Modeling of human liver diseases in mice by liver repopulation with hepatocytes derived from patient-specific induced pluripotent stem cells

Hypothesis and Specific Aims

The development of new therapies for severe liver disease has been hampered by the lack of effective disease models. Many human liver diseases cannot be faithfully modeled in mice because a genetic cause has not been identified or because of species-specific differences. Repopulating the livers of immune-deficient mice suffering from liver failure with human hepatocytes has emerged as a promising alternative. However, viable human hepatocytes cannot be safely obtained from patients in sufficient numbers. A potential solution to this problem is to generate disease-specific hepatocytes for mouse liver repopulation using induced pluripotent stem (iPS) cell technology. Unfortunately, we and others have found that current in vitro differentiation protocols produce human iPS cell-derived hepatocytes that resemble primary human hepatocytes in phenotype and function, but fail to repopulate the livers of mice.

We hypothesize that the natural ability of primary human hepatocytes to engraft and expand in the mouse liver is either underdeveloped or lost in human iPS cell-derived hepatocytes due to inadequacies of the in vitro differentiation protocols. To answer this question, we will transplant human iPS cell-derived liver cells of different maturation stages, corresponding to immature liver progenitors or mature hepatocytes, into an immunodeficient liver repopulation mouse model (see Approach) and determine whether the two cell populations exhibit differences in survival, engraftment, and long-term function. After identifying the specific reason(s) for current findings of failed liver repopulation with human iPS cell-derived hepatocytes, we will use targeted strategies to overcome them.

Specific Aims:

1. Compare the survival, engraftment, and function of immature liver progenitors and mature hepatocytes, both derived by in vitro differentiation of human iPS cells, after transplantation into an immunodeficient liver repopulation mouse model.

Previous reports showed that beta cells capable of therapeutically effective function in vivo can only be generated from human embryonic stem cells if cells resembling pancreatic progenitors are transplanted, not cells that have been fully differentiated in vitro (*1*). Our systematic analysis will reveal whether the stage of differentiation established in vitro also determines the liver repopulation capacity of iPS cell-derived liver cells. Moreover, this analysis will identify the specific issues that need to be addressed to achieve liver repopulation with iPS cell-derived liver cells in mice, and potentially also in humans.

2. Deliver liver progenitors or mature hepatocytes derived from human iPS cells directly into the liver parenchyma using an extracellular matrix-like substrate.

Current hepatocyte transplantation methods involve splenic or portal injection of cells, which then become entrapped in the liver sinusoids before migrating into the liver parenchyma. This method may pose a problem for delivery of iPS cell-derived hepatocytes due to poor migration of cells across the sinusoids or elimination by liver macrophages, a cell type preserved in most immunodeficient liver repopulation mouse models (see Approach). By delivering cells directly to the liver parenchyma, we can exclude factors associated with the current transplant method, which may be contributing to poor engraftment. By measuring survival, engraftment and function of primary human hepatocytes delivered in this alternative fashion, we can further define any specific issues that need adjustment to allow engraftment of human iPS cell-derived hepatocytes. Lessons learned from this approach will also have an impact on the development of cell delivery systems for cell therapy in the clinical setting.

Career Plan

I have completed three years of General Surgery residency, and I plan on pursuing additional training and a career in transplantation after completing residency. As a surgical resident interested in transplantation and academic surgery, skills enabling me to contribute to the field of transplantation in the form of basic and translational research are very important to me. Stem cell research, in particular if using iPS cells derived from patient biopsy specimens, offers exciting new opportunities for liver disease modeling and therapy. Academic surgeons trained in techniques related to stem cell research will be instrumental in translating research advances into clinical applications. By carrying out the proposed project, I will receive training relevant to both basic and translational research. Therefore, I will not only have the immediate opportunity to contribute to advancing the field, but will also be a strong candidate for future career development and research funding from the National Institutes of Health and the California Institute for Regenerative Medicine. In summary, I hope this award will allow me to begin to contribute to the field of liver cell therapy in a significant way, and act as a stepping-stone to a successful career as a surgeon/scientist and principal investigator.

Background and Innovation

Creating chimeric mice with human livers has been performed to investigate infection with human hepatotropic viruses and drug toxicity. Currently, however, only primary human hepatocytes have been successfully used to repopulate the livers of immunodeficient mice (*2-8*). Modeling of human liver diseases in such mice has been hampered by the limited availability of patient-specific hepatocytes. A solution to this problem would be to use induced pluripotent stem (iPS) cells, which can be generated from human biopsy specimens including skin fibroblasts (*9*). These cells could be expanded and differentiated *in vitro* and transplanted into immunodeficient mice (*10*), allowing repopulation with human hepatocytes genetically identical to those from the donor. Thus far, however, repopulation of a mouse liver with hepatocytes derived from iPS cells has not been achieved. Simply using a greater quantity of cells delivered by splenic injection has not been sufficient to overcome this problem (*11*), and clinically there is a limit on how many cells can be injected due to transient portal hypertension that results from portal vein injection. For these reasons, a systematic investigation of the underlying reasons and likely a new system of cell delivery is needed in order to repopulate a mouse liver with human iPS cell-derived hepatocytes.

Potential reasons that hepatocytes derived from iPS cells are not able to efficiently engraft and repopulate a mouse liver are 1) decreased survival of iPS cell-derived hepatocytes following transplantation due to the transplantation technique or elimination by liver macrophages, 2) poor migration of iPS cell-derived hepatocytes across the liver sinusoids into the liver parenchyma and 3) insufficient replication of iPS cell-derived hepatocytes following successful engraftment.

The vast majority of laboratories working on generating human iPS cell-derived hepatocytes for liver disease modeling and liver cell therapy are currently struggling due to failure of these cells to repopulate the livers of mice, despite near-normal function and appearance *in vitro*. Thus, it is surprising that a systematic analysis of the underlying issues, such as proposed here, has not been carried out.

We anticipate that identifying limitations specific for the maturation stage of the transplanted iPS cell-derived hepatocytes or characteristics of the current method of transplantation that prevent their efficient and stable engraftment may lead to the development of new methods to allow liver repopulation. Additionally, tissue-engineering techniques using degradable extracellular matrices as carrier materials are expected to aid in creating a delivery system that circumvents the problems associated with current techniques by delivering newly transplanted hepatocytes directly into the liver parenchyma (*12-15*).

Our laboratory has generated multiple iPS cell lines from liver disease patients, which are equivalent to the widely used human embryonic stem (ES) cell line H9 in pluripotency as measured by marker analysis and teratoma assays (data not shown). We and others also found that human iPS cells and human ES cells can be differentiated into hepatocytes with similar efficiency and that derivatives of both fail to achieve significant liver repopulation after transplantation into mice (*16*). Therefore, we will focus on human iPS cells in the proposed experiments.

Approach

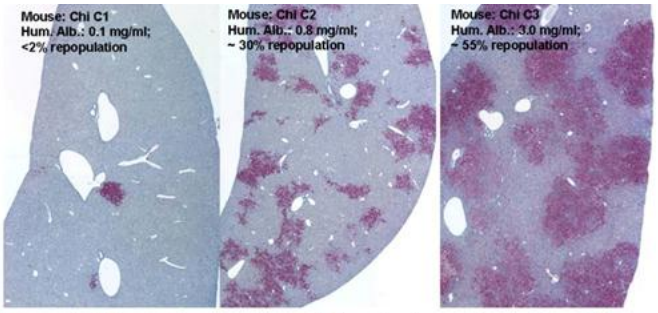

Figure 1: Mouse liver sections following transplantation with primary human hepatocytes. Staining for FAH, present only in transplanted human cells, shows engraftment and progressive expansion of human hepatocytes within the mouse liver parenchyma.

The mice we will use for liver repopulation are immunodeficient and carry a genetic defect that causes hepatocyte injury. In these mice, genetic deficiency of fumarylacetoacetate hydrolase (FAH), a tyrosine degrading enzyme expressed specifically in hepatocytes, leads to tyrosinemia type I and results in liver failure (*10*). The drug 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) is protective in FAH-deficient mice, and severe hepatocyte damage and liver failure only occur when the NTBC is withdrawn (*17*). This confers a selective growth advantage to wildtype hepatocytes transplanted into Fah-deficient mice. In order to allow engraftment of human hepatocytes in mice, we will use severely immunodeficient FAH-deficient mice with additional knockout mutations of *Rag2* and *Il2rg* (resulting in absence of B, T and natural killer cells, while macrophages are present) (*2*). This mouse model allows transplants of primary human hepatocytes to be efficiently expanded in the recipient livers by withdrawal of NTBC.

Our laboratory routinely repopulates *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice with primary human hepatocytes. Figure 1 shows engraftment and clonal expansion of human hepatocytes in these mice's livers, resulting in stable human albumin secretion into the blood.

Sequential differentiation of undifferentiated iPS cells into immature liver progenitors and further into mature hepatocytes can be achieved with growth factors acting at corresponding stages in mouse liver development (*18, 19*). Our laboratory routinely uses a protocol that, at day 25, produces a homogeneous population of near-mature hepatocytes from human iPS cells (Figure 2). In work related to Aim 1, we will transplant these cells and cells obtained at day 8, which resemble liver progenitors (Figure 2), using splenic injection. In this procedure, a single cell suspension is injected into the mouse spleen via a left flank incision. From the spleen, the hepatocytes travel via the portal vein into the liver, where they become entrapped within the sinusoids before migration into the liver parenchyma (*20, 21*). We will measure cell survival and engraftment following transplantation by liver sectioning and staining of the mouse livers for FAH, which is specific for hepatocytes and not expressed in the recipient cells. Hepatocyte function will be assessed by measurement of human albumin in the mouse blood, and transition from a mouse to a human lipid profile (Table 1).

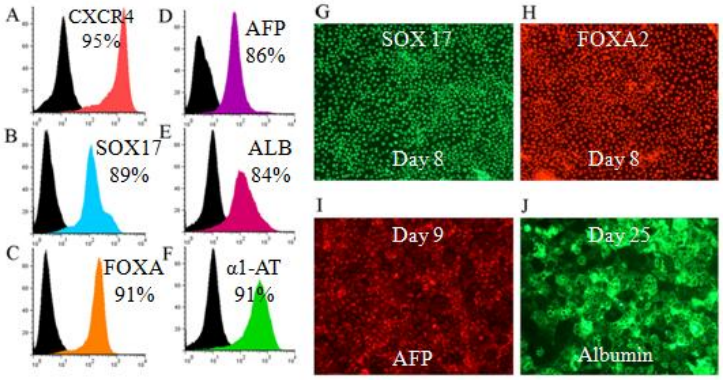

Figure 2: Differentiation of iPS cells to hepatocytes. Flow cytometry at day 5 showing markers of definitive endoderm (A-C) and mature hepatocytes (D-F), and immunostaining (G-I) showing markers of hepatocyte differentiation.

Table 1: iPS Cell Derived Hepatocytes

	Survival	Engraftment	Hepatocyte function
Measure	Number of human iPS cell derivatives in the liver parenchyma relative to the number of injected cells	Number of human iPS cell derivatives in the liver parenchyma relative to the number of surviving cells	Functional output (serum human albumin level) relative to the same number of primary human hepatocytes
Time Point	3 days	7 days	>2 weeks

By comparing mature and immature hepatocyte survival, engraftment and function, we will be able to identify specific problems related to the current cell delivery method.

During hepatocyte transplantation via the splenic vein, cells become lodged within the liver sinusoids prior to migration into the hepatic parenchyma. This causes an ischemia-reperfusion like injury as blood flow

through the sinusoids is disrupted by entrapped cells (*22*). This inflammatory reaction leads to activation of liver macrophages (Kupffer cells), which participate in early clearance of transplanted hepatocytes within the sinusoids (*23*). If we find that immediate hepatocyte survival is a limiting factor in cell engraftment, administration of anti-inflammatory agents at the time of transplantation may prevent Kupffer cell activation and lead to enhanced survival of transplanted iPS cell-derived hepatocytes. Subsequent hepatocyte transplants will be done with concurrent administration of anti-inflammatory or macrophage-targeted immunosuppressive agents to test if Kupffer cell activation can be overcome to improve hepatocyte survival.

Specific membrane molecules may be required for successful migration from the sinusoids into the liver parenchyma (*20*), and migration of hepatocytes into the hepatic parenchyma from the sinusoids is critical for cell survival (*21*). Liver progenitors derived from iPS cells may lack the necessary complement of surface molecules necessary for efficient migration into the liver parenchyma; therefore determining the ideal stage of differentiation for cell transplantation is critical. If migration of iPS cell-derived hepatocytes is a problem, treatment with agents to disrupt the sinusoidal endothelium (*24*) may enhance engraftment of transplanted cells in future experiments.

Our prior unsuccessful attempts at engrafting hepatocytes derived from iPS cells have utilized fully differentiated hepatocytes. Recent observations have shown that human iPS cell-derived liver progenitors are able to complete differentiation following engraftment within the liver (*25*). We know from *in vitro* experiments that mature hepatocytes derived from iPS cells have the functional ability to produce human albumin, but immature hepatocyte progenitors may have even better functional capacity after transplantation due to completion of differentiation within the hepatic parenchyma *in vivo*. A similar phenomenon is seen with endoderm differentiation into beta cells in the pancreas, where pancreatic endoderm derived from embryonic stem cells differentiated into glucose-responsive endocrine cells after implantation into mice (*1*). Also, differentiated cells derived from iPS cells exhibit limited expansion and early senescence (*26*), and this is a potential factor limiting their ability to repopulate the liver. We expect that liver progenitors will have greater liver repopulation potential by avoiding the potential problem of senescence. Therefore, transplanting partially differentiated liver progenitors, derived from human iPS cells, may confer a greater ability to replicate once engrafted in the mouse liver, and may have equal or better function *in vivo*.

Another method to overcome the problems of poor cell survival during entrapment within the sinusoids, and poor migration from the sinusoids to the liver parenchyma is to use an alternative transplantation technique. In work related to Aim 2, we will deliver hepatocytes directly into the liver parenchyma using a hydrogel substrate. Hydrogels are 3-dimensional networks of insoluble polymers that replicate the physical characteristics of extracellular matrix (ECM). A variety of natural and synthetic polymers have been used to fabricate hydrogels such as collagen, hyaluronic acid, chondroitin sulfate, fibrin, fibronectin, alginate, agarose, chitosan, and silk (*13*). One of the benefits of hydrogel as a substrate for cell delivery is that providing the adhesive cues for hepatocytes will prevent apoptosis and encourage survival of transplanted cells prior to engraftment (*12*). We plan to use a semi-fluid hydrogel matrix composed of hyaluronic acid, which provides both the necessary adhesive cues to cultured cells necessary to maintain viability, and a vehicle for transplantation (*27, 28*). iPS cells will be cultured and differentiated into hepatocytes in this substrate, then transplanted within the substrate. This will avoid any stress to the cells from manipulation, such as utilization of trypsin for cell detachment, prior to transplantation. This hydrogel matrix has a low enough viscosity to be directly injected into the liver parenchyma, creating a method of cell delivery that avoids the pitfalls and low efficiency of splenic injection. This delivery method is expected to improve survival of delivered cells by providing a temporary ECM following transplantation, and allow delivery of large numbers of viable cells directly into the anatomic location needed to support cell expansion.

Following transplantation, NTBC therapy will be withdrawn allowing transplanted iPS cell-derived hepatocytes to repopulate the liver of the recipient mice. We will measure survival, engraftment, and function of transplanted hepatocytes in the same fashion. We expect that 1) mice transplanted with sufficient quantities of hepatocytes derived from iPS cells will survive as transplanted hepatocytes assume hepatic function within the native liver, and 2) transplanted iPS cell-derived hepatocytes will repopulate the native liver architecture of the mouse creating a chimeric mouse with a humanized liver.

If successful, this work will facilitate the generation of new accurate human liver disease models and provide an in vivo assay to test the efficacy of iPS cell-derived hepatocytes for human liver cell therapy.

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