World Journal of Gastroenterology

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DOI: 10.3748/wjg.v21.i21.6427

World J Gastroenterol 2015 June 7; 21(21): 6427-6433
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Cell therapy from bench to bedside: Hepatocytes from fibroblasts - the truth and myth of transdifferentiation

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Author contributions: Sanal MG conceived the issues which formed the content of the manuscript and wrote the manuscript.

Supported by IIP fellowship (2013-2014), Albert Einstein College of Medicine, New York, through the generosity of the Gruss Lipper Family Foundation.

Conflict-of-interest: The author has no conflict of interests.

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Received: December 4, 2014
Peer-review started: December 5, 2014
First decision: March 10, 2015
Revised: March 24, 2015
Accepted: May 7, 2015
Article in press: May 7, 2015
Published online: June 7, 2015

Abstract

Hepatocyte transplantation is an alternative to liver transplantation in certain disorders such as inherited liver diseases and liver failure. It is a relatively less complicated surgical procedure, and has the advantage that it can be repeated several times if unsuccessful. Another advantage is that hepatocytes can be isolated from partly damaged livers which are not suitable for liver transplantation. Despite these advantages hepatocyte transplantation is less popular. Important issues are poor engraftment of the transplanted cells and the scarcity of donor hepatocytes. Generation of "hepatocyte like cells"/iHeps from embryonic stem cells (ES) and induced pluripotent stem cells (iPSCs) by directed differentiation is an emerging solution to the latter issue. Direct conversation or trans-differentiation of fibroblasts to "hepatocyte like cells" is another way which is, being explored. However this method has several inherent and technical disadvantages compared to the directed differentiation from ES or iPSC. There are several methods claiming to be "highly efficient" for generating "highly functional" "hepatocyte like cells". Currently different groups are working independently and coming up with differentiation protocols and each group claiming an advantage for their protocol. Directed differentiation protocols need to be designed, compared, analyzed and tweaked systematically and logically than empirically. There is a need for a well-coordinated global initiative comparable to the Human Genome Project to achieve this goal in the near future.

Key words: Trans differentiation; i-Heps; Fibroblasts; Induced pluripotent stem cells; Embryonic stem cells; Hepatocyte like cells; Telomere/telomerase; Hepatocyte transplantation; differentiation; Inherited/genetic liver disease; Cell therapy; Gene therapy

Core tip: Hepatocyte transplantation is an alternative for liver transplantation in chronic liver disease patients for a long term cure. There is a scarcity of donor liver and hepatocytes. Induced pluripotent stem cells (iPSC) derived hepatocytes and hepatocytes generated by

transdifferentiation are two possibilities. iPSC derived hepatocytes often fail to engraft upon transplantation. We need to define methods to evaluate and compare efficiency of differentiation, standards and clear quality definition for hepatocyte like cells. More comprehensive analysis of the RNAs and proteome is required. Methods to compare and analyze the expression profiles, standards and references to be compared with need to be defined. There is a need for a well-coordinated global initiative comparable to the scale of the Human Genome Project to achieve this goal in the near future.

Sanal MG. Cell therapy from bench to bedside: Hepatocytes from fibroblasts - the truth and myth of transdifferentiation. *World J Gastroenterol* 2015; 21(21): 6427-6433 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i21/6427.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i21.6427

HEPATOCYTE TRANSPLANTATION OVER LIVER TRANSPLANTATION

Liver transplantation is the only long term option in some inherited metabolic liver diseases, acute liver failure and most end stage liver diseases[1,2]. Hepatocyte transplantation is an alternative for these patients for a long term cure if not as a bridge to regeneration or liver transplantation[3,4]. Hepatocyte transplantation has the advantage that it can be performed more than once on the same patient. Hepatocytes for this procedure can be isolated from cadaveric liver or living donor liver tissue which are not suitable for liver transplantation for various reasons such as damage to the blood vessels, localized hypoxic damage or local lesions. Another advantage of hepatocyte transplantation is that the procedure is less complicated and the mortality and morbidity is expected to be much lower compared to liver transplantation. Moreover, even if the patient receives the hepatocyte transplant and it does not work, they can be considered for orthotopic liver transplantation as if they never received the hepatocyte transplant. Shortage of donor liver is one of the major limiting factors in both liver transplantation and hepatocyte transplantation[3].

APPLICATIONS OF HEPATOCYTE TRANSPLANTATION

Conditions benefitting from hepatocyte transplantation can be classified into two major categories (1) where transplanted cells rescue the liver function and help the patient survive (example: acute liver failure resulting from toxins). Here hepatocyte transplantation might actually work because the host liver suffered a massive damage and loss of hepatocytes and transplanted hepatocyte help with the liver functions; and (2) an inherited disease of the liver. Example: Hemophilia, Wilson's disease, Urea cycle disorders, α1 antitrypsin deficiency, Crigler Najjar syndrome. Here the patient's liver cells have a gene defect which does not allow them to synthesize a protein in its correct form or function. Here the disease can be cured only if we can replace certain percentage the patient's defective hepatocytes with healthy hepatocytes or gene corrected (example by gene therapy using viral vectors or genome editing tools such as ZFN, TALENs[5] or CRISPR-Cas9) hepatocytes in sufficient quantities to allow normal liver functions. This is a very difficult task to achieve and hence a major road block in the technique of hepatocyte transplantation because the transplanted cells would engraft and repopulate the host liver only if we could provide them a selective advantage. This is usually achieved by inflicting a physical or chemical damage to the host liver. However, the methods for damaging hepatocytes are not safe and therefore not clinically acceptable.

HEPATOCYTES FROM PLURIPOTENT STEMS CELLS AND FIBROBLASTS - THE TRUTH AND MYTH

Shortage of donor hepatocytes is another major issue in hepatocyte transplantation. Generating hepatocytes from embryonic stem cells (ES) or induced pluripotent stem cells (iPSCs) (or even less known entities such as very small embryonic-like stem cells) is an exciting solution to this conundrum[4-7]. There are several protocols which claim generation of hepatocyte like cells from directed differentiation of ES, iPSC or other stem cell types[6,8-23]. There is no doubt about the future promise of hepatocytes derived from pluripotent stem cells (such as ES, iPSCs or SCNT/iPSCNT), however worldwide there is no unambiguous data to support the usefulness of ES or iPSC derived "hepatocyte like cells" in their current form (using the "highly efficient" and "state of the art" protocols), in animal models or humans[4,5,23-28]. This is because despite the "extensive" and "excellent" *in vitro* characterized cellular, biochemical, metabolic, physiological and microscopic properties (such as various surface and structural proteins, transcriptional factors, secretion of albumin, clotting factors, liver enzymes, active bile acid and drug transporters, lipoprotein mediated lipid uptake/secretion, glycogen accumulation, mild to moderate cytochrome activity, electron microscopic demonstration of subcellular structures characteristic for hepatocytes) these cells fail to integrate in host livers upon transplantation except under extreme selection pressure in certain limited animal models such as genetically engineered fumaryl hydroxylase deficient knock-out (FAH-KO) immunodeficient mice[29,30], urokinase-type plasminogen activator-severe combined immunodeficiency [uPA(+/+)-SCID] mice[31]. Alternatively host hepatocytes are

intentionally damaged by chemical agents such as dimethylnitrosamine[32] or physical agents such as radiation[33] such that the transplanted hepatocytes will have a selective advantage over the host hepatocytes. Thus host liver damage would facilitate engraftment and repopulation of the transplanted cells. All these techniques will induce chronic liver damage and therefore have a very limited clinical value (*e.g.*, clinical trial # NCT01465100)[34].

The current hepatocyte like cells are likely to be a mixture of immature cells which express several markers belonging predominantly to the endodermal lineage which includes many liver transcription factors and liver genes. Many of the current iPSC protocols claim high efficiency, however there is no standard means to compare various protocols. Many of these protocols decide efficiency of differentiation by calculating the percentage of cells expressing one or two hepatocyte markers such as albumin, HNF-4α and ASGPR1. This method is not entirely correct because only a few markers are evaluated and many investigators do not typically look for the quantification and co-expression of various factors. Similarly, many investigators do not look for markers which are not typically expressed in liver (for example pancreas or lung specific markers). It is possible that these ES/iPSC derived hepatocytes are somewhere "lost" with respect to their identity along their way to hepatocytes[22]. Finally, much of the published work is dependent on immunofluorescent techniques for the determination of differentiation efficiency, however, this can be inherently flawed as many immunofluorescent techniques are associated with errors from various sources such as, nonspecific binding, variability in fixation procedures, lack of proper controls and observer bias[35].

POPULAR ANIMAL MODELS FOR HEPATOCYTE REPOPULATION - THE ISSUES

In FAH model, part of the mechanism of engraftment and repopulation is the fusion of the transplanted cells with host cells which are deficient in a critical enzyme necessary for hepatocyte survival. Similarly, the uPA model suffers from spontaneous (or cell fusion induced deletion upon xenotransplantation) deletion of the offending uPA gene[36-39]. This implies that non-liver cells can fuse with host hepatocytes (resulting in a compensated phenotype) and repopulate the host liver. The fusion is expected to result in unstable or metastable intermediate stages which may acquire some degree of genomic stability by spontaneous deletions, duplications or recombination of the genetic material. Therefore repopulation of FAH-KO/UPA mouse liver cannot be considered as a proof of hepatocyte identity or quality and one needs to be skeptical towards the different claims for "highly efficient" generation of hepatocyte like cells from ES/iPSC. Spontaneous repopulation of liver with transplanted hepatocytes was reported in mutant α1-antitrypsin protein (AAT-Z) expressing mice even in the absence of severe liver injury[40]. However there is little information available in the literature on the post-repopulation genetic/epigenetic changes in transplanted cells.

HEPATOCYTE LIKE CELLS - TRANSDIFFERENTIATED FIBROBLASTS *VS* IPSC/ES

It is amidst these unsubstantiated claims of iPSC derived "highly functional" hepatocyte like cells, claims of trans-differentiated hepatocytes rose to the limelight[19,35,41-51]. The proponents of transdifferentiation (from fibroblasts, the preferred source for most investigators) claim 'highly efficient conversion of fibroblasts to hepatocytes' by ectopic expression of a combination of transcription factors (or using a cocktail of small molecules, growth factors and cytokines). It has been claimed that these trans-differentiated fibroblasts are better than hepatocyte like cells from iPSC because they are less likely to form tumors (such as teratomas). They point to the potential of iPSCs to form teratomas. Another reason they cite is that oncogenes such as c-myc are used in the generation of iPSCs. These are pithless arguments for the following reasons: (1) it is the essential and natural property of all pluripotent stem cells to form teratomas and teratomas are benign tumors[52]. All the pluripotent cells in a morula which would give rise to a healthy offspring have the potential to form teratomas. In fact teratoma formation is the gold standard for the quality of pluripotency (in humans)[53]; and (2) all oncogenes are essential genes for normal development and function of an organism. Oncogenes can be oncogenic or anti-oncogenic depending on the cellular and extracellular genetic and epigenetic context which is partly dictated by the microenvironment. For example c-Myc can induce apoptosis in hepatocytes instead of proliferation[54,55]. Another argument favoring the trans-differentiated hepatocytes is that fibroblasts the common "raw material" for the generation of "trans-differentiated hepatocyte like cells" are easily available. This is essentially wrong because ES/iPSC can proliferate indefinitely (by definition) *in-vitro* and therefore several fold more fibroblasts are required to generate a similar quantity of "hepatocyte like cells" through transdifferentiation considering the fact that fibroblast proliferation is limited by Hayflick's limit. I would expect hepatocyte like cells generated from iPSC to be better than directly trans-differentiated cells because during iPSC generation the somatic cells are brought down to a ground state and this brings more epigenetic uniformity compared to transdifferentiation where hepatocytes

might carry a stronger epigenetic memory of the parent cell. This can be explained by the simple analogy that scrap metal can be melted and remolded to new goods. New items can also be made by compression at high temperatures without going through the melting step. We also know that items made by compression will be less homogenous and will retain some properties of the material from which it originated. List below are the reasons why hepatocyte like cells derived from iPSC are expected to be better than those derived directly from fibroblasts: (1) fibroblasts are likely to retain an epigenetic memory which could be stronger compared to iPSC derived i-Heps because during the process of generation of iPSCs, cells are pushed to the ground state (at least partially if not completely); (2) several generations of iPSC (passages) will improve and ensure uniformity and quality (compared to trans-differentiated fibroblasts as once they become hepatocyte like cells, they will either not proliferate or their proliferation is limited because any differentiated cell would eventually undergo senescence). This could be the reason why extended passaging of iPSC clones improves the efficiency of differentiation[56]; (3) iPSC culture can be scaled up to industrial levels using bioreactors[5] but this is not possible with fibroblasts because they will undergo senescence issue; (4) iPSCs are similar to ES cells in their pluripotency and clonal nature. The clonal nature and pluripotent stem cell properties of iPSC will allow intense screening- morphological, physiological, genetic, epigenetic and functional, of iPSC cells to insure that the best clones are selected based on the screening. Thus the uniformity, genetic quality and safety can be assured for cells used in transplantation/ clinics[57,58]. This is not possible with hepatocytes from trans-differentiated fibroblasts because their proliferative capacity is limited (and they are not clonal) and are highly heterogenous to start with. Single cell genomics and epigenomics are making tremendous progress and the sequencing costs seems to follow the Moore's law[59]; (5) telomere "resetting" happens in iPSC but not in trans-differentiated cells. This will help them from becoming senescent[60]; (6) huge number of starting fibroblasts are required to make trans-differentiated hepatocytes. More starting cells means more heterogeneity and more somatic mutations (see below)[57,58]. As mentioned before, fibroblasts cannot be expanded beyond Hayflick's limit but iPSC has no such limit[5,61]; (7) as noted earlier, heterogeneity and mutation content of trans-differentiated hepatocyte like cells will be more because they originate from more fibroblasts[58]. Theoretical mutation rate is 10^{-7} per gene per cell[62,63]. One can screen iPSC for potentially harmful mutations, as noted before, because they can be clonally selected and expanded without a limit in practice; and (8) the cell cycle of stem cells, especially pluripotent stem cells are different and has special mechanisms for more faithful DNA replication and repair than somatic cells ensuring better genetic reproducibility and hence lesser number of mutations[64-69].

To conclude we have a long way to our goal of generating hepatocytes by directed differentiation from ES/iPSCs or by transdifferentiation from somatic cells. Currently for many reasons iPSC derived hepatocytes are superior to hepatocyte like cells from transdifferentiated fibroblasts or other somatic cells.

CALL FOR A UNIFIED EFFORT SIMILAR TO THE HUMAN GENOME PROJECT FOR ELUCIDATING DEVELOPMENTAL PATHWAYS AND SIGNALING

Before we compare and promote one method or the other for generating hepatocyte like cells, we need to define methods/protocols to evaluate and compare efficiency of differentiation, we need to define the standards and more importantly a clear definition for hepatocyte like cells. A more comprehensive analysis of the epigenome, RNAs and proteome of the different hepatocyte like cells are required to set the standards- the gold standard of comparison being human primary hepatocytes. Methods to compare and analyze the expression profiles, standards and references to be compared with need to be defined.

Currently different groups are working independently and coming up with differentiation protocols and each group claiming an advantage for their protocol. Directed differentiation protocols need to be compared, analyzed and tweaked systematically and logically than empirically. This is true not only for hepatocytes but also for several other cell types such as cardiomyocytes, neurons, retinal cells, cartilage, macrophages, endometrial or germ cells, which could eventually be used in clinics. There is a need for a well-coordinated global initiative comparable to the scale of the Human Genome Project[70] to achieve this goal in the near future.

ACKNOWLEDGMENTS

The author is thankful to Dr. Mitradas Panicker for the useful discussions. The author is indebted to Mr. Alan Alfieri for carefully reading the manuscript and improving it remarkably.

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ISSN 1007-9327
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