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Amelioration of Hyperbilirubinemia in Gunn Rats after Transplantation of Human Induced Pluripotent Stem Cell-Derived Hepatocytes

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SUMMARY

Hepatocyte transplantation has the potential to cure inherited liver diseases, but its application is impeded by a scarcity of donor livers. Therefore, we explored whether transplantation of hepatocyte-like cells (iHeps) differentiated from human induced pluripotent stem cells (iPSCs) could ameliorate inherited liver diseases. iPSCs reprogrammed from human skin fibroblasts were differentiated to iHeps, which were transplanted into livers of uridinediphosphoglucuronate glucuronosyltransferase-1 (UGT1A1)-deficient Gunn rats, a model of Crigler-Najjar syndrome 1 (CN1), where elevated unconjugated bilirubin causes brain injury and death. To promote iHep proliferation, 30% of the recipient liver was X-irradiated before transplantation, and hepatocyte growth factor was expressed. After transplantation, UGT1A1$^+$ iHep clusters constituted 2.5%–7.5% of the preconditioned liver lobe. A decline of serum bilirubin by 30%–60% and biliary excretion of bilirubin glucuronides indicated that transplanted iHeps expressed UGT1A1 activity, a postnatal function of hepatocytes. Therefore, iHeps warrant further exploration as a renewable source of hepatocytes for treating inherited liver diseases.

INTRODUCTION

Stem cells offer enormous promise as a source of differentiated cells for curing human diseases. Although liver transplantation is curative for life-threatening metabolic liver disorders (Åberg et al., 2011), minimally invasive catheter infusion of isolated hepatocytes into the liver can partially correct metabolic liver diseases and can greatly reduce the risk of fatal complications (Fox et al., 1998; Lysy et al., 2008; Roy-Chowdhury et al., 2009; Fisher and Strom, 2006). Host conditioning regimens developed in our laboratories (Guha et al., 2002) allow the expansion of engrafted donor hepatocytes, leading to complete cures of animal models of metabolic liver diseases. This host conditioning strategy is now being tested in a clinical hepatocyte transplant trial (University of Pittsburgh, Institutional Review Board [IRB] number PRO09040497).

Clinical application of hepatocyte transplantation has been impeded by the scarcity of donor livers, which are prioritized for organ transplantation. Pioneering studies have shown that somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) that resemble embryonic stem cells (ESCs) (Takahashi and Yamanaka, 2006; Yu et al., 2007). Recent successes in differentiating ESCs and iPSCs into hepatocyte-like cells (iHeps) (Basma et al., 2009; Lavon et al., 2004; Schwartz et al., 2005; Cai et al., 2007; Duan et al., 2010; Si-Tayeb et al., 2010; Song et al., 2009) has opened the possibility of using iPSCs as a renewable source of human and, possibly, autologous hepatocytes.

Although iPSCs at various stages of differentiation have been reported to engraft and improve the survival of mice with severe toxic injury of the liver, neither the cause of death from the toxic liver injury nor the correction of any specific liver function by the engrafted cells has been demonstrated (Liu et al., 2011). In this study, we examined the efficacy of transplanting human iHep cells into Gunn rats, a well characterized animal model of Crigler-Najjar syndrome 1 (CN1) (Roy-Chowdhury et al., 1991, 1993). CN1 is an autosomal recessive disorder in which genetic lesions of *UGT1A1* cause life-long unconjugated hyperbilirubinemia because of a lack of uridinediphosphoglucuronate glucuronosyltransferase 1A1-mediated bilirubin glucuronidation by hepatocytes (Bosma et al., 1992). CN1 is lethal unless treated with life-long daily phototherapy to reduce bilirubin levels. Even with aggressive therapy, patients remain at risk of bilirubin encephalopathy and death. Liver transplantation is the only definitive therapy (Ozçay et al.,

2009). Gunn rats are well characterized animal models of CN1, with genetic and metabolic abnormalities similar to CN1 patients.

In this study, we transplanted human iHeps into the livers of Gunn rats. Proliferation of the transplanted cells was induced by preconditioning a single liver lobe by hepatic X-irradiation (HIR). HIR enhances the engraftment of transplanted cells by transiently disrupting the sinusoidal endothelial barrier. Additionally, reduction of the mitotic capacity of the irradiated host hepatocytes provides a competitive proliferative advantage to the engrafted cells (Guha et al., 2002; Yamanouchi et al., 2009). Here, as with patients, to increase the safety of HIR, we treated only one liver lobe, representing 30% of the liver mass, to achieve regional hepatic repopulation by the transplanted cells.

RESULTS AND DISCUSSION

Characteristics of Adult Human Skin Fibroblast-Derived iPSCs

iPSCs had a typical ESC-like morphology; expressed pluripotency markers (Figure S1A) at levels similar to those of the H1 hESC line (WiCell); differentiated spontaneously to cells of mesodermal, endodermal, and ectodermal origin (Figure S1B); and gave rise to teratomas after injection in severe combined immunodeficiency (SCID) mice (Figure S1C). iPSCs were diploid and contained 46 intact chromosomes (Figure S1D), and RT-PCR showed that exogenous *OCT-4*, *SOX2*, *KLF4*, and *c-MYC* were silenced (data not shown).

Directed Differentiation of Human iPSCs and Characteristics of iHeps

Undifferentiated iPSCs (Figure 1) expressed OCT-4 but there was very little expression of hepatocyte-preferred genes. After exposure to activin A and bFGF, OCT4 expression declined markedly, and the definitive endoderm marker SOX17 and the nuclear factor FOXA2 were expressed in 70%–85% of cells. The fetal hepatocyte marker α-fetoprotein (AFP) was expressed in 30%–50% of cells. After culturing with HGF and DMSO, AFP expression increased and human serum albumin (HSA) and CK18 were expressed. Following exposure to dexamethasone, the morphology of a majority of the cells changed toward that of primary human hepatocytes (Figures 2A–2C). A majority of these cells, termed iHeps, expressed HSA and cytokeratin 18, and less than half of the cells also expressed the asialoglycoprotein receptor (ASGPR), a marker of mature hepatocytes. AFP expression declined but was still seen in 1%–5% of cells (Figure 1). mRNA content measured by qRT-PCR showed gene expression of transcription factors that are important in hepatocyte development (e.g., HEX and PROX1) and maturation (e.g., HNF4α and C/EBPα) and secreted proteins as well as cytosolic, ER, plasma membrane, and peroxisomal proteins (Figure S2A). Confocal microscopy showed that ASGPR was distributed in both the plasma membranes and the cytoplasm (Figures S2B–S2D), whereas HSA was present in the cytoplasm (Figures S2E–S2G). In this cell population, some albumin-negative cells stained positive for SOX17, an early endodermal marker. SOX17 staining was absent or very faint in albumin-expressing cells (Figures S2H–S2K). Flow cytometry showed that 60%–90% of cells expressed HSA, whereas 28%–40% of cells expressed ASGPR (Figure 2D). At all stages, differentiation of iPSCs toward iHeps was similar to that seen with human embryonic stem cells (hESCs) (Basma et al., 2009). The iHeps exhibited hepatocyte-like characteristics, including glycogen storage (by periodic acid Schiff staining) (Figures 2E and 2F) and indocyanin green uptake (Figure 2I). They also showed uptake of dioctadecylindocarbocyanine (DiI)-labeled low-density lipoproteins (Figures 2G and 2H). Western blot analysis of cell homogenates confirmed that AFP appeared after iPSC exposure to activin A plus bFGF and increased after exposure to HGF and DMSO (Figure 2J). After exposure to dexamethasone, AFP decreased markedly. AFP was undetectable in mature primary hepatocytes. A trace of HSA was found in iPSCs and at the definitive endoderm stage (Figure 2J). HSA expression increased markedly after exposure to HGF plus DMSO. After exposure to dexamethasone, the HSA content reached 25% of that in freshly isolated primary human hepatocytes (Figure 2L).

In contrast to AFP and HSA, significant levels of UGT1A1 appeared only after exposure to HGF plus DMSO. Developmentally, UGT1A1 is expressed only with hepatocyte maturation after birth. After exposure to dexamethasone, the UGT1A1 content in iHeps increased markedly (Figure 2J), and UGT1A1 activity toward bilirubin was 18.8 ± 3.4 to 25.00 ± 4.6 pmole/min/mg protein (mean ± SD, n = 4), which is 18%–22% of the mean UGT1A1 activity in isolated normal primary human hepatocytes (Figure 2K).

As assessed by ELISA, the iHeps secreted the liver-specific proteins HSA, transferrin, and α-1-antitrypsin (AAT) into the culture medium at 33%, 46%, and 60% of the rate of secretion by primary human hepatocytes under identical conditions (Figures 2L–2N). iHeps secreted urea at approximately half the rate of primary human hepatocytes (Figure 2O). Undifferentiated iPSCs did not secrete HSA, AAT, transferrin, or urea (Figures 2K–2O).

Transplantation and Repopulation of Gunn Rat Livers with iHeps

Gunn rats were transplanted with 2×10^6 viable HSA-positive iHeps by intrasplenic injection as described