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The road to regenerative liver therapies: The triumphs, trials and tribulations

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ABSTRACT

The liver is one of the few organs that possess a high capacity to regenerate after liver failure or liver damage. The parenchymal cells of the liver, hepatocytes, contribute to the majority of the regeneration process. Thus, hepatocyte transplantation presents an alternative method to treating liver damage. However, shortage of hepatocytes and difficulties in maintaining primary hepatocytes still remain key obstacles that researchers must overcome before hepatocyte transplantation can be used in clinical practice. The unique properties of pluripotent stem cells (PSCs) and induced pluripotent stem cells (iPSCs) have provided an alternative approach to generating enough functional hepatocytes for cellular therapy. In this review, we will present a brief overview on the current state of hepatocyte differentiation from PSCs and iPSCs. Studies of liver regenerative processes using different cell sources (adult liver stem cells, hepatoblasts, hepatic progenitor cells, *etc.*) will be described in detail as well as how this knowledge can be applied towards optimizing culture conditions for the maintenance and differentiation of these cells towards hepatocytes. As the outlook of stem cell-derived therapy begins to look more plausible, researchers will need to address the challenges we must overcome in order to translate stem cell research to clinical applications.

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1. Introduction

The liver is the largest internal organ in an adult organism. It performs many important functions that sustain the organism's vitality. Liver metabolizes nutrients from ingested food and regulates glucose levels by converting excess glucose to glycogen for storage and releasing it when the blood glucose level is low. It detoxifies xenobiotics and harmful metabolites, and synthesizes many proteins in the blood (Lemaigre and Zaret, 2004). Most of these functions of the liver are carried out by the parenchymal cell type, hepatocytes, that comprise approximately 70% of the adult liver mass.

Liver failure may arise from many causes, including cirrhosis, viral infections and drug overdoses. Typically, the liver has a tremendous regenerative capacity to repair itself. After partial hepatectomy to remove two-thirds of the liver surgically, it is capable of regaining its original mass over time. However, liver disorders can compromise its inherent regenerative capacity and result in complete liver failure leading to death. Although treatment of the symptoms can alleviate the severity of liver failure, organ transplantation is the only curative treatment. However, a severe shortage of donors has limited the access of liver transplants for many patients. As of 2012, there are approximately 17,000 people on the waitlist for liver transplantation in the United States alone, while only half the number of transplantations were performed annually because of the shortage of donor organs (United Network for Organ Sharing: http://optn.transplant.hrsa.gov).

Extracorporeal liver devices have been explored as a treatment to sustain patients until successful liver regeneration, or until a donor organ becomes available. These extracorporeal devices comprise of hepatocytes from a variety of cell source (porcine, human, *etc.*) as well as mechanical components to provide temporary assistance (Carpentier et al., 2009; Gerlach et al., 2002). The mechanical components of the device employ filtration, adsorption or dialysis to remove small molecular weight toxic metabolites from the patient's blood, while hepatic cells provide the bio-transformative and biosynthetic functions (Carpentier et al., 2009; Nyberg, 2012). Other approaches of liver failure treatments include transplantation of dissociated hepatocytes from organs and implantation of tissue engineered liver analogs to augment liver's regenerative capacity (Fukumitsu et al., 2011; Hughes et al., 2012; Soltys et al., 2010).

For applications involving liver cells such as extracorporeal devices, cell transplantation and tissue engineering, primary human hepatocytes have been the preferred cell source because of its low risk of immunogenicity. However, difficulties in expanding and maintaining primary hepatocytes in culture still remain a major hurdle in this field. Even with

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the use of isolated liver cells to expand the pool of hepatocytes, the need still exceeds the availability of hepatocytes. Furthermore, functional capabilities of hepatocytes in culture decrease rapidly during *in vitro* culture (Soltys et al., 2010). Thus, in addition to maintaining our desired cell type, we need to also address the need for large quantities of primary cells that are needed for future cellular therapy or other therapeutic applications.

Hepatocytes isolated from other species, primarily porcine, may provide an alternative cell source, however, these cells also suffer from rapid decrease in functional activities when cultured *in vitro* similar to primary human hepatocytes. Moreover, the differences in drug metabolism and other hepatic functions compared with human hepatocytes, along with potential immunogenic concerns, render these xenogeneic hepatocytes less than desirable compared to human sources (Nyberg, 2012).

For future medical applications of liver cells, including cellular therapy and extracorporeal liver assist devices, *in vitro* cultivation is most likely to be employed to expand the supply of human cells. These expanded cell population can then be guided to differentiate to the desired cell type for specific applications. In the past few years, stem cell research has made significant advances; stem cells and progenitors cells can now be isolated from various sources, and further expanded and differentiated towards the liver lineage. This has brightened the prospect of generating large numbers of functional hepatocytes for applications in hepatic cell transplantation, extracorporeal liver-assist devices and liver tissue engineering. In this article, we will highlight those advances and the path forward for transforming these protocols into standard clinical therapies.

1.1. Embryonic liver development — The guide for in vitro culture processes

In this section, we will describe the development of mouse liver, as an example of mammalian development, being mindful that the development in mouse and man differs in certain aspects.

In early embryo development, the blastocyst consists of an inner cell mass and an outer layer of trophoblast cells. The inner cell mass develops from the primitive blastocyst after the cells undergo polarization and are subjected to certain signaling pathway cues (Johnson et al., 1986; Yamanaka et al., 2010). During this developmental stage, embryonic stem cells can be isolated from the inner cell mass which can give rise to all three germ layers. The inner cell mass will further differentiate to two specialized cell type, hypoblast and epiblast cells (Gardner and Rossant, 1979). Hypoblast cells will give rise to extraembryonic tissues, while epiblast cells will make up all the tissues in an adult by first differentiating to ectoderm and mesoendoderm (De Miguel et al., 2009). During this time, the formation of the primitive streak will set in place the bilateral symmetry and anterior–posterior axis indicating the start of gastrulation (Liu et al., 1999). This event marks the beginning of the delineation between the three germ layers, ectoderm, endoderm, and mesoderm, leading up to organogenesis.

The differentiation of the intermediate stage, mesoendoderm, is driven primarily by *Nodal* signaling, a member of the transforming growth factor (TGF-β) family, as demonstrated in an explant model (Conlon et al., 1994; Feldman et al., 2000; Gritsman et al., 1999). The *Nodal* protein acts as a morphogen; high levels promote endoderm formation and low levels promote mesoderm formation. In the developing mouse embryo, *Nodal* is produced at the anterior region of the primitive streak where it can exert its effect through a number of downstream transcription factors, including *Foxa2*, *Sox17*, *Gata4-6*, *Mixl1* and *Eomesodermin* (Kim et al., 2011). The expression level of the genes regulated by these transcription factors delineates the difference between endoderm and mesoderm. The importance of *Nodal* signaling for endoderm commitment was demonstrated in multiple transplantation studies that showed ectopic regions expressing *Nodal* signaling can induce cells to express endoderm markers and differentiate further into endodermal derivatives (David and Rosa, 2001; Schier, 2003).

The epithelial layer of endoderm, in close contact with a thick layer of mesenchymal cells called the septum transversum, will give rise to the digestive and respiratory organs, including the liver. The processes by which cells undergo a massive transformation into a multi-layered group of cells from the blastula are regulated by several signaling pathways. The most widely studied pathway, FGF signaling, can cause cells to undergo an epithelial to mesenchymal (EMT) transition by decreasing the amount of cell–cell adhesion (Ciruna and Rossant, 2001; Rossant et al., 1997). The decrease in adhesion allows cells to expand and spread out to form new layers of cells. The initial stage of gastrulation involves the invagination of the epithelium which results in the cell movement to subdivide the gut tube into foregut, midgut and hind gut regions. In the ventral region of the foregut, the cardiogenic mesenchymal cells secrete several fibroblast growth factors (FGFs), in conjunction with bone morphogenic proteins (BMP) (also members of TGF-superfamily) produced by the septum transversum mesenchyme (STM) cells, to induce hepatic specification (Douarin, 1975; Fukuda-Taira, 1981; Jung, 1999; Rossi et al., 2001; Zhang et al., 2004). These growth signals stimulate the formation of fast proliferating hepatoblasts and commit this segment of the endoderm to develop into the liver bud.

Hepatic endoderm cells or hepatoblasts are bi-potential and can undergo differentiation to hepatocytes or biliary epithelial cells that line the lumen of the intrahepatic bile ducts. Hepatoblasts are capable of proliferating extensively and invading the surrounding septum transversum. Endothelial cells then interact with these hepatoblasts by providing specific growth factors needed for hepatoblasts maintenance and proliferation (Matsumoto et al., 2001). Between E10-15 in mice, the formation of the liver bud undergoes rapid growth and vascularization. By day E14-15, the liver bud is a highly vascularized tissue. During this stage of liver development, the STM continues to provide BMP signaling, and additionally expresses hepatocyte growth factor (HGF), while the hepatoblasts express C-Met, the HGF receptor (Ishikawa et al., 2001). HGF acts as a suppressor for apoptosis of hepatoblasts by promoting hepatoblasts proliferation through Wnt3a *via* the Wnt-Beta-Catenin pathway (Hussain et al., 2004). It has been speculated that both HGF and BMP provide growth signals, perhaps through parallel pathways. In addition to HGF, Oncostatin is released by the hematopoietic cells which promotes hepatocyte differentiation and maturation through the JAK/STAT3 signaling pathway through activation of the gp130 receptor (Kamiya et al., 1999). The functional cells will slowly undergo maturation and the biliary network will form as the liver attains the appropriate tissue architecture and functional capability (Lemaigre and Zaret, 2004; Zaret, 2002; Zorn, 2008). The detailed mechanisms behind liver bud development and subsequent maturation of the hepatocytes have been investigated further using microarray analysis on samples taken through various time points of mouse embryonic development (Li et al., 2009). The importance of these findings will be illustrated in the next few sections when we discuss the selective growth factors for *in vitro* differentiation.

1.2. Fetal liver cells

Progenitor cells derived from the fetal liver may be a promising source of hepatocytes for liver cell based therapy. Hepatoblasts, a common progenitor population during liver development, can give rise to both hepatocytes and cholangiocytes. These hepatoblasts exhibit a larger proliferative potential compared to primary hepatocytes because of its less differentiated state. Furthermore, they are less prone to de-differentiation in culture than primary hepatocytes and are more suitable for transplantation (Kung and Forbes, 2009). Thus there has been a long sustained interest in their isolation and *in vitro* cultivation. However, because these cells are isolated from fetal livers, the studies have mostly been performed in animal models.

Hepatic progenitor cells have been isolated from fetal rodent livers around E11 to E14.5 by flow cytometry or magnetic activated sorting of dissociated liver cells based on the expression of one or more combination of surface markers. Early cell sorting studies used the absence of

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