml bFGF, 10 ng/ml BMP4, 10 μM LY294002, 3 μM CHIR99021 (only for 24 h)	2	SOX17, FOXA2 and HHEX	(56)
	followed by100 ng/ml Activin A, 100 ng/ml bFGF-1 day and 50 ng/ml Activin A	3		
	2 μg/ml CHIR99021 and 100ng/ml Activin A	1	CXCR4, Ckit, SOX17, FOXA1	(57)
	followed by 10.5 ng/ml BMP4, 10 ng/ml FGF2, 100 ng/ml Activin A and 10 ng/ml VEGF	4		
	100 ng/ml Activin A	6	HNF3B, CXCR4	(58)
	100 ng/ml Activin A	5	GATA4, SOX17, FOXA2	(59)
Hepatoblast	250 nM sodium butyrate and 0.5% DMSO	6-8	FOXA2, AFP, ALB, HNF4A, CK18, CK19	(53)
	10 ng/ml FGF-2 and 50 ng/ml BMP-4	4	FOXA2, AFP, ALB,	(53,54)
	followed by 50 ng/ml FGF1, 10 ng/ml FGF 4 and 25 ng/ml FGF8b	4	HNF4A, CK18, CK19,	
	1% DMSO	5	AFP, CEBPA, FOXA2, GATA4, HNF4A PROX1, TBX3, TTR	(46,55)
	20 ng/ml BMP4, 10 ng/ml FGF10	4	HNF4A, PROX1, HHEX, AFP, TBX3, FOXA2, AFP, ALB	(56)
	50 ng/ml BMP4, 10 ng/ml FGF2, 10 ng/ml VEGF, 10 ng/ml EGF, 20 ng/ml TGFα, 100 ng/ml HGF and 0.1 μM Dex	6	FOXA2, AFP, ALB	(57)
	5 ng/ml FGF4, 10 ng/ml BMP2, 5 ng/ml FGF4, 10 ng/ml BMP2, 10 ng/ml BMP4	4	HNF4A	(58)
	20 ng/ml BMP4, 10 ng/ml FGF2 followed by 20 ng/ml HGF	5 5 (at 4% O_2)	FOXA2, HNF4A, AFP	(59)
Hepatic maturation	1 μM SB431542 and 1% DMSO	8	ALB, A1AT, ASGPR, MRP2, CYP3A4, CYP1A1, CYP1A2, urea	(53)
	20 ng/ml HGF and 100 ng/ml Follistatin	6	ALB, A1AT, ASGPR, MRP2, CYP3A4, CYP1A1,	(53,54)
	100 nM DiHexa, 100 nM Dex	10	ALB, HNF4A, A1AT, CYP3A4, CYP1A2, Fibronectin	(55)

Table I. Continued.

	Signalling molecules	Duration (days)	Markers	Refs.
Hepatic maturation	100 ng/ml HGF, 20 ng/ml OSM, 10 μM HC	18-20	ALB, ECAD, CYP3A4, CYP2D6, MRP1, HNF4A	(46)
	50 ng/ml HGF, 30 ng/ml OSM	15	ALB, CK18, A1AT, CYP3A4, LDL uptake, TAT, TTR	(56)
	10 ng/ml FGF2, 10 ng/ml VEGF, 10 ng/ml EGF, 100 ng/ml HGF, 1.5 μM γ secretase inhibitor, 0.1 μM Dex, 1% DMSO	6	A1AT, ALB, AFP, TTR, fibronectin, transferrin, CYP450	(56)
	followed by 100 ng/ml HGF, 20 ng/ml OSM 6 μg/ml Vitamin K, 0.1 μM Dex	6		
	10 ng/ml HGF, 10 ng/ml OSM, 0.1 μM Dex	6	ALB, AAT, CK19, CK8, CK18, AFP,	(58)
	followed by 0.1 μM Dex	5	CYP3A4, CYP1A2	
	20 ng/ml OSM	5	ALB, AFP, HNF4A, FOXA2	(59)

bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; BMP, bone morphogenetic protein; VEGF, vasculalr endothelial growth factor; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; HGF, hepatocyte growth factor; Dex, dexamethasone; DiHexa, N-hexanoic-Tyr-Ile-(6) aminohexanoicamide; OSM, oncostatin M; HC, hydrocortisone.

4. Generating hepatocytes from iPS cells

Under *in vivo* conditions, stem cells undergo a complex set of chemical interactions, switching on or off of various signalling molecules, cell-cell and cell-ECM interactions, during the process of becoming terminally differentiated cells. It is difficult to mimic many of these cues under experimental conditions. Some cell types, such as cardiomyocytes, develop spontaneously in stem cell culture (at low frequency) while other cell types, such as hepatocytes and renal tubular cells, require significant technical manoeuvres. Pluripotent stem cells certainly appear to progress more efficiently than mesenchymal stem cells toward a hepatocyte fate (37).

During embryogenesis, Nodal and Wnt signalling triggers endoderm development. Later on in the differentiation process, the switching on or off of this signalling regulates endodermal tube patterning (34,35). Mesodermal cells surrounding the endoderm tube are involved in the hepatic commitment of foregut cells (mainly through fibroblast growth factor and bone morphogenetic protein signalling) and liver bud formation. Later, endothelial cells colonise the liver bud and hepatoblasts start to differentiate to biliary cells (hepatoblasts near the portal vein) and hepatocytes (38,39).

Researchers have tried to differentiate pluripotent stem cells in cell aggregates or as a monolayer. Pluripotent stem cells can be aggregated to form embryoid bodies and can be differentiated by exposure to specific cytokine and growth factor cocktails (40). However, all the existing embryoid body-based protocols suffer from low differentiation efficiency and spontaneous differentiation which gives rise to unwanted cell lineages (41-43). As monolayer culture can be adjusted to avoid most of these issues, it has been utilised widely and is capable of producing relatively pure populations of cells with hepatic function. Among the different approaches used to generate hepatocyte-like cells as monolayers, methods mimicking the embryonic developmental stages of the liver seem to be the more promising (Table I). Unfortunately, the outcome of such approaches varies between different research groups for a variety of reasons, including variation in cell lines and the conditions used for culture. Efforts have been made to optimise the protocol by eliminating the use of poorly defined components such as serum, feeder cells, undefined culture medium and extracellular matrix to promote consistent results (44-46). A key to the success of these protocols is the ability to generate definitive endoderm cells with high efficiency, mainly by using activin A and Wnt3A (47,48), and this approach has yielded functionally improved hepatocyte-like cells. Efforts to differentiate iPS cells under 3D dynamic environments also helped to enhance the function, but were also more like foetal hepatocytes and lacked maturity (49).

Stem cell-derived hepatocytes have been reported to express phase I and II metabolising enzymes, hepatic morphology and polarisation, but are still not comparable to primary hepatocytes. Hepatic differentiation protocols, evaluation strategies and phenotypic or functional outcomes vary widely between different laboratories (50). A direct comparison of the efficiency of pluripotent stem cell lines to generate functional hepatocytes is therefore difficult. As a general observation, hiPSC lines present more variable hepatocyte differentiation and performance compared with ESC lines,

particularly the H9 line. This may be due to the fact that the available differentiation protocols have been developed with ESC lines, and these protocols do not address the potential retained epigenetic memory or aberrations that iPSC lines may possess, making certain that iPSC lines respond weakly to hepatic differentiation signals. Moreover, *in vitro* culture itself can induce aberrations in pluripotent cells, and can result in significant changes in the differentiation potential within different passages of the same cell line (51,52).

5. iPSC-derived hepatocyte-like cells for industry and research

The pharmaceutical industries are, at present, reliant primarily on animal tests to establish the safety of any new products. These tests often fail to predict human toxicity accurately due to the physiological differences between humans and animals. The advent of iPSCs have offered a new opportunities and one would anticipate that in the near future it will be possible to perform toxicity screens using iPSC-derived healthy or diseased cells where safety, efficacy, dosage studies and the effect of genetics could be studied in human cells before a clinical trial.

Severe perturbance in key proteins of metabolic pathways can upset hepatocyte homeostasis and can promote liver disease (60). Using iPSC technology, it is now possible to study, at the cellular level, the pathobiology of genetic liver diseases under *in vitro* conditions. Disease-specific hiPSCs of α1-antitrypsin deficiency, familial hypercholesterolemia and glycogen storage disease type 1a generated from patients presented key pathological features of the diseases in *in vitro* culture (61). A later study from the same group demonstrated that genetic correction of an α1-antitrypsin-deficient iPSC line by gene editing could restore the structure and function of α1-antitrypsin in the derived hepatocytes both *in vitro* and *in vivo* (62), providing hope for future gene therapy. A number of iPSC lines from patients suffering from tyrosinemia, glycogen storage disease, progressive familial hereditary cholestasis and Crigler-Najjar syndrome have also been generated successfully (63). Wilson's disease-specific iPSC lines with the R778L hotspot mutation in the ATP7B gene were able to produce hepatocytes with defective copper transport in culture (64). The modelling of other inherited diseases, such as hemochromatosis, hepatobiliary cystic fibrosis and idiosyncratic drug reactions would be helpful for understanding the respective disease processes and devising clinical interventions. In relation to this, the gene editing of iPS cells, not only to correct mutations (62), but also to introduce mutations of interest, is an important development in disease modelling. Of particular interest in this regard is the developing use of CRISPR technology in iPSCs to edit genes involved in drug metabolism, thereby providing *in vitro* models relevant to drug development.

6. Discussion

Although significant progress has been made in iPSC technology, a full adoption of iPSC-derived hepatocytes by industry will require more robust, consistent and cost effective protocols, scale-up and comparative studies with primary hepatocytes (65). At present, the state-of-the-art hepatic differentiation protocols produce cells, which are foetal in both phenotype and function (66). There remains insufficient data demonstrating that iPSC-derived hepatocyte-like cells are comparable to existing primary hepatocyte systems or hepatic cell lines, although improved protocols for generating hepatocytes are progressing rapidly. Following the establishment of improved hepatocyte differentiation protocols, the technology for cost-effective scale-up conditions that can maintain phenotype, function, batch-to-batch consistency and reproducibility will be required. Given the intensive ongoing research efforts, iPSCs will soon be utilised for generating highly predictable human hepatic cells of use in determining drug safety, as well as for studying the mechanism of action of drugs or small molecules and for the generation of new cellular disease models.

Acknowledgements

The present study is supported by the Innovative Medicines Initiative Joint Undertaking (grant no. 115439), resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (grant no. FP7/2007-2013) and EFPIA companies in kind contribution. This publication reflects only the author's views and neither the IMI JU nor EFPIA, nor the European Commission, are liable for any use that may be made of the information contained therein.

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