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Review Article

Human embryoid bodies to hepatocyte-like clusters: Preparing for translation

Giuseppe Pettinato, Melissa T. Thompson, Robert A. Fisher*

Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

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ABSTRACT

End-stage liver disease and acute liver failure are some of the most common causes of death worldwide, affecting over 40,000 patients in the United States, for most of whom liver transplantation is the only treatment. Transplantable livers are obtained primarily from deceased donors and living donors in the east, with demand outstripping supply, leading to thousands of deaths each year for those on the transplant waiting lists.

As a functional liver support bridge to liver transplantation, human primary hepatocytes have been transplanted with low success, owing to their inability to grow and expand *in vitro*, their high sensitivity to cold storage-induced damage, and their dedifferentiation following 2D culture. In the past decade, human induced pluripotent stem cells (hiPSCs) have been studied as a potential alternative to liver and primary hepatocyte transplantation through their differentiation into hepatocyte-like cells (HLCs). Differentiation of hiPSCs into HLCs is limited by the low percentage of differentiated cells that reach a mature hepatic phenotype, poor reproducibility of existing differentiation protocols, and inadequate long-term viability and function *in vitro* and *in vivo*. In this review, we will discuss the mechanisms of the various techniques that aim to improve the hepatic differentiation of hiPSCs into mature and genotypically stable HLCs for use in drug studies, as a functional liver support bridge for liver transplantation after liver failure or as therapy for liver regeneration and replacement.

1. Introduction

In the United States, more than 40,000 patients die of end-stage liver disease and liver failure, and an additional 2000 patients suffer from acute fulminant hepatic failure.[1] The time period is in one year. The only available treatment for most of these patients is liver transplantation. Livers from brain death and heart death human donors account for 96% of the transplanted liver pool and represent the most common source for transplantation in the Western world. However, the high demand of donor livers creates an imbalance in which it outstrips supply. Therefore, finding alternatives to solid liver transplantation is an important strategy to increase the number of transplants and improve patient outcomes.

Hepatocytes have been used widely in drug evaluation and liver disease studies *in vitro* and for transplantation. Although the techniques for isolating human hepatocytes are well established, it is the shortage of human liver tissue that is the major supply problem that limits this cellular therapy. Moreover, hepatocytes cannot be maintained easily in culture for extended periods. Furthermore, hepatocytes have limited ability to expand *in vitro,* even when specific growth factors are given (e.g., hepatocyte growth factor).[2] Hepatocytes are also difficult to cryopreserve, because they are highly sensitive to freeze-thaw injury.[3] Therefore, alternative sources of hepatocytes are being examined to solve the dilemma.

Human induced pluripotent stem cells (hiPSCs) represent a promising option in regenerative medicine, based on their pluripotency, high proliferative capacity, and absence of ethical controversy. hiPSCs can be generated by retro-engineering a patient's cells into a pluripotent state through the addition of various stemness factors.[4–6] Differentiation of hiPSCs into Hepatocyte-like cells (HLCs) is a potential cell therapy strategy for liver failure, bioengineered livers, and pharmaceutical testing.[7] However, the translational potential of stem cell-derived HLCs is limited by their

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* Corresponding author.
E-mail address: rafisher@bidmc.harvard.edu (R.A. Fisher).

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scalability, immature genotypes post-differentiation, and poor long-term function after transplantation.[8–10] Recent studies have shown the potential of HLCs that are generated from hiPSCs.[11,12] These cells acquire the ability to secrete human albumin and alpha-1-antitrypsin (A1AT), synthesize urea, and regulate cytochrome P450 (CYP) enzymes *in vitro*.

This review will focus on the latest research on HLCs that are derived from hiPSCs and the promising technology of human embryoid bodies (EBs). The formation of human embryoid bodies is particularly important, because a fundamental characteristic of living organisms is their cellular organization in a three-dimensional structure, allowing adequate polarization and tissue organization in defined functional structures.

2. Embryoid body formation for three-dimensional culture

The dedifferentiation of hepatocytes into a two-dimensional cell culture (monolayer culture) is a well-described phenomenon that is accompanied by a reduction in hepatocyte function, such as its detoxification properties and decreased plasma protein production (i.e., albumin).[13] Two-dimensional culture forces adherent cells to modify their cytoskeleton toward a flattened morphology. This change in morphology limits cell–cell and cell-matrix interactions, diminishing cell polarization and impairing signaling pathways that are needed for normal hepatocyte function.[13] Such alterations are especially pertinent to hepatocytes, which are polygonal and have a multi-polarized structure with at least two basolateral and two apical surfaces.[14] Maintaining liver parenchymal function *ex vivo* is needed for fully functional hepatocytes for use in toxicology screens,[13] for primary hepatocyte transplantation into patients, and the creation of bioartificial liver devices. The concept of full inducible hepato-cellular function has to be taken into consideration when using hiPSCs to differentiate HLCs *in vitro*.

The adequate and controlled differentiation of hiPSCs into a specific cell lineage with high throughput is fundamental for therapeutic purposes, especially when a considerable amount of one or more specific cell populations is needed. A common technique for differentiating hiPSCs requires the production of human hEBs. EBs consist of a three-dimensional cell aggregate that resembles the structure of a developing embryo.[15] EBs allow us to generate cells from all three germ layers, and their differentiation relies on the nature of the EBs, which is influenced by the media,[16] the number of cells that constitute the EBs, and their size.[17–19] For instance, small EBs cannot survive differentiation, whereas those that are too large can result in core necrosis.[19]

Based on recent advances, EBs can be obtained using several methods: i) by spontaneous self-aggregation in non-adhesive wells/dishes under static conditions,[20] ii) in a hanging drop,[21] or iii) by agitation (rotary culture, rocked culture, bioreactor)[22] or through microcavities and agarose micromolds.[23–26]

Conventional techniques for EB formation that are based on mechanical dissection of colonies have generated colony-derived EBs with a heterogeneous population that have not been reproducible with regard to size and cell type.[27] To ensure that EBs from hiPSCs are developed in a synchronized manner, the use of singularized hiPSCs is optimal, allowing strict control of the cell seeding density in every EB to manage its dimensions and consistency.

Several bioreactors have been constructed to produce hEBs and differentiate them in a precise scalable manner.[28,29] Regardless of the benefits of this technology, the transplantation of differentiated cells using bioreactors has not shown any advantages for tissue replacement.[30] When hiPSCs are dissociated, they are at greater risk of apoptosis than when they are maintained as part of a colony, decreasing the rate of hEB formation from individualized hiPSCs.[31] Small molecules, such as the Rho-associated kinase (ROCK) inhibitor Y-27632, ensures the viability of singularized hiPSCs, most likely by preventing anoikis or promoting cell–cell contact to facilitate their aggregation.[31] Although ROCK inhibitor (ROCK-i) promotes the aggregation of dissociated hiPSCs, this small molecule might preclude the use of differentiated hiPSCs in a clinical setting.[32] Another method of promoting aggregation in a suspension of single hiPSCs is centrifugation (i.e., the spin EB method).[33] This technique can induce damage in the hiPSC and might be an obstacle to automated scalable production of hEBs.[34]

In order to avoid the use of ROCK-i and centrifugation, our group developed a new technology that has allowed us to produce uniform hEBs from dissociated hiPSCs by using an agarose micromold.[24,25] Through precise control of the cell seeding density, we obtained homogeneous and synchronized hEBs in a scalable manner. Starting from a homogeneous pool of EBs, it was possible to effect more synchronous differentiation, such that all EBs could respond similarly to various growth factors.

3. Differentiation strategies

Before being considered for clinical application, HLCs that are obtained from hiPSCs have to be compared with primary hepatocytes and have similar morphology and function. In the past 10 years, many differentiation protocols have been published on the generation of HLCs from hESCs and hiPSCs.[11,35–40] All of these studies have shown that the differentiation and culture homogeneity are dependent on several variables in the culture system, including how the hiPSCs are cultured (2D vs. 3D), the differentiation protocol, and the scalability of the resulting differentiated cells. The best way to efficiently differentiate HLCs using hESCs and hiPSCs is by recapitulating *in vitro* the proper signaling pathways that are observed in *in vivo* embryo development studies.

Liver development in the embryo follows three steps: the formation of the definitive endoderm (DE), hepatoblast formation and proliferation, and the differentiation of hepatoblasts into mature, functional hepatocytes. Hepatoblasts are bi-potential stem cells that give rise to the main cell lineages of the liver: hepatocytes and biliary epithelial cells (cholangiocytes).[41]

A sequence of signals *in vivo* drives this process, which ultimately leads to liver organogenesis. In particular, specification of the mesendoderm, from which the mesenchyme and endoderm arise, is propelled by the nodal, bone morphogenetic protein (BMP), and activin signaling pathways.[42,43] In conjunction with activin-A, the stimulation of other pathways has also been demonstrated to promote endoderm formation, including fibroblast growth factor (FGF) and Wnt signaling.[44,45] In certain protocols, low doses of serum are required for activin-A to facilitate endoderm differentiation.[11,42,46]

Additional signaling from the FGF and BMP families, specifically by BMP4, FGF2, and FGF4, leads to the differentiation of hepatoblasts.[11,47,48] Following the formation of the liver bud, the inferential signals of hepatocyte growth factor (HGF) and oncostatin drive hepatoblasts to differentiate into hepatocytes.[49]

Despite the sequential administration of growth factors that are involved in hepatogenesis to differentiate hiPSCs through various stages, no hepatic differentiation protocol has addressed inhibition of the Wnt pathway, which occurs during *in vivo* liver development.[12,35,50–53] The effects of Wnt/β-catenin signaling on cell differentiation into specific lineages, including hepatocytes, is widespread during embryogenesis across species,[54] and its influence on liver embryogenesis is highly regulated over time.[55,56] In the first stages of liver development, β-catenin expression is elevated at E10-E12, declining after E16.[57,58] During hepatogenesis, Wnt pathway regulation occurs late in cell differentiation and, in association with β-catenin, is fundamental in mediating the

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