# HUMAN INDUCED PLURIPOTENT STEM CELLS DERIVED FROM ADULT AND FETAL HEPATOCYTES FOR THE STUDY AND TREATMENT OF LIVER METABOLIC DISEASES

by

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**HUMAN INDUCED PLURIPOTENT STEM CELLS DERIVED FROM ADULT AND FETAL HEPATOCYTES FOR THE STUDY AND TREATMENT OF LIVER METABOLIC DISEASES**

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Hepatocyte transplantation has been used to treat liver disease. The availability of cells for these procedures is quite limited. hESCs and hiPSCs may be a useful source of hepatocytes for basic research and transplantation if efficient and effective differentiation protocols were developed and problems with tumorigenicity could be overcome. Recent evidence suggests that the cell of origin may affect hiPSC differentiation. Thus, hiPSCs generated from hepatocytes may differentiate back to hepatocytes more efficiently than hiPSCs from other cell types. We examined the efficiency of reprogramming adult and fetal human hepatocytes. The present studies report the generation of 40 hiPSC lines from primary human hepatocytes under feeder-free conditions (37 from fetal hepatocytes, 2 from normal adult hepatocytes and 1 from adult hepatocytes of a patient with Crigler-Najjar Syndrome, Type-1). All lines were confirmed reprogrammed and expressed markers of pluripotency by gene expression, flow cytometry, immunofluorescence, and teratoma formation. Fetal hepatocytes were reprogrammed at a frequency over 50-fold higher than adult hepatocytes. Adult hepatocytes were only reprogrammed with 6 factors, while fetal hepatocytes could be reprogrammed with 3 or 4 factors. The increased reprogramming efficiency of fetal cells was not due to increased transduction efficiency or vector toxicity.

We also report the transplantation and differentiation of human fetal hepatocyte-derived iPSCs. We show preliminary data that undifferentiated cells can engraft in mouse livers of FRG

and NOD/SCID mice. Engraftment was based on human DNA presence in liver tissue. Furthermore we differentiated these cells to definitive endoderm and transplanted them to FRG mice. Human DNA and human albumin were present in mouse livers and mouse serum respectively. Finally, full hepatic differentiation was performed, although we show limited results in terms of the cells' ability to express liver specific genes and perform liver-specific metabolism. Taken together, these studies confirm that hiPSCs can be generated from adult and fetal hepatocytes, including those with genetic diseases, and differentiated back to the hepatocyte lineage. Fetal hepatocytes reprogram much more efficiently than adult, although both could serve as useful sources of hiPSC-derived hepatocytes for basic research or transplantation if an efficient hepatic differentiation protocol could be developed.

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## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

| | |
|---|---|
| ActA | Activin A |
| AFP | Alpha-fetoprotein |
| ALB | Albumin |
| ALK-R | Activin-like kinase receptor |
| B27 | B27 supplement |
| BAF155 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 |
| BDESMG | BD embryonic stem cell-qualified matrigel |
| BMP4 | Bone morphogenetic protein-4 |
| BRG1 | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 |
| BSA | Bovine serum albumin |
| $CCl_4$ | Carbon tetrachloride |
| c-MYC | Myelocytomatosis viral oncogene homolog |
| CN-1 | Crigler-Najjar Syndrome Type-1 |
| CXCR4 | Chemokine (C-X-C motif) receptor 4 |
| CYC | Cyclophilin |

| | |
|---|---|
| CYP1A1 | Cytochrome P450, family 1, subfamily A, polypeptide 1 |
| CYP1A2 | Cytochrome P450, family 1, subfamily A, polypeptide 2 |
| CYP2B6 | Cytochrome P450, family 2, subfamily B, polypeptide 6 |
| CYP3A4 | Cytochrome P450, family 3, subfamily A, polypeptide 4 |
| CYP3A7 | Cytochrome P450, family 3, subfamily A, polypeptide 7 |
| DMEM | Dulbecco’s modified eagle’s medium |
| DMSO | Dimethyl sulfoxide |
| DNMT3B | DNA (cytosine-5-)-methyltransferase 3 beta |
| EB | Embryoid body |
| EGF | Epidermal growth factor |
| FBS | Fetal bovine serum |
| FGF1 | Fibroblast growth factor-1 |
| FGF2 | Fibroblast growth factor-2 |
| FGF4 | Fibroblast growth factor-4 |
| FGF8 | Fibroblast growth factor-8 |
| FGF10 | Fibroblast growth factor-10 |
| GDF | Growth differentiation factor |
| GFP | Green fluorescent protein |
| hESC | Human embryonic stem cell |
| HBM | Hepatocyte basal medium |
| Hep | Hepatocyte / hepatic |
| HGF | Hepatocyte growth factor |
| hiPSC | Human induced pluripotent stem cell |

| | |
|---|---|
| HMM | Hepatocyte maintenance medium |
| HNF1α | Hepatocyte nuclear factor 1 alpha |
| HNF3β | Hepatocyte nuclear factor 3 beta |
| HNF4α | Hepatocyte nuclear factor 4 alpha |
| HSC | Hepatic stellate cell |
| hTERT | Human telomerase reverse transcriptase |
| IF | Immunofluorescence |
| IHL | Intrahepatic lymphocyte |
| IL2 | Interleukin-2 |
| IP | Intraperitoneal |
| IS | Intrasplenic |
| ITS | Insulin, transferrin, selenium supplement |
| KC | Kupffer cell |
| KLF4 | Kruppel-like factor 4 |
| KGF | Keratinocyte growth factor |
| LDH | Lactate dehydrogenase |
| LY294002 | PI3K inhibitor |
| MOI | Multiplicity of infection |
| N2 | N2 supplement |
| NANOG | Nanog homeobox |
| NaBut | Sodium butyrate |
| NOD/SCID | Non obese diabetic severe combined immunodeficient |
| OCT4 | Octamer 4 |

| | |
|---|---|
| OSM | Oncostatin M |
| OTC | Ornithine Transcarbamylase |
| PBS | Phosphate buffered saline |
| PFA | Paraformaldehyde |
| PI3K | Phosophoinositide 3-kinase |
| PV | Portal vein |
| RA | Retinoic acid |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SB431542 | Inhibitor of ALK-R |
| SEC | Sinusoidal endothelial cell |
| SEM | Standard error of mean |
| SNAP | S-nitrosoAcetylPenicillamine |
| SOX2 | SRY (sex determining region Y)-box 2 |
| SOX17 | SRY (sex determining region Y)-box 17 |
| SSEA | Stage specific embryonic antigen |
| SubQ | Subcutaneous |
| TRA | Tumor rejection antigen |
| Wnt3a | Wingless-type MMTV integration site family, member 3A |

# 1.0 INTRODUCTION

## 1.1 GENERAL LIVER BACKGROUND

The liver is one of the largest, most complex organs in the human body and is essential for life. It is located below the diaphragm within the abdominal pelvic region of the abdomen and weighs approximately 1500-1800 grams. It is made up of a spongy mass of wedge-shaped lobes [1]. Functions of the liver include, but are not limited to: processing of carbohydrates, fats, and proteins, vitamin storage, detoxification of substances in the blood, metabolism, protein synthesis, glycogen storage, decomposition of red blood cells, plasma protein synthesis, clotting factor synthesis, hormone production, bile production and it regulates high volume biochemical reactions (synthesis and breakdown) of complex molecules necessary for normal physiology and homeostasis [1, 2].

The liver receives blood inflow from the portal vein and hepatic artery. This arterial and venous blood mix together and travel through the specialized liver sinusoids perfusing the entire organ with blood that eventually will drain into the hepatic vein. The hepatic sinusoids perfuse the liver with both oxygen-rich blood from the hepatic artery and venous blood from the portal vein. The portal vein inflow blood has already circulated through the gut, pancreas, spleen and other associated organs and is enriched with toxins and other substances needed to be handled by the specialized cells of the liver for normal body physiology to occur.

The different lobes of the human liver consist of parenchymal cells known as hepatocytes and various non-parenchymal cells. Hepatocytes make up 80% of the total liver volume whereas non-parenchymal cells only make up 6.5%; however 40% of the cells within the liver are of the non-parenchymal type [2]. The walls of the hepatic sinusoid are lined by the following non-parenchymal cells: sinusoidal endothelial cells (SECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) and intrahepatic lymphocytes (IHLs) such as pit cells. Under both normal and pathological conditions there is cross-talk between both hepatocytes and their non-parenchymal cell counterparts through paracrine effects that influence cell behavior and function within the liver microenvironment [2].

SECs line the specialized hepatic sinusoid and their major function is to filter the blood that perfuses the entire liver. SECs have specialized fenestrations that allow free diffusion of blood to the surfaces of hepatocytes allowing the parenchymal cells to perform their various functions to maintain normal body physiology [2].

KCs are liver tissue specific macrophages that have strong endocytic and phagocytic abilities. They secrete potent mediators of the inflammatory response and therefore play an important role in early phase liver inflammation and innate immune defense [2].

HSCs are characterized by their intracytoplasmic fat droplets and well-branched cytoplasmic processes. Under normal liver conditions HSCs store vitamin A, control turnover of liver ECM and regulate sinusoidal contractility and therefore sinusoidal blood flow. When liver damage occurs quiescent HSCs transform to myofibroblast-like cells that play a major role in the development of liver fibrosis and eventually cirrhosis [2].

## 1.2 ORTHOTOPIC LIVER TRANSPLANTATION

The first experimental trials of auxiliary liver transplantation occurred in homotransplantation canine models in 1956 by Goodrich and colleagues [3]. These successful trials laid the groundwork for the first clinical liver transplant in humans to be performed by Thomas E. Starzl in 1963 at the University of Pittsburgh [4]. The report described orthotopic liver transplant (OLT) in 3 patients, one of which died on the operating table (patient 1) and the other two survived 22 and 7.5 days respectively with their grafts (patients 2 and 3). Patient 1 was a 3 year old white male with congenital biliary atresia, patients 2 was a 48 year old African American male with Laenne's cirrhosis and a primary hepatoma and patient 3 was a 67 year old white male with progressive jaundice and an intrahepatic duct cell carcinoma. Long term survival in these studies was limited by the lack of an effective immunosuppressive drug. In 1983 the development of immunosuppressive drug azathioprine allowed OLT to become an established treatment for hepatic failure [5]. The introduction of cyclosporine and FK506 a few years later further solidified the procedure in the medical community [5]. Table 1 summarizes the liver diseases currently treated by OLT in the United States.

**Table 1.** Summary of the Liver Diseases Currently Treated by OLT
Table adapted from the OPTN/SRTR 2010 Annual Data Report [6].

| **Liver Diseases Treated by OLT** |
|---|
| Non-cholestatic Cirrhosis |
| Cholestatic Liver Disease/Cirrhosis |
| Biliary Atresia |
| Acute Hepatic Necrosis |

| |
|---|
| Metabolic Diseases:<br>Wilson's Disease, Hemochromatosis-Hemosiderosis, Tyrosinemia, Primary Oxalosis, Glycogen Storage Diseases, Hyperlipidemia, Crigler-Najjar Syndrome, Carbamoyl Phosphate Synthase Type-1 Deficiency, Ornithine Transcarbamylase Deficiency, Clotting Factor Deficiencies, Familial Hypercholesterolemia, Infantile Refsum's Disease, Progressive Familial Intrahepatic Cholestasis, Argininosuccinate Lyase Deficiency, Citrullinemia |
| Malignant Neoplasms |
| Other:<br>Cystic Fibrosis, Budd-Chiari Syndrome, Neonatal Hepatitis, Congenital Hepatic Fibrosis, Graft vs. Host Disease, Chronic or Acute Liver Failure, Benign Tumor |

Since the initial 3 OLT surgeries, there has been significant advancement in surgical techniques, immunosuppression and overall patient management to the point where OLT is a routine procedure, albeit with the potential complications and risks associated with a whole organ transplant. Currently, the only curative therapy for acute and chronic liver failure and inborn errors in metabolism is OLT. There are three different types of OLT surgeries practiced in the United States today: whole liver, reduced liver and liver segment [1]. Whole liver transplants involve removing a patient's entire liver and replacing it with a whole liver from an organ donor, first performed in 1963 [4]. Since the liver is known to regenerate *in vivo*, surgeons speculated that smaller patients could be transplanted with organs that could be tailored made in size to the patients and have the graft grow with patients as they grow. This observation led to the establishment of the following two liver transplant techniques. Reduced liver transplants are performed when the surgeon makes a donor liver smaller for the recipient, first performed in 1984 [7]. And finally liver segment transplants are surgeries that allow one cadaveric donor liver to transplant two patients, first performed in 1989 and 1990 [8, 9]. Liver segment transplants can also occur with living donor donations as well. Since partial transplantation of the liver to treat patients is possible it seems likely that cell transplantation may also be possible, if issues with

engraftment, proliferation of the graft, immunosuppression and patient management are overcome. Cells can be given to patients with liver failure to allow for metabolic support and potential liver failure reversal. Cells can also be given to patients with inborn errors in metabolism because transplanted cells will be competent in the enzyme or protein that is deficient in the patient with the metabolic disorder.

## 1.3 CELL TRANSPLANT AS A POTENTIAL THERAPY – REASONS FOR DEVELOPMENT

An important question that needs to be answered is whether or not cell transplant, either primary-cell or stem cell-based, is a viable therapy to treat liver disease. The need for cellular transplant to become a viable therapy to treat liver diseases is obvious. According to the most recent annual report performed by the Organ Procurement and Transplantation Network (OPTN) and the Scientific Registry of Transplant Recipients (SRTR), in 2009 there were almost 26,000 people on the liver transplant waiting list and only a total of 6,320 OLTs performed (Figure 1) [6]. This resulted in 1,723 deaths on the waitlist and 15,682 people on the waitlist at year's end (Figure1) [6].

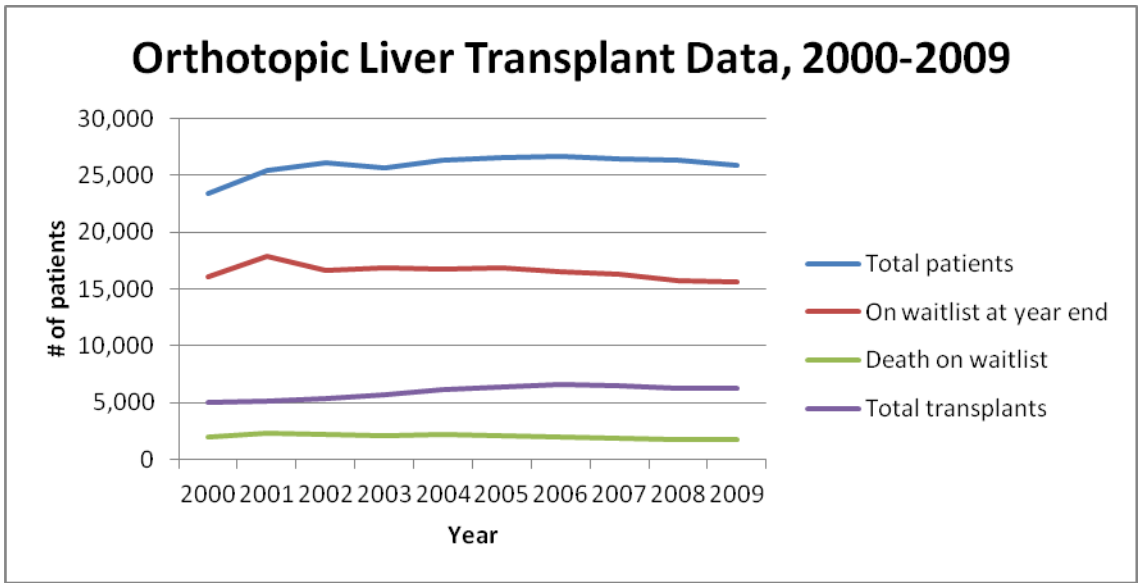


**Figure 1.** Orthotopic Liver Transplant Data, 2000-2009
Number of total patients on the liver transplant waitlist (blue), number of patients on waitlist at year end (red), number of patient deaths on waitlist (green) and total patients transplanted (purple) from 2000-2009. Reproduced from the OPTN/SRTR Annual Report.

Over the course of the past 10 years of collated data, one can see a trend that is continuing. The total number of patients requiring a transplant is staying stagnant and so is the number of total transplants being performed. This is due to the lack of donor livers available for transplant. In order to transplant more patients an alternative source of liver tissue is necessary. When a surgeon performs an OLT there is one donor liver for one patient (in most cases). If cellular transplant can be used to treat patients there can be 1 donor for many recipients, depending on the number of cells necessary to treat the patient and the overall viable cell yield from the isolation. Furthermore, one can increase the amount of tissue used for transplant if cells can be isolated from liver tissues that are normally discarded. Therefore one can identify liver diseases that can be managed or cured by cellular transplant and treat those patients with cells and leave the whole organ transplants solely for diseases that can only be treated by OLT. This

would greatly reduce the burden on the liver transplant waiting list and allow surgeons to decrease the number of patients on the waitlist while increasing the number of total transplants performed. Also, if cell transplant can be shown to manage patient symptoms for certain underlying liver pathologies requiring an OLT, these patients can be given cells as a “bridge to transplant” and be sustained until an organ becomes available for OLT. This procedure would reduce the number of deaths on the transplant waitlist. Moreover, with the emerging fields of human adult stem cell, human embryonic stem cell (hESC) and human induced pluripotent stem cell (hiPSC) research there is the possibility of alternative cell sources to treat liver diseases, which would further reduce the burden on the liver transplant waiting list.

Although OLT is a routine procedure, it still is a major surgery with a high incidence of surgical complications (graft vs. host disease, ischemia/reperfusion injury, graft failure, etc.). Furthermore the expenses associated with the surgery and therapies to maintain the graft for the life of a patient are extremely high. To compound issues further, there are limited donors and the timing of the transplant is critical. In contrast, cell transplantation is less invasive and less costly with fewer complications and risks associated with the procedure. Moreover, cryopreserved cells can be made available on demand from cell banks with cell expansion possible to treat a greater number of patients with the same amount of donor tissue. New cell sources for liver tissue to transplant are also becoming a reality with the discovery of different stem cells (hESCs, hiPSCs and adult stem cells) and the ability to turn these cells into hepatocyte-like cells. These alternative cell sources would solve the problem of the shortage of donor liver tissue to isolate primary hepatocytes from. Taken together this information shows the need to investigate if cellular transplantation to treat different liver diseases is possible. Table 2 summarizes the benefits of cell transplantation over OLT.

**Table 2.** Benefits of Cell Transplantation over OLT
Adapted from Dhawan et al. [5].

| **OLT** | **Cell Tx** |
|---|---|
| One or two patients maximum per one donor liver | Multiple patients can be treated from one donor liver |
| Major surgery | Less invasive surgery compared to OLT |
| Organs have to be immediately transplanted | Hepatocytes can be cryopreserved for long term storage and transplantation on demand |
| Graft failure is life threatening | Graft failure reverts patient to pre-transplantation state; not immediately life threatening |
| Native liver removed | Native liver remains, allowing for its potential recovery |
| Curative treatment, but requires patient to be on life-long immunosuppression (in most cases) | Patients can be potentially weaned off immunosuppression if methods become available |

## 1.4 PRIMARY HEPATOCYTE TRANSPLANT RESEARCH – FROM TISSUES TO CELLS IN ANIMAL MODELS

In 1964 investigators performed the first heterotopic partial autotransplantation of pieces of rat livers [10]. This crude liver graft was demonstrated to have structure and function when transplanted to the kidney capsule or subcutaneous space. This idea of transplanting pieces of liver was further solidified to be viable when sliced rat liver tissue was autologously transplanted into rats subcutaneously [11]. These autografts were approximately 1/10 of the original size and found alive in subcutaneous tissue 1 year after transplant. Partial hepatectomy was performed on the rats prior to transplantation to see if this would increase the size of the graft, however this did not lead to an increase at the end of the 1 year study. Therefore if pieces of liver can be transplanted and shown to have function why can't cells be transplanted and function as well?

Two important studies that paved the way for hepatocyte transplants becoming a potential reality to treat liver diseases occurred in the pancreas and diabetes field. In 1967 a method for the isolation of intact islet of Langerhans from rat pancreas was reported [12] and then in 1972 these rat islets were transplanted into diabetic rats [13]. This cell transplant improved blood glucose levels though intraperitoneal or subcutaneous injection of the cells, thus demonstrating that transplanted cells can function within a recipient. Now a procedure to isolate viable hepatocytes needed to be discovered in order for the hepatocyte transplant field to push forward. This was accomplished in 1969 by Berry and Friend, who described a high-yield preparation of isolated rat liver parenchymal cells using a collagenase perfusion method [14]. Since then this procedure has been refined and optimized with human tissue. Gramignoli and colleagues have developed a purified tissue dissociation enzyme mixture for the isolation of human hepatocytes where the isolated cells can be used in a clinical transplantation setting [15].

Now that it is possible to isolate highly viable and functioning human hepatocytes, researchers need to demonstrate that the isolated cells can be transplanted into liver metabolic disease animal models and function enough to improve blood chemistries and overall symptoms associated with disease. In 1970 cells from a clonal strain of a rat hepatoma were transplanted subcutaneously into Gunn rats that are deficient in the UGT1A1 enzyme [16]. This enzyme deficiency causes animals to become hyperbilirubinemic and subsequently jaundiced. The Gunn rat models CN-1 in humans, which is characterized by the inability to metabolize bilirubin. Bilirubin forms in the blood serum through the metabolism of heme oxygenases released by the breakdown of hemoglobin in old erythrocytes or any other cells that contain hemoproteins. Patients suffering from this disorder experience altered senses, incoordination, slurring of speech, weakness, brain damage and possible coma due to high amounts of unconjugated

bilirubin in the blood serum. Although the cells transplanted were not hepatocytes, they had been previously shown to metabolize bilirubin *in vitro* [17] and were readily available. This was the first report to demonstrate transplanted cells can provide metabolic support, in this case helping to reduce bilirubin levels in rats modeling a known human metabolic disease. Matas and colleagues further developed this research in the CN-1 rat model by transplanting mechanically isolated liver fragments and demonstrating that they too reduce bilirubin levels in blood serum [18]. Finally in 1977 the first actual hepatocyte transplant occurred when investigators took isolated rat hepatocytes and transplanted them via the portal vein into the Gunn rat [19]. They were able to show correction of hyperbilirubinemia. More specifically, they observed a 21% decrease in bilirubin levels at day 10 with a maximum reduction of 35% at day 28. An important caveat of this experiment was the usage of immunosuppression through the administration of cyclophosphamide, which allowed the graft to stabilize within the recipient.

Further evidence that cell transplantation can be used to treat metabolic disease occurred when cryopreserved human hepatocytes were attached to collagen-coated microcarriers and injected IP into athymic-UGT1A1 deficient Gunn rats and athymic-analbuminemic Nagase rats [20]. These Nagase rats are deficient in albumin synthesis. Animals were made athymic in order to be deficient in T-cells to help with stabilization of the graft. Cells formed aggregates on the surface of the pancreas. Gunn rats excreted bilirubin glucuronides (conjugated bilirubins) in bile for 30 days and overall serum bilirubin levels were reduced. Nagase rats had plasma albumin levels increased from a low of 0.024 mg/ml to a high of 4.8 mg/ml 8 days post transplant and remained near this high level for the duration of the 30 day experiment. This study was one of the first to demonstrate that human cells have the ability to engraft after transplant and function metabolically. More importantly this study demonstrated that when you transplant 1-2% liver

mass you get a 15 fold increase in albumin and when you transplant 2-4% you elevate it 60%. 1-2% engraftment does not equal 1-2% increase in albumin synthesis. Therefore a large amount of cellular engraftment is not needed to get a large effect and reversal of pathology and symptoms. This observation is extremely important because it demonstrates that a large supply of viable hepatocytes is unnecessary if issues with engraftment and graft stability can be overcome.

A study performed on the Eizai hyperbilirubinemic rat, which models the human Dubin-Johnson Syndrome, demonstrated that transplanted fully competent rat hepatocytes can engraft and successfully integrate with the hepato-biliary system. These rats have impaired canalicular excretory transport of organic anions, bile acid glucuronides and sulfate conjugates therefore causing conjugated hyperbilirubinemia. Animals were treated with intraportal injections of wild type rat hepatocytes along with a 68% partial hepatectomy to promote hepatic proliferation *in vivo*. This therapy resulted in reduction of serum bilirubin level and bile excretion became possible demonstrated by biliary transport of indocyanine and sulfobromophthalein into bile. Furthermore it shows that hepatic transport of bile acid conjugates can be restored by hepatocyte transplant. Additionally, it is a second report that showed only a transplant of 1-2% of liver mass results in a 50% reduction in bilirubin levels. It appears that one does not need large engraftment to make a major physiological impact within the transplant recipient.

Multi-locational allogenic hepatocyte transplant for the treatment of ascorbic acid deficiency in rats was shown to be possible [21]. Cells were transplanted in the portal vein, spleen omentum and mesentery. Ascorbic acid was found in the serum of recipients up to 3 months after transplant. This report demonstrates that transplanted hepatocytes do not need to engraft into the liver architecture to provide metabolic support.

Further evidence that hepatocyte transplant is a viable method to treat liver metabolic disease occurred when investigators corrected a mouse model of progressive familial intrahepatic cholestasis type-3 (PFIC-3) [22]. Patients that have PFIC-3 have a mutation in the MDR3 gene that encodes the hepatocanalicular phospholipid translocator that results in the absence of phospholipids in the bile. This absence causes chronic bile salt-induced damage to hepatocytes and bile duct epithelium. The mdr2-knockout mouse is the animal model for the human disease. Transplanted mdr2-competent hepatocytes repopulated the liver and were shown to restore phospholipid secretion and diminish liver pathology. More importantly, liver engraftment was shown to be enhanced by administering a high cholate diet to further intensify the liver damage. The thought here is that enhancing liver damage will give the transplanted cells more room to engraft in the liver. However, the animals given a high cholate diet developed multiple hepatic tumors and biliary phospholipid secretion decreased, most likely due to the diet causing enhanced pathology. Animals fed the control diet had slower repopulation; however these transplant recipients were still able to completely abrogate pathology without tumor formation. Overall, these data demonstrate that hepatocyte transplantation could be useful to treat PFIC in humans.

Reduction of serum cholesterol in Watanabe rabbits has also been accomplished by porcine hepatocyte transplant [23]. This is the animal model for the human homozygous familial hypercholesterolemia, an LDL-receptor defect. Investigators demonstrated that the transplanted cells initially localize within the hepatic sinusoids and can over time migrate out of vessels and integrate into the parenchyma. These engrafted cells provided functional LDL receptors to lower serum cholesterol by 30-60% for the 100 day study. This report determined that they transplanted 10% of liver mass, which resulted in 1-5% survival of hepatocytes. Therefore 0.1-

0.5% of liver mass corrects cholesterol by 30-60%, thus one does not need large engraftment to correct disease phenotype.

Wilson's disease was corrected by hepatocyte transplant in a rat model [24]. Long-Evans Cinnamon rats have excessive accumulation of copper within hepatocytes and therefore, are a good model of human Wilson's disease. These animals have the atp7b gene mutation, an ATP-dependent copper transporter, which results in copper cytotoxicity and decreased biliary excretion of copper. The investigators pretreated all rat recipients with retrorsine to inhibit proliferation of native hepatocytes and gave the animals a partial hepatectomy to promote liver repopulation. The liver was shown to have repopulation of transplanted rat cells. This repopulation was <25% at 6 weeks, 26-40% at 4 months and 74-100% at 6 months and longer. The highly repopulated animals restored biliary copper excretion and lowered intrahepatic copper levels. Furthermore the liver histology was completely normal compared to extensive damage in non-transplanted animals. Wilson's disease was also shown to be reversed using hepatocyte transplant as an early intervention to treat the underlying disease pathologies [25]. Rat hepatocytes were transplanted into the same rat modeled mentioned previously via intrasplenic injections. 71% of the transplanted animals became highly repopulated. Copper levels were virtually normal in 60% of these animals and the remaining 40% had substantially less copper compared to control non-transplanted animals. There were positive changes in ceruloplasmin levels; bile copper excretion capacity and liver histology were maintained as expected with the decreases in liver copper levels. This report demonstrated the efficacy of cell transplant to treat inborn errors in metabolism at an early age before extensive liver damage occurs.

Rat hepatocytes have been shown to engraft, survive and proliferate in rat livers that have $CCl_4$ and phenobarbital-induced cirrhosis [26]. This demonstrates that cell transplant is a viable option to treat liver failure and cirrhosis. This was further corroborated when rat hepatocytes were shown to rescue rats with D-galactosamine-induced acute liver failure [27]. Intrasplenic injections of cells and liver cell culture supernatants were shown to lead to a survival rate of 47.1% and 42.9% respectively. Injections were performed 20-28 hours after poisoning. These reports demonstrated that hepatocytes themselves, and the soluble factors that they release, improve survival of the recipient either through engrafting within the liver to provide metabolic support or by stimulating liver regeneration of the recipient's native cells or both

Cell transplant has also been shown to treat chronic liver failure as well. A rat model of $CCl_4$ and phenobarbital-induced chronic liver failure utilized intrasplenic injection of rat hepatocytes to treat the disease [28]. The rats in this model display irreversible liver cirrhosis. Animals receiving the cells showed significant improvements in liver function and prolonged survival compare to non-transplanted controls. These data further demonstrated the utility of hepatocyte transplant to treat liver disease.

Hepatocytes have been shown to provide metabolic support, in a glucose-related function, when transplanted to rats that have received a 90% hepatectomy [29]. Rat hepatocytes were attached to collagen coated microcarriers and were transplanted IP 3 days prior to surgery. Non-transplanted animals died within 48 hours from hypoglycemia. Rats that received cells had significantly higher blood glucose levels and 40% of them survived longer than 28 days. All control rats died within 5 days. When cells were injected IP immediately after the surgery all rats became hypoglycemic and died within 48 hours, suggesting that engraftment is required for the transplanted hepatocytes to provide metabolic support and improve survival.

Primary mouse hepatocyte transplantation improved the phenotype and extended survival in a murine model of intermediate maple syrup urine disease [30, 31]. This was also true in a mouse model of tyrosinemia type-1 [32]. Table 3 summarizes some of the hepatocyte transplants that have been performed to treat different liver diseases within relevant animal models. This table includes the disease treated, transplant site, donor cell species and recipient species.

**Table 3.** Hepatocyte Transplants Performed in Relevant Liver Disease Animal Models
Table summarizes some of the hepatocyte transplants that have been performed in relevant liver disease animal models. Information includes disease treated, transplant site, donor cell species, recipient species and reference of paper according to first author last name.

| Disease Treated | TX Site | Donor Species | Recipient Species | References |
|---|---|---|---|---|
| CN-1 | SubQ | Rat | Rat | Rugstad et al.[17], Matas et al. [18] |
| CN-1 | Portal vein | Rat | Rat | Groth et al. [19] |
| CN-1 | IP | Human | Rat | Moscioni et al. [20] |
| Analbuminemia | IP | Human | Rat | Moscioni et al. [20] |
| Dubin Johnson Syndrome | Portal Vein | Rat | Rat | Hamaguchi et al. [33] |
| Ascorbic Acid Deficiency | Portal vein, spleen, omentum, mesentery | Rat | Rat | Nakazawa et al. [21] |
| PFIC (type 3) | Spleen | Mice | Mice | De Vree et al. [22] |
| Homozygous familial Hypercholesterolemia | Portal vein | Porcine | Rabbit | Gunsalus et al. [23] |
| Wilson's Disease | Spleen | Rat | Rat | Irani et al. [24], Malhi et al. [25] |
| Maple Syrup Urine Disease | Direct Liver | Mouse | Mouse | Skvorak et al. [30], Skvorak et al. [31] |

| | | | | |
|---|---|---|---|---|
| Tyrosinemia Type-1 | Spleen | Human | Mouse | Azuma et al. [32] |
| Acute Liver Failure | Spleen | Rat | Rat | Gagandeep et al. [26], Baumgartner et al. [27] |
| Chronic Liver Failure | Spleen | Rat | Rat | Kobayashi et al. [28] |
| Acute Liver Insufficiency | IP | Rat | Rat | Demetriou et al.[29] |

## 1.5 INTEGRATION AND INTERACTION OF TRANSPLANTED CELLS WITHIN RECIPIENT

The integration and interaction of transplanted hepatocytes in the host has been determined in a rat model [34]. When cells are transplanted via the portal vein they reach the distal portal spaces and sinusoids within 1 hour after injection. During this process they occlude the portal vein flow and thus increase the portal pressure and cause initial damage of the recipient liver. This was inferred from increases in AST, ALT and LDH 1-2 hours after transplant. The cells then traverse the endothelial barrier through mechanical disruption. The transplanted cells lose their membrane-bound gap junctions facilitated by connexin-32 down-regulation during the migration through the sinusoids. Integration of the graft in the liver parenchyma and bile canalicular activity requires up to 5 days and is associated with connexin-32 up-regulation. MMP-2 degrades the ECM in close proximity to donor cells, which allows space for cell proliferation of the graft. Cells that remain in the portal vein are removed by polymorphonuclear leukocytes and macrophages.

Primary hepatocytes have been transplanted via many different avenues. These include: peritoneal cavity, omentum, mesentery, spleen, portal vein, kidney capsule and lung. Table 3 summarizes some of the animal models of liver disease that have been corrected by hepatocyte transplant.

## 1.6 METHODS TO INCREASE REPOPULATION – GIVING DONOR CELLS PROLIFERATIVE ADVANTAGE

There have been many studies that have investigated ways to increase repopulation of the recipient liver with donor hepatocytes. Methods to increase repopulation include: genetic manipulation of the recipient through delivery of a virus expressing urokinase plasminogen activator to breakdown the ECM and give the transplanted cells more space to engraft [32], growth factor administration in concordance with cell transplantation (VEGF) to help increase migration past endothelial cells [35], pretreatment with partial hepatectomy to stimulate expression of soluble factors in the body that lead to liver regeneration, pretreatment with retrorsine which inhibits proliferation of the native hepatocytes through alkylation of DNA without extensive toxicity and allows transplanted cells to be the only cells that can proliferate, pretreatment with $CCl_4$ or radiation to damage the host liver to allow transplanted cells to have a growth advantage over native cells, administration of vasodilators to promote migration through liver sinusoids, immunosuppression [19], preconditioning with radiation and ischemia-reperfusion [36] to damage native cells and promote proliferation of transplanted cells through oxidative DNA damage and finally hepatic stimulatory substances obtained from regenerating

porcine livers [37]. Table 4 summarizes some of the methods to increase donor cell repopulation and the species of the donor and recipient.

**Table 4.** Methods to Increase Cellular Engraftment in Relevant Animal Models
Table summarizes some of the methods to increase cellular engraftment in relevant liver disease animal models. Information includes method to increase engraftment, donor cell species, recipient species and reference of paper according to first author last name.

| **Method to Increase Engraftment** | **Donor Species** | **Recipient Species** | **References** |
|---|---|---|---|
| Removal of thymus | Human | Rat | Moscioni et al. [20] |
| Partial hepatectomy | Rat | Rat | Hamaguchi et al. [33], Irani et al. [24] |
| Retrorsine | Rat | Rat | Irani et al. [24] |
| $CCl_4$ | Rat | Rat | Gagandeep et al. [26] |
| Phenobarbital | Rat | Rat | Gagandeep et al. [26] |
| D-galactosamine | Rat | Rat | Baumgartner et al. [27] |
| uPA expressing virus | Human | Mouse | Azuma et al. [32] |
| Immunosuppression | Mouse | Mouse | Groth et al. [19] |
| Radiation | Rat | Rat | Malhi et al. [36] |
| Ischemia/Reperfusion | Rat | Rat | Malhi et al. [36] |
| Hepatic Stimulatory Substances | Rat | Rat | Jiang et al. [37] |

Some liver-based inborn errors in metabolism may provide a built in selective growth advantage of donor cells that may make the liver more susceptible to donor hepatocyte engraftment and repopulation. This is due to the nature of the disease and the associated hepatocellular damage to native cells. These diseases include: tyrosinemia, progressive familial cholestasis type 2 and 3, Wilson's disease and alpha-1-antitrypsin disease.

## 1.7 HEPATOCYTE TRANSPLANTS IN HUMANS – THE CLINICAL EXPERIENCE

Hepatocyte transplantation was transitioned to the clinic to see if patients would respond to this new therapy after showing promise in various liver-based metabolic disease and liver failure animal models. Intraportal injection is the preferred method of delivery for clinical hepatocyte transplantation; however alternative sites include the spleen, pancreas, peritoneal cavity and subrenal capsule [5]. Transplantation of cells through the portal vein has shown to lead to portal hypertension and formation of hepatocytes thrombi; however this can be minimized by limiting the number of cells per infusion and the rate at which the cells are delivered. To increase the total number of cells given to a patient multiple infusions of cells can be performed [5].

Initially researchers believed transplantation of cells would not be as immunogenic as OLT; however this is not the case. There is no consensus as to what is the best immunosuppressive protocol but most transplants follow the protocol used for OLT, which includes combination of tacrolimius (with or without steroids) and monoclonal antibodies, such as interleukin 2 receptor antibodies [5].

In animals, engraftment of the transplanted cells can be assessed rather easily by making sure the transplanted cells have a unique reporter protein the recipient liver does not and identifying the transplanted cells by ICC, in situ hybridization, real time RT-PCR or flow cytometry. In humans, it is more difficult to assess engraftment. Indirect evidence includes positive changes in the symptoms of the patients and changes in enzyme activity. For example patients with urea cycle disorders show extended decreases in serum ammonia levels and patients with CN-1 show extended decreases in serum bilirubin levels after hepatocyte transplant. Direct evidence includes the identification of donor sex chromosomes or HLA antigens in cases of donor-recipient sex mismatch or HLA mismatch. In patients that fit this

category who receive eventual OLT, livers can be obtained to perform ICC, in situ hybridization or real time RT-PCR to test for the presence of donor-derived cells.

Theoretically, all patients that are afflicted by liver-based metabolic disorders resulting from a mutation within an important metabolic protein can benefit from hepatocyte transplant. Transplanted cells will be genetically competent in whatever protein is mutated within the patient and when they repopulate the recipient liver they will provide metabolic support that results in the symptoms of the disease being alleviated. The indication for hepatocyte transplantation is currently based on severity of disease or quality of life, with the aim being to avoid or postpone liver transplantation whether due to genetic defects or acute or chronic liver failure [5]. The following paragraphs will describe all reports of clinical hepatocyte transplants that have occurred worldwide since 1993. Most transplants have been performed with adult hepatocytes, however some have been performed with hepatocytes isolated from livers of aborted fetuses. The first report of adult hepatocyte transplant occurred when Mito and colleagues treated 9 patients with cirrhosis and 1 patient with chronic active hepatitis [38]. Autotransplantation of cells from cirrhotic individuals took place. Investigators reported the presence of cells in the spleens of 8 patients up to 11 months post transplant. One patient's ascites resolved.

CN-1 is characterized by patients who experience hyperbilirubinemia caused by a mutation in the UDP-glucuronosyltransferase 1A1 (UGT1A1) gene. This gene encodes a protein that is responsible for glucuronidating bilirubin found in the serum, which allows for the excretion of the molecule through the bile. If it is not excreted and builds up to high enough levels in the serum, bilirubin can cross the blood-brain barrier and cause brain damage. Therefore it needs to be removed from the blood. Bilirubin is formed from the breakdown of hemoproteins,

such as hemoglobin. Bilirubin is water insoluble and therefore cannot be cleared from the body unless it becomes water soluble. This is accomplished by hepatocytes that express the UGT1A1 enzyme. This enzyme is responsible for glucuronidating bilirubin (conjugated bilirubin). Glucuronidation of the propionic acid carboxyls of bilirubin disrupts hydrogen bonding and makes the molecule water soluble and rapidly excretable in the bile. The only current therapy available for patients is phototherapy. This therapy has patients sit underneath bilirubin lamps that emit a blue light (420-470 nm). This light can penetrate the skin and help convert bilirubin to its conjugated form so it can be excreted in the urine and feces. This therapy becomes less and less effective as the patients become older as their surface area to body mass ratio decreases. Currently the only curative treatment for CN-1 is OLT. However, since 1998, 10 patients ranging from 1 to 11 years have been treated with 15 million to 7.5 billion hepatocytes (1 patient received CD326+ fetal hepatocytes) [39-46]. Patients saw a 20-50% reduction in serum bilirubin, increased the amount of conjugated bilirubin and decreased their phototherapy. Only 1 of 10 patients did not benefit from the transplant. However no patients saw complete reversal. This resulted in patients either receiving OLTs or remaining on the OLT wait list at the end of all studies.

Familial hypercholesterolemia (FH) is characterized by patients with abnormally high levels of low density lipoprotein (LDL) in their blood serum. Patients generally have mutations in the LDLR gene that encodes the LDL receptor protein. LDLR is responsible for removing LDL from the circulation. Heterozygous FH can lead to early onset cardiovascular disease around the age of 30 to 40 and can be treated with statins, bile acid sequestrants or other hypolipidemic agents. On the other hand, homozygous FH can cause severe cardiovascular disease in early childhood and often does not respond to conventional therapies and may have to

be treated with LDL apheresis or eventual OLT. In 1995 Grossman and colleagues transplanted 5 patients from the ages of 7 to 41 with 1 to 3.2 billion hepatocytes [47]. Researchers saw up to a 20% reduction in LDL in 3 patients. The other 2 patients did not respond to therapy.

Factor VII deficiency is a disorder caused by the absence of this coagulation factor, which is one of the critical components of the coagulation cascade that results in the clotting of blood. Patients that have this deficiency have a mutation within the factor VII protein that makes it non-functional. Only homozygotes or compound heterozygotes (2 different mutations within the gene) develop hemorrhagic syndrome, heterozygotes are asymptomatic. Patients develop intracerebral hemorrhages, hemarthroses, cutaeneous-mucosal hemorrhages or hemorrhages provoked by a surgical intervention. Severity of disease depends on type of mutation within the factor VII gene [48]. In 2004 Dhawan et al. transplanted 1 3-month old patient and 1 35-month old patient with 1.1 and 2.2 billion cells respectively [49]. The doctors saw a 70% reduction in factor VII requirements after 6 months; however both patients eventually received an OLT.

Glycogen storage disease is characterized by a defect in the processing of glycogen. This inborn error in metabolism results from a mutation in one of the enzymes responsible for the breakdown of glycogen causing impaired protein activity. One type of glycogen storage disease is caused by a mutation in the G6Pase gene, which is responsible for the process of glycogen degradation and gluconeogensis in the liver. This mutation prevents the production of free glucose molecules and leads to hypoglycemia, abnormal blood sugar levels between meals without constant feedings, the buildup of lactate, uric acids and triglycerides, hepatomegaly, developmental delay and seizures leading to impaired breathing, coma and death. Patients with this disorder must adhere to a strict dietary regimen with constant feeding every one to four hours. Children often have a nasogastric feeding tube placed on them for the use of a continuous

feeding pump; this is especially true at night. The strict diet is so the body does not receive glucose. If it does, the body will store the glucose as glycogen, which will end up building up in high amounts in hepatocytes because it cannot be broken down. This hyper-glycogen state can cause hepatomegaly and eventual liver damage. OLT is the only known curative treatment. Severity of the disease depends on what gene is mutated and the type of mutation. Two patients have been treated for glycogen storage disease with hepatocyte transplant [50, 51]. Patients were 18 and 47 years old and received 6 and 2 billion cells respectively. They were able to be put on a normal diet and their fasting time increased after transplantation. The 18 year old had normal G6Pase activity up to 7 months after transplant.

Infantile Refsum's disease is characterized by mutations in genes that encode peroxins, which are important proteins for normal peroxisome assembly and function, therefore making this disease a peroxisome biogenesis disorder. The most common mutations for the disease occur in the PEX1, PEX3, PEX6, PEX12 and PEX26 genes and severity of the disease depends on what gene is mutated and where the mutation is within the gene. As a result of mutation, these proteins become less active or completely inactive altogether. Since one of the major functions of the peroxisome is the catabolism of very long chain and branched chain fatty acids, non functioning PEX genes lead to the accumulation of these lipids within cells and tissues. Abnormal peroxisome biogenesis can lead to a reduction of myelin in the central nervous system (CNS) leading to abnormal CNS function and development as well as hepatomegaly, eventually causing liver damage. There is currently no standard treatment for this disease. In 2003 a four year old female received 2 billion hepatocytes to treat her underlying Infantile Refsum's disease [52]. She saw a 40% reduction in pipecholic acid at 18 months post transplant, thus demonstrating indirect evidence of hepatocyte engraftment and function.

Progressive familial intrahepatic cholestasis (PFIC) is a disease characterized by defects in genes that encode proteins of biliary epithelial transporters. These defects cause problems in the export of bile out of hepatocytes and eventually lead to progressive cholestasis that presents in early childhood. This leads to eventual hepatic failure and need for OLT. Specifically PFIC-2 is caused by mutation in the bile salt export pump (BSEP) that results in the retention of bile salts within hepatocytes causing hepatocelluar damage through cholestasis. 2 patients with PFIC-2 were transplanted in 2006 to treat their underlying disease [42]. These patients were 16 and 32 months old and were transplanted with 400 million and 200 million cells respectively. Both of these patients showed no biochemical benefit from the transplantation and the disease-state progressed eventually resulting in cirrhosis that called for OLT.

Biliary atresia is a congenital or acquired disease associated with the common bile duct between the liver and the small intestine being blocked or absent leading to progressive cholestasis and conjugated hyperbilirubinemia. The acquired type most often occurs due to an underlying autoimmune disease, this being one of the main causes of rejection after OLT. As mentioned earlier, patients have progressive cholestasis with conjugated hyperbilirubinemia, which causes classical liver failure symptoms: jaundice, malabsorption with developmental delays, fat-soluble vitamin deficiencies, and hyperlipidemia eventually causing cirrhosis and liver failure. Since the bilirubin is conjugated it cannot gross the blood brain barrier, thus brain damage caused by kernicterus is not a concern. The cause of congenital biliary atresia is unknown and the only curative treatment is OLT. In 2008, 1 1-year old patient was treated for biliary atresia by the transplantation of 12 million CD326+ fetal hepatocytes. The patient saw a 3-fold reduction in total bilirubin and an 8-fold reduction in conjugated bilirubin. Furthermore,

ALT levels decreased and hepatobiliary scintigraphy showed an increase in liver cell function at 2 months.

Ornithine Transcarbamylase (OTC) deficiency is a urea cycle disorder characterized by a mutation in the OTC gene that encodes the OTC protein essential for the clearance of ammonia from the blood. Ammonia appears in the blood as a normal toxic breakdown product of the body's use of protein and amino acids. Therefore patients that have a mutation within their OTC gene have low functioning or completely non-functioning OTC enzyme activity leading to hyperammonemia and a buildup of orotic acid. The buildup of orotic acid is due to increasing concentration of carbamoyl phosphate entering the pyrimidine synthesis pathway; if the urea cycle was working properly carbamoyl phosphate concentration would remain constant and thus would not enter this alternative biochemical pathway. Hyperammonemia, if left untreated, can cause seizures, loss of appetite, lack of energy, developmental delay, brain damage, irrational behavior, mood swings, coma or death. Management of OTC is done mainly through the administration of a low protein diet. The only curative treatment is OLT. From 1997 to 2009 6 patients with OTC have been treated for their disease by hepatocyte transplant [53-57]. Age of patients ranged from 6 hours to 5 years and they received anywhere from 600 million to 9 billion cells. All patients saw a decrease in serum ammonia levels associated with psychomotor improvement and an increase in protein tolerance administered by a normal diet. One patient was confirmed to have 0.5% normal liver OTC activity once his liver was explanted when death occurred at day 42 post transplant. Cause of death in this patient was not due to cell transplant. Some patients were also confirmed to have normal urinary orotic acid concentrations. All patients at the end of the various studies either received an OLT or were still on the OLT waiting list.

Argininosuccinate lyase (ASL) deficiency is a urea cycle disorder characterized by a mutation in the ASL gene that encodes the ASL protein essential for the clearance of ammonia from the blood. ASL deficiency leads to hyperammonemia with symptoms and consequences very similar to other urea cycle disorders. ASL is an enzyme that cleaves argininosuccinate to produce a molecule of fumarate and arginine in the urea cycle. A mutation in the ASL protein will lead to elevated levels of argininosuccinate resulting in argininosuccinic aciduria and clearance through the urine. Elevated argininosuccinate in the urine is a main symptom of disease. Although argininosuccinate contains two waste nitrogen molecules normally found in urea, patients still experience urea cycle disorder systems. OLT is the only curative treatment. 1 42-month old patient was transplanted with 4.7 billion hepatocytes to treat the underlying ASL deficiency [58]. The patient saw a decrease in serum ammonia levels associated with psychomotor catch up. Although OLT was performed 18 months post cell transplant, donor cells were still detected in the liver.

Carbamoyl phosphate synthetase (CPS) deficiency type-1 is a urea cycle disorder characterized by a mutation in the CPS gene that encodes the CPS protein essential for the clearance of ammonia from the blood. CPS-1 deficiency leads to hyperammonemia with symptoms and consequences very similar to other urea cycle disorders. CPS-1 is the enzyme that catalyzes the reaction of 2 molecules of ammonia and 2 molecules of bicarbonate to form carbamoyl phosphate. This is the initial reaction in the urea cycle to clear waste nitrogen from the body through the production of urea. Thus patients with mutation in the CPS-1 gene have inactive CPS-1 enzyme activity resulting in ammonia accumulation in the serum. OLT is the only curative treatment. In 2009, 1 2.5-month old patient was treated for CPS-1 by the transplantation of 1.4 billion hepatocytes [54]. The patient saw a decrease in serum ammonia

levels and an increase in urea production for 11 months. At the end of the study the patient was still on the OLT waiting list.

Citrullinemia is a urea cycle disorder characterized by a mutation in the argininosuccinate synthetase (ASS) gene that encodes the ASS protein essential for the clearance of ammonia from the blood. Citrullinemia leads to hyperammonemia with symptoms and consequences very similar to other urea cycle disorders. ASS is the enzyme that catalyzes the reaction of citrulline and aspartate to form argininosuccinate. Thus patients with mutation in the ASS gene have inactive enzyme activity resulting in ammonia accumulation in the serum. OLT is the only curative treatment. In 2009, 1 36-month old patient was treated for citrullinemia by the transplantation of 1.5 billion hepatocytes [54]. The patient saw a return to normal ammonia blood serum levels as well as a 40% increase in urea production with an increase in protein intake due to dietary changes.

Liver failure is the inability of the liver to perform its normal synthetic and metabolic function to maintain normal physiology and homeostasis and comes in two forms: acute and chronic. Acute liver failure (ALF) is defined as the rapid development of hepatocellular dysfunction and chronic liver failure usually occurs in the context of cirrhosis (liver fibrosis). Since 1993 the following types of liver failure have been treated by hepatocyte transplant: drug-induced ALF (16 patients; 5 received fetal hepatocytes), viral-induced ALF (10 patients; 1 received fetal hepatocytes), idiopathic ALF (4 patients), mushroom poisoning-induced ALF (1 patient), post surgical ALF (1 patient) and ALF induced by acute fatty liver of pregnancy (1 patient). Routes of hepatocytes transplant in these patients include intraperitoneal, intrasplenic and portal vein. Some patients died from their underlying disease, some survived until an organ became available for OLT and in rare instances some of the patient's liver failure resolved.

Table 5 summarizes all current reports, to the knowledge of this investigator, of clinical hepatocyte transplants performed worldwide since 1993 broken down by disease. Table includes the specific reference where all data can be found along with the age of patient, total number of cells transplanted, the findings associated with the study and the outcomes of the patients.

**Table 5.** Clinical Hepatocyte Transplants in Patients Worldwide
Table summarizes all reports of clinical hepatocyte transplants performed worldwide since 1993 to treat various liver-based inborn errors of metabolism and acute liver failure. Table is broken down by each disease that has been treated. Cells transplanted are hepatocytes unless specified otherwise. PV = portal vein; IS = intrasplenic; IP = intraperitoneal. Adapted from Dhawan et al. [5].

**Disease: CN-1**

| **Reference** | **Age of Patient** | **# Cells TX ($X10^9$)** | **Findings** | **Outcome** |
|---|---|---|---|---|
| Fox et al. [43] | 10 years | 7.5 | ↓ 50% bilirubin, UGT1A1 activity in liver | OLT after 4 years |
| Darwish et al. [41] | 8 years | 7.5 | ↓ 40% bilirubin for 6 months | OLT after 20 months |
| Ambrosino et al. [40] | 9 years | 7.5 | ↓ 30% bilirubin for a few weeks | OLT after 5 months |
| Dhawan et al. [42] | 18 months<br><br>42 months | 4.3<br><br>2.1 | ↓ 40% bilirubin<br><br>No clear benefit | OLT after 8 months<br><br>NA |
| Allen et al. [39] | 8 years | 1.4 | ↓ 30% bilirubin, ↓ phototherapy | OLT after 11 months |
| Lysy et al. [45] | 9 years<br><br>1 year | 6.1<br><br>2.6 | ↓ 35% bilirubin for 6 months<br><br>↓25% bilirubin, ↓ phototherapy for 4 months | OLT waiting list<br><br>OLT after 4 months |
| Khan et al. [44] | 2 years | 0.015<br><br>CD326+ Fetal hepatocytes | ↓ 50% bilirubin, ↑ 5-fold conjugated bilirubin | NA |
| Meyburg et al. [46] | 11 years | 7.2 | ↓ 20% bilirubin | OLT waiting list |

**Disease: Familial Hypercholesterolemia**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Grossman et al. [47] | Five total w/ages between 7 and 41 years | 1.0-3.2 | Up to ↓ 20% LDL in 3 patients | NA |

**Disease: Factor VII Deficiency**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Dhawan et al. [49] | 3 months | 1.1 | ↓ 70% rFVII requirement for 6 months | OLT after 7 months |
| | 35 months | 2.2 | ↓ 70% rFVII requirement for 6 months | OLT after 8 months |

**Disease: Glycogen Storage Disease Type-1**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Muraca et al. [51] | 47 years | 2.0 | Normal diet, ↑ fasting time | NA |
| Lee et al. [50] | 18 years | 6.0 | Normal G6Pase activity up to 7 months | NA |

**Disease: Infantile Refsum's Disease**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Sokal et al. [52] | 4 years | 2.0 | ↓ 40% pipecholic acid after 18 months | NA |

**Disease: Progressive Familial Intrahepatic Cholestasis Type-2**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Dhawan et al. [42] | 32 months | 0.2 | No benefit, cirrhosis established | OLT after 5 months |

| | | | | |
|---|---|---|---|---|
| | 16 months | 0.4 | No benefit, cirrhosis established | OLT after 14 months |

**Disease: Biliary Atresia**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Khan et al. [59] | 1 year | 0.012<br><br>CD326+ Fetal hepatocytes | ↓ 3-fold total bilirubin, ↓ 8-fold conjugated bilirubin, ↓2.5-fold ALT levels and hepatobiliary scintigraphy showed ↑ liver cell function at 2 months | NA |

**Disease: Ornithine Transcarbamylase Deficiency**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Out come |
|---|---|---|---|---|
| Strom et al. [57] | 5 years | 1.0 | ↓ $NH_3$, 0.5% of normal liver OTC activity | Death 42 days after TX |
| Horslen et al. [53] | 10 hours | 9.0 | ↓ $NH_3$, ↑ protein tolerance for a short period | OLT after 6 months |
| Stephenne et al. [56] | 14 months | 2.4 | ↓ $NH_3$, ↑ urea, psychomotor improvement | OLT after 6 months |
| Puppi et al. [55] | 1 day | 1.6 | ↓$NH_3$, ↑ urea under normal protein diet | APOLT after 7 months |
| Meyburg et al. [54] | 6 hours<br><br>9 days | 0.6<br><br>0.6 | ↓ $NH_3$, ↑ urea, normal urinary orotic acid excretion<br><br>↓ $NH_3$, ↑ protein intake, urinary ortoic acid normal at 6 months | Death 4 months after TX<br><br>OLT waiting list |

**Disease: ASL Deficiency**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Stephenne et al. [58] | 42 months | 4.7 | ↓$NH_3$, psychomotor catch-up, donor cells detected in liver | OLT after 18 months |

**Disease: CPS-1 Deficiency**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Meyburg et al. [54] | 2.5 months | 1.4 | ↓ $NH_3$, ↑ urea for 11 months | OLT waiting list |

**Disease: Citrullinemia**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Findings | Outcome |
|---|---|---|---|---|
| Meyburg et al. [54] | 36 months | 1.5 | Normal $NH_3$, ↑ 40% urea, ↑ protein intake | NA |

**Disease: Drug-induced Acute Liver Failure**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Route of delivery | Outcome |
|---|---|---|---|---|
| Bilir et al. [60] | 32 years | 1.3 | IS | Death on day 14 |
| | 35 years | 10 | IS | Death on day 20 |
| | 55 years | 39 | IS | Death in 6 hours |
| Strom et al. [61] | 13 years | 1.0 | PV | Death on day 4 |
| | 43 years | NA | NA | Death on day 35 |
| Fisher et al. [62] | 27 years | 0.03 | IS | OLT on day 10 |
| | 26 years | 1.2 | IS | OLT on day 2 |

| | | | | |
|---|---|---|---|---|
| | 21 years | 0.94 | IS | Death on day 1 |
| | 35 years | 5.4 | PV | Death on day 18 |
| | 35 years | 3.7 | PV | Full recovery |
| | 51 years | 3.9 | PV | Death on day 3 |
| Habibullah et al. [63] | 32 years | 0.06/kg | IP | Death in 30 hours |
| | 29 years | 0.06/kg | IP | Death in 37 hours |
| | 20 years | 0.06/kg | IP | Death in 48 hours |
| | 20 years | 0.06/kg | IP | Full recovery |
| | 24 years | 0.06/kg Fetal Hepatocytes | IP | Full recovery |

**Disease: Viral-induced Acute Liver Failure**

| **Reference** | **Age of Patient** | **# Cells TX ($X10^9$)** | **Route of delivery** | **Outcome** |
|---|---|---|---|---|
| Meyburg et al. [46] | 3 weeks | 1.7 | PV | Death on day 11 |
| Fisher et al.[62] | 4 years | 3.4 | PV | Death on day 2 |
| | 54 years | 6.6 | PV | Death on day 7 |
| Bilir et al. [60] | 29 years | 10 | PV and IS | Death in 18 hours |
| | 65 years | 30 | PV and IS | Death on day 52 |
| Strom et al. [61] | 28 years | 0.17 | IS | OLT on day 3 |

| | | | | |
|---|---|---|---|---|
| | 37 years<br><br>43 years | 0.12<br><br>0.73 | IS<br><br>PV | Death on day 5<br><br>OLT on day 1 |
| Fisher et al. [64] | 37 years | 0.88 | IS | Full recovery |
| Habibullah et al. [63] | 40 years | 0.06/kg<br><br>Fetal Hepatocytes | IP | Death in 13 hours |

**Disease: Idiopathic Acute Liver Failure**

| **Reference** | **Age of Patient** | **# Cells TX ($X10^9$)** | **Route of delivery** | **Outcome** |
|---|---|---|---|---|
| Fisher et al. [62] | 3.5 months<br><br>23 years<br><br>48 years | 0.18<br><br>0.44<br><br>0.75 | PV<br><br>IS<br><br>PV | OLT on day 1<br><br>OLT on day 5 and death on day 13<br><br>Death on day 1 |
| Habibullah et al. [63] | 8 years | 0.06/kg<br><br>Fetal Hepatocytes | IP | Full recovery |

**Disease: Mushroom-poisoning-induced Acute Liver Failure**

| **Reference** | **Age of Patient** | **# Cells TX ($X10^9$)** | **Route of delivery** | **Outcome** |
|---|---|---|---|---|
| Schneider et al. [65] | 64 years | 4.9 | PV | Full recovery |

**Disease: Postsurgical Acute Liver Failure**

| **Reference** | **Age of Patient** | **# Cells TX ($X10^9$)** | **Route of delivery** | **Outcome** |
|---|---|---|---|---|
| Strom et al. [61] | 69 years | 0.53 | IS | Death on day 2 |

**Disease: Acute Liver Failure Induced by Acute Fatty Liver of Pregnancy**

| Reference | Age of Patient | # Cells TX ($X10^9$) | Route of delivery | Outcome |
|---|---|---|---|---|
| Khan et al. [66] | 26 years | 0.3<br><br>Fetal hepatocytes | IP | Full recovery |

It has been concluded that cell therapy shows promise in reversing clinical symptoms associated with liver failure and many metabolic diseases; however issues with cell source, size, viability, cryopreservation, engraftment, immunosuppression and family compliance with disease specific therapy need to be addressed. Moreover the lack of controlled trials during the clinical hepatocyte transplants performed to date makes it difficult to interpret findings and compare different studies. No patients to date have been completely cured of their disease; however cell transplant has shown the best outcomes in patients with liver-based inborn errors in metabolism. These patients demonstrate obvious positive effects on various disease specific symptoms. Cell-based therapy in some cases helped to reverse liver failure, however once again the lack of proper controls make it difficult to say patient's liver failure was reversed due to hepatocyte transplant.

Hepatocyte transplant has been shown to have potential to serve as a "bridge to transplant" for both metabolic liver-based diseases and liver failure therefore taking some of the burden off the OLT waiting list and allowing patients to survive longer until an organ for transplant becomes available.

Current sources of hepatocytes for transplant are scarce and tissues that are available are livers that are unused or organs unsuitable for OLT. Livers not suitable for whole organ transplant include tissues with greater than 30% steatosis and extended cold ischemic times. This

fact demonstrates the limited supply of donor tissue that can provide high quality cells, thus leading to the transplantation of hepatocytes isolated from marginal livers. To compound issues, isolated primary adult human hepatocytes rarely, if ever, proliferate *in vitro* after cellular isolation. This means isolated cells cannot be expanded to treat more patients.

Taken together, alternative cell sources and new methods to improve cell engraftment and liver repopulation need to be investigated in order to make cell-based therapeutics to treat liver diseases a regular clinical procedure. Alternative cell sources for transplant will increase the number of patients that can be treated and protocols to increase engraftment will allow for fewer cells to be delivered to patients and therefore more patients to be treated overall. Stem cells, specifically hESCs and hiPSCs, are an attractive cell source for hepatocyte transplant because they represent a potential constant and unlimited source of cells to transplant. They are easier to cryopreserve and smaller in size and thus easier to transplant.

## 1.8 INTRODUCTION TO STEM CELLS

The concept of one cell type that can give rise to all cells of the human body is very exciting to the medical and scientific world. This totipotent cell could theoretically be used to make an unlimited amount of terminally differentiated cells. These cells could be used for transplant to potentially correct a disease or stabilize a patient until an organ for transplant becomes available. These cells could also be used for *in vitro* drug discovery and screening. Furthermore, if stem cells could be isolated from patients with genetic diseases, the cells could be differentiated to the cell type affected by the disease and used to model the disease and understand disease-related mechanisms. Disease-specific cells could also be genetically corrected and then used for cell

transplantation to correct the phenotype. Isolation, characterization, and propagation of pluripotent stem cells can come from embryonic sources, such as human embryonic stem cells (hESCs). They can also come from somatic cells by forced expression of specific transcription factors, somatic cell nuclear transfer to an unfertilized oocyte, or fusion of a somatic cell with a hESC.

## 1.9 HESC DERIVATION

hESCs are derived from the inner cell mass of the blastocyst. They have normal karyotypes, express high levels of telomerase activity, express cell surface markers that are found on primate ESCs, and maintain the developmental potential to form trophoblast and all 3 embryonic germ layers [67]. This was the first report of derivation and characterization of hESCs. The next step in the research process would be to determine how to differentiate these cells to become cell types of terminally differentiated cells that can be used for transplant or *in vitro* analysis.

## 1.10 ESC IN VITRO DIFFERENTIATION TO ENDODERM

Researchers began determining ways to differentiate hESCs *in vitro* to desired cell types by exploiting observations made by developmental biologists during the study of normal embryonic development. Chemically defined culture conditions were determined by mimicking the same signaling events that occur during normal development. Therefore the first approach to generating functional hepatocytes from hESCs was to prime the cells to become endoderm, the

embryonic derivative from which hepatocytes are developed. It has been shown that hESC differentiation toward hepatic endoderm requires activin A and Wnt3a signaling [68] as well as FGF signaling [69]. Furthermore, researchers have determined that activin A will only specify definitive endoderm from hESCs when phosphatidylinositol 3-kinase (PI3K) signaling is suppressed, thus establishing the PI3K inhibitor LY294002 as an important small molecule to use during endoderm differentiation [70]. PI3K signaling is usually active by insulin/IGF induction. It has been reported that inhibition of glycogen synthase kinase 3 (GSK-3) is also important in forming definite endoderm from hESCs [71]. Furthermore this group showed prolonged treatment with their novel GSK inhibitor generated a population of cells that displayed hepatoblast characteristics such as expression of α-fetoprotein and HNF4α, with further treatment leading to the generation of hepatocyte-like cells capable of producing albumin. Small molecules have also been demonstrated to direct hESCs to definitive endoderm at a higher efficiency than that achieved by activin A [72]. Finally, researchers have been able to establish endoderm progenitors through constitutive expression of SOX17 via a Cre-inducible expression vector [73]. These cell lines represent a constant source of endoderm that can be differentiated on demand to endoderm derivatives that could be used for cell transplant or *in vitro* analysis, such as hepatocytes. Some of the papers that have made important observations and advancements for *in vitro* endoderm formation are summarized in Table 6 [68-74].

**Table 6.** Formation of Endoderm from hESCs
This table summarizes some of the important observations, advancements and protocols for *in vitro* formation of endoderm from hESCs.

| **Reference** | **Species** | **Importance for *in vitro* Endoderm Formation** |
|---|---|---|
| Hay et al. [68] | Human | Activin A and Wnt3a signaling |
| Morrison et al. [69] | Mouse | FGF signaling |
| McLean et al. [70] | Human | PI3K inhibition |

| | | |
|---|---|---|
| Bone et al. [71] | Human | GSK-3 inhibition |
| Borowiak et al. [72] | Mouse and Human | Novel small molecules to induce endoderm |
| Seguin et al. [73] | Human | Endoderm cell lines established by constitutive SOX17 expression |
| D'Amour et al. [74] | Human | Activin A signaling and low serum |

It is important to note that these papers fail to address the issue of which protocol forms the definitive endoderm that is best suited to be differentiated toward the hepatocyte lineage that is both reproducible and efficient.

## 1.11 ESC IN VITRO DIFFERENTIATION TO HEPATOCYTES

The next step to generating stem cell-derived hepatocytes from hESCs is to form hepatic progenitors and then mature those progenitor cells to cells molecularly, functionally, and morphologically similar to primary hepatocytes. Many protocols have been established using this stepwise approach with chemically defined media mimicking the molecular signaling events during normal embryonic development of the liver [75-84]. All of these protocols have varying degrees of success in terms of generating hESC-derived hepatocytes that have liver specific gene expression, protein expression, protein secretion, CYP450 metabolism and induction and glycogen storage comparable to primary human hepatocytes. Mature hepatic function has yet to be accomplished through *in vitro* differentiation of hESCs, however it has been determined that HNF4α is essential for specification of hepatic progenitors from human pluripotent stem cells by establishing the expression network of transcription factors that control hepatocyte cell fate decisions [85]. Researchers were able to investigate each stage of the hepatic differentiation

process by depleting expression of various genes using shRNA technology and lentiviruses. Table 7 summarizes the various protocols that have been published regarding differentiation of hESCs to hepatocytes.

**Table 7.** Various Reports of ESC Differentiation to Hepatocytes
This table summarizes the various reports within the literature on the generation of ESC-derived hepatocytes from different species' cells. Table includes the reference, species of cell used and a brief summary of the growth factors, cytokines and culture techniques used within each reference's differentiation protocol. The forward slashes in the "summary of protocol" column denote a change in culture conditions consisting of a basal medium or growth factor change.

| Reference | Species | Summary of Protocol |
|---|---|---|
| Agarwal et al. [75] | Human | ActA / FGF4, HGF / BSA, FGF4, HGF / FGF4, HGF, OSM, DEX |
| Basma et al. [76] | Human | EB formation / ActA, FGF2 / HGF, DMSO / DEX |
| Bukong et al. [77] | Human | ActA, FGF2 / FGF4, BMP2, LY294002 / FGF10, RA, FGF4, HGF, EGF, SB431542 / FGF10, RA, FGF4, HGF, EGF, SB431542, OSM, DEX |
| Cai et al. [78] | Human | ActA / FGF4, BMP2 / HGF / OSM, DEX |
| Duan et al. [79] | Human | ActA / FGF4, HGF, BMP2, BMP4 / FGF4, HGF, BMP2, BMP4, DMSO / FGF4, HGF, OSM, DEX, DMSO |
| Hay et al. [80] | Human | ActA, NaBut / ActA, NaBut / DMSO / HGF, OSM |
| Medine et al. [81] | Human | ActA, Wnt3a / DMSO / HGF, OSM |
| Sharma et al. [82] | Human | DMSO / NaBut / SNAP |
| Touboul et al. [83] | Human | ActA, FGF2 / LY294002, ActA, BMP4, FGF2 / FGF10 / FGF10, RA, SB431542 / FGF4, HGF, EGF |
| Tuleuova et al. [84] | Mouse | Use of various growth factor arrays and micropatterned co-cultures to induce hepatic differentiation |

A formidable issue holding back the use of hESCs for regenerative medicine and *in vitro* drug discovery is the ethical dilemma behind the formation of the various cell lines. The creation of each hESC line involves developing a fertilized egg to the pre-implantation blastocyst stage

and isolating the cells from the inner cell mass, which will eventually become the hESC line. Some believe this process to be destruction of a human life, therefore making this topic a huge political debate around the world. However, if an alternative pluripotent cell source were to become available that is identical to the proliferative and developmental potential of hESCs, there would no longer be an ethical issue. The discovery and creation of hiPSCs circumvents this ethical issue.

## 1.12 PLURIPOTENCY AS A TWO-WAY STREET

Mammalian embryogenesis begins with the fertilization of the female oocyte by a male sperm to form a single cell that contains the proper amount of genetic material. Successive cell divisions lead to the formation of the pre-implantation blastocyst that contains the outer trophectoderm and inner cell mass. Directed differentiation of the cells within the blastocyst to their appropriate cell type is coordinated by highly specific molecularly controlled signaling events that are spatially and temporally controlled. These observations indicate that development potential is controlled by manipulation of global DNA gene expression within the cell rather than direct genetic code alterations.

One attempt to prove this observation experimentally, in mammalian cells, occurred when researchers successfully performed the first somatic cell nuclear transfer experiments in lambs [86]. Researchers transferred a somatic cell nucleus to an enucleated unfertilized egg and implanted the egg within a host uterus eventually leading to the birth of live lambs. This experiment provided evidence that differentiation of a cell toward a somatic state is not accomplished by irreversible genetic manipulation. A cell becomes specified not by changing its

DNA sequence, but by controlling the expression of certain genes through specific controls. Cell differentiation and pluripotency was no longer seen as a one way road. This somatic cell nuclear transfer approach has yet to be successfully accomplished in human cells.

## 1.13 IPSC DERIVATION

Since the successful somatic cell nuclear transfer experiments in lambs, researchers have determined ways to manipulate a cell's developmental potential back to an embryonic-like state where, theoretically, developmental potential is at its highest. One approach to generate pluripotent stem cells from somatic cells was discovered when mouse embryonic and adult fibroblasts were shown to be reprogrammed to an ESC-like state by forced transcription of Oct3/4, Sox2, c-Myc, and Klf4 by lentiviral induction [87]. These cells were similar to ESCs in morphology, growth properties, expression of ESC marker genes, teratoma formation and injection into a blastocyst yielded cells that contributed to multiple tissues during mouse development, including the germ line. Further characteristics of iPSCs include DNA demethylation of key pluripotency genes and regulators, endogenous expression of pluripotency genes and down-regulation of viral genes used to induce pluripotency. These cells were coined "induced pluripotent stem cells" (iPSCs). Generation of iPSCs was confirmed to be possible in adult human dermal fibroblasts a year later by the same method as was performed in mice [88] and by forced expression of a new set of 4 factors: Oct4, Sox2, Nanog, and Lin28 [89].

In order to make iPSC technology clinically relevant researchers needed to figure out how to induce pluripotency without viral DNA integration into the target cell genome, which can create unwanted mutations because DNA is randomly inserted and copy number cannot be

controlled. Mouse fibroblasts and hepatocytes were shown to be successfully reprogrammed by using non integrating adenoviruses to deliver the reprogramming factors [90]. This observation demonstrated that reprogramming to pluripotency could be achieved through transient expression of viral genes. Another report of using virus to generate hiPSCs occurred when researchers demonstrated that temperature-sensitive Sendai viral vectors can be used resulting in hiPSC free of transgenes [91]. Vector integration-free human iPSCs have been derived by using nonviral techniques such as episomes derived from the Epstein-Barr virus [92] and transient transfection of plasmid DNA encoding reprogramming factors [93]. Furthermore it has been demonstrated that a novel nonviral minicircle vector free of bacterial DNA can be used to induce pluripotency [94]. This report is important because it decreases the amount of vector being produced, as there is one vector containing all the genes necessary to form hiPSCs. These five reports demonstrate that somatic cell reprogramming does not require transgene genomic integration or continued expression of exogenous DNA. Other non-integrating methods used to generate hiPSCs include using mRNA transfection [95, 96] and direct delivery of reprogramming proteins [97]. Investigators have also reported being able to use a bacteriophage ΦC31 integrase to reprogram cells with only a single integration site with locations favoring intron regions, thus producing cells with undisturbed endogenous gene function [98]. It has also been shown that poly-β-amino esters can be used to transfect plasmids, although this technique still resulted in hiPSCs that contain transgenes, however it did not require the use of a virus to deliver the genes [99]. Researchers were able to induce pluripotency without the use of Oct4 under entirely feeder-free conditions, albeit still using integrating lentiviral vectors [100]. Finally, a report demonstrated that reprogramming can be achieved using Dox-inducible lentiviral vectors [101]. This report is important because it provides a platform for researchers to control when the reprogramming

process is initiated by viral gene expression. This allows for the characterization of the reprogramming process and screens to increase reprogramming efficiencies or replace individual factors used to induce pluripotency.

The usage of the oncogenes c-Myc and Klf4 to induce pluripotency also had to be solved in order to make iPSCs more clinically relevant. Recent studies have shown that reprogramming could be successful without using c-Myc and Klf4. Human fetal gut mesentery-derived cells and human amnion using Oct4, Sox2, and Nanog [102, 103] and primary human fibroblasts using only Oct4 and Sox2 [104] were shown to be successfully reprogrammed to pluripotency. It is important to point out that although iPSCs can be derived without the use of Klf4 or c-Myc the efficiency of reprogramming is drastically reduced. Table 8 summarizes the first reports of the different ways to induce pluripotency and if that method leads to transgene-free cells.

**Table 8.** Methods to Generate iPSCs
This table summarizes the first reports of different ways to induce pluripotency, species of cells that were used and if that method leads to transgene-free cells.

| Reference | Species | Method | Transgene-free? |
|---|---|---|---|
| Takashashi et al. [87] | Mouse | Lentivirus | No |
| Takahashi et al. [88], Yu et al. [89] | Human | Lentivirus | No |
| Stadtfeld et al. [90] | Mouse | Adenovirus | Yes |
| Ban et al. [91] | Human | Sendai Virus | Yes |
| Yu et al. [92] | Human | Episomal | Yes |
| Si-Tayeb et al. [93] | Human | Plasmid DNA | Yes |
| Jia et al. [94] | Human | Novel 'minicircle' DNA | Yes |
| Warren et al. [95], Yakubov et al. [96] | Human | mRNA transfection | Yes |
| Kim et al. [97] | Human | Protein transfection | Yes |
| Montserrat et al. [99] | Human | Poly-β-amino esters to transfect plasmids | No |

| | | | |
|---|---|---|---|
| Ye et al. [98] | Mouse and Human | Bacteriophage ΦC31 integrase | No |
| Hockemeyer et al. [101] | Human | Drug inducible lentivirus | No |
| Li et al. [102] and Zhao et al. [103] | Human | Lentivirus delivering only Oct4, Sox2 and Nanog | No |
| Huangfu et al. [104] | Human | Retrovirus delivering only Oct4 and Sox2 along with valproic acid treatment | No |
| Montserrat et al. [100] | Pig | Lentivirus delivering only Sox2, Klf4 and c-Myc in feeder-free conditions | No |

Since iPSC technology has the potential to be very useful in the regenerative medicine field as well as drug discovery research, investigators became interested in seeing which cell types of the body can be reprogrammed to pluripotency. This was important because researchers postulated that perhaps the cell of origin of the iPSC will influence developmental potentials due to retention of epigenetic memory of the parent cell. Researchers have confirmed iPSC induction in the following cell types: fetal lung fibroblasts, neonatal foreskin fibroblasts, mesenchymal stem cells and dermal fibroblasts taken from healthy patients [105], adult mouse liver and stomach cells [106], primary human adult hepatocytes [107], fetal gut mesentery-derived cells [102], human and mouse extra-embryonic cells [108], human mesenchymal cells from the umbilicial cord matrix and amniotic membrane [109], human amnion [103], juvenile human primary keratinocytes [110], mouse hematopoietic and myogenic cells [111], and mouse fetal hepatocytes [112, 113]. Table 9 summarizes the first reports of reprogramming of different somatic cell types and what species the iPSCs came from.

**Table 9.** Generation of iPSCs from Various Somatic Cell Sources
Table 9 summarizes some of the first reports of iPSCs generated from various somatic cell sources and species.

| Reference | Species | Cell Type |
|---|---|---|
| Park et al. [105] | Human | Fetal lung fibroblasts, neonatal skin fibroblasts, mesenchymal stem cells and dermal fibroblasts |
| Aoi et al. [106] | Mouse | Adult mouse liver and stomach cells |
| Liu et al. [107] | Human | Adult Hepatocytes |
| Li et al. [102] | Human | Fetal gut mesentery-derived cells |
| Nagata et al. [108] | Human and Mouse | Extra-embryonic cells |
| Cai et al. [109] | Human | Mesenchymal cells from the umbilical cord matrix and amniotic membrane |
| Zhao et al. [103] | Human | Amnion |
| Aasen et al. [110] | Human | Keratinocytes |
| Polo et al. [111] | Mouse | Hematopoietic and Myogenic cells |
| Kleger et al. [112] and Lee et al. [113] | Mouse | Fetal Hepatocytes |

The observation that different somatic cells could be reverted back to an embryonic state was very important; however there were no data that indicated if cell source affected the differentiation potentials of the generated iPSCs.

## 1.14 DISEASE-SPECIFIC IPSC GENERATION

Now that iPSC technology has been well established, investigators are trying to generate disease-specific iPSCs to study disease both *in vitro* and *in vivo*. These disease specific iPSCs will allow investigators to have an unlimited supply of disease specific cells if the iPSCs can be

differentiated to the cell type affected by the disease. These differentiated cells can be used for disease modeling, drug discovery and autologous cell replacement therapies after genetic correction. This genetic correction is possible through the usage of genome modifying zinc-finger nucleases (ZFNs) [114]. These ZFNs allow for more efficient and less time-consuming gene targeting. They generate site-specific double strand breaks, leading to insertions or deletions via DNA repair by the non-homologous end joining pathway. ZFNs have corrected genetic defects in hiPSCs from disease specific cells, specifically in those generated from patients with sickle cell anemia [115] and alpha-1-antitrypsin deficiency [116]. In short, disease specific iPSCs represent an invaluable opportunity to recapitulate both normal and pathologic human tissue formation *in vitro,* thereby enabling disease investigation and drug development.

Some successful reports of generating disease-specific iPSCs include: adenosine deaminase deficiency-related severe combined immunodeficiency [117], Shwachman-Bodian-Diamond syndrome [117], Gaucher disease type III [117], muscular dystrophy [117, 118], Parkinson disease [117, 119], Huntington disease [117], juvenile-onset type 1 diabetes mellitus [117], down syndrome/trisomy 21 [117], carrier state of Lesch-Nyhan syndrome [117], spinal muscular atrophy [120], familial dysautonomia [121], lysosomal storage disorders [122], Timothy syndrome [123], Fanconi anemia [124], and sickle cell anemia [115]. iPSCs have also been generated from skin fibroblasts of an 82 year old woman diagnosed with amyotrophic lateral sclerosis and subsequently differentiated back into motor neurons [125]. This report demonstrates that iPSC technology can even be used to generate cells from patients who are in the latter stages of life.

Finally, iPSCs have been generated from patients with many liver-based genetic inborn errors in metabolism. These diseases include: $\alpha_1$-antitrypsin deficiency [116, 126], familial

hypercholesterolemia [126], glycogen storage disease [126, 127], CN-1 [126, 127], tyrosinemia [126, 127], progressive familial hereditary cholestasis [127] and Wilson's disease [128]. Table 10 summarizes the various disease-specific iPSCs that have been generated.

**Table 10.** Summary of Various Reports of Disease-specific iPSC Generation
This table is a summary of the various diseases that have had iPSCs generated from patient-specific cells.

| Reference | Species | Disease |
|---|---|---|
| Park et al. [117] | Human | adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, muscular dystrophy, Parkinson disease, Huntington disease, juvenile-onset type 1 diabetes mellitus, down syndrome/trisomy 21, carrier state of Lesch-Nyhan syndrome |
| Kazuki et al. [118] | Mouse and Human | muscular dystrophy |
| Soldner et al. [119] | Human | Parkinson's disease (transgene-free) |
| Ebert et al. [120] | Human | spinal muscular atrophy |
| Lee et al. [121] | Human | familial dysautonomia |
| Meng et al. [122] | Mouse | lysosomal storage disorders |
| Pasca et al. [123] | Human | Timothy syndrome |
| Raya et al. [124] | Human | Fanconi anemia |
| Sebastiano et al. [115] | Human | sickle cell anemia |
| Dimos et al. [125] | Human | Amyotrophic lateral sclerosis |
| Rashid et al. [126] | Human | $\alpha_1$-antitrypsin deficiency, familial hypercholesterolemia, glycogen storage disease, CN-1, tyrosinemia |
| Ghodsizadeh et al. [127] | Human | Tyrosinemia, glycogen storage disease, progressive familial hereditary cholestasis, CN-1 |
| Yusa et al. [116] | Human | $\alpha_1$-antitrypsin deficiency |
| Zhang et al. [128] | Human | Wilson's disease |

## 1.15 INCREASING REPROGRAMMING EFFICIENCIES

In order to make hiPSC technology clinically relevant for both *in vitro* diagnostics or cell transplantation for regenerative medicine purposes, hiPSCs need to be efficiently and reproducibly generated in a low-cost and non-labor intensive manner. Therefore investigators began determining ways to increase reprogramming efficiencies of somatic cell reprogramming.

It is known that reprogramming is enhanced by p53 deficiency and that p21, a downstream target of p53, contributes to p53-dependent repression of iPSC formation. This led researchers to postulate that p53 targets may help to hold back cells from becoming reprogrammed. miR-34 microRNAs (miRNAs) are downstream targets of p53 that are induced during iPSC development. Researchers determined that miR-34 deficiency, in mice, significantly increases reprogramming efficiency, kinetics and iPSC generation [129]. Furthermore, unlike p53 deficiency, which enhances reprogramming at the expense of iPSC differentiation potential, miR-34 deficiency increases iPSC formation without compromising self renewal or differentiation. Therefore miR-34 inhibitors can be used to help increase the reprogramming efficiency and therefore decrease the time for establishing iPSC lines and increasing the amount of lines that can be selected for to determine the best line to differentiate to the desired cell type of choice.

Transfection of somatic cells with protein-encoding RNA of reprogramming genes is an attractive method to produce clinically relevant iPSCs free of genomic modification. However this is a very low efficiency method and it can become labor intensive, resulting in few iPSC lines to select for the best ones that differentiate to the investigator's cell type of choice. It has been determined that multiple rounds of RNA transfection trigger an innate immune response that leads to growth arrest and eventual cell death. Therefore researchers hypothesized that if

they desensitize cells to exogenous RNA with various siRNA cocktails targeting the innate immune response they can enable multiple rounds of RNA transfection without causing growth arrest and cell death. Specifically researchers used siRNA to knockdown interferon-β, Eif2ak2 and Stat2 to rescue transfected cells from the innate immune response triggers by frequent RNA transfection [130]. Therefore reprogramming efficiency can be increased if siRNA cocktails are used to target these three genes during RNA-mediated reprogramming of somatic cells.

It has also been determined that reprogramming efficiency can be enhanced through forcing the transcription of all six transcription factors, rather than just using the Yamanaka four or the Thomson four [131]. This was shown using lentiviruses, however it is likely that this concept would be applicable to all types of reprogramming methods. Moreover, using chemical compounds that inhibit the TGFβ and MEK-ERK pathways have increased reprogramming efficiency of human fibroblasts and decrease the time to cell line establishment [132]. This was determined because it is known that TGFβ and MEK-ERK pathway inhibitors promote mesenchymal to epithelial transition, which is important to the reprogramming process in fibroblasts.

Other ways to increase the reprogramming process include: adding SV40 large T antigen (which inactivates p53) to either set of 4 reprogramming genes enhances colony formation up to 70-fold higher with formation 1-2 weeks earlier [133], co-expressing RAR-γ and Lrh-1 with the Yamanaka factors [134], culturing cells being reprogrammed under hypoxic conditions [135], using UTF1 forced transcription with p53 siRNA along with the Yamanaka factors [136] and enrichment of the cell population for progenitor cells [112]. Table 11 summarizes the different strategies to increase reprogramming efficiencies and iPSC colony formation.

**Table 11.** Summary of the Different Strategies to Increase Reprogramming Efficiencies and hiPSC Colony Formation

This table summarizes the various ways researchers have increased reprogramming efficiencies and hiPSC colony formation. Table includes the specific reference associated with the method and the species of cells used. Method described in table is what is included in addition to the usual forced transcription of the reprogramming genes.

| Reference | Species | Method |
|---|---|---|
| Choi et al. [129] | Mouse | miR-34 deficiency |
| Angel et al. [130] | Human | Innate immune suppression |
| Liao et al. [131] | Human | Combination of six transcription factors |
| Yoshida et al. [135] | Mouse and Human | Hypoxia |
| Lin et al. [132] | Human | TGFβ and MEK-ERK pathway inhibition |
| Mali et al. [133] | Human | Addition of SV40 large T antigen |
| Wang et al. [134] | Mouse and Human | Co-expression of RAR-γ and Lrh-1 |
| Zhao et al. [136] | Human | Addition of UTF1 forced transcription and p53 siRNA |
| Kleger et al. [112] | Mouse | Enrichment of the cell population for progenitor cells |

## 1.16 MECHANISMS OF REPROGRAMMING – A BRIEF OVERVIEW OF IMPORTANT PATHWAYS

It is important to know the molecular mechanisms associated with the reprogramming process. This information will enable researchers to determine more efficient and reproducible ways to generate iPSCs. It will also help in assessing if iPSCs are safe to use in a clinical setting. This is based on what pathways are being manipulated and if this can potentially lead to complications if the cells are transplanted into a patient. Important pathways and events involved in the reprogramming process include: p53 and its various downstream targets [129, 133, 136], innate

immune response pathways and regulators [130], pathways downstream of hypoxia inducible factors [135], MEK-ERK and TGFβ pathways [132], retinoic acid signaling [134], AID-dependent DNA demethylation [137], downstream targets of the orphan nuclear receptor Esrrb [138], and up-regulation of epithelial cell adhesion molecule complex proteins [139]. Furthermore it has been determined that reprogramming factor stoichiometry influences the epigenetic state of resulting iPSCs [140]. Different epigenetic states of iPSCs influence the resulting pluripotency and developmental potential of generated iPSCs and can affect the tumorigenicity of cells when transplanted *in vivo.* Finally, through the use of high resolution time-lapsed imagining it was determined that successfully reprogrammed cells undergo a rapid shift in their proliferative rate that coincides with a reduction in cell size [141]. This observation suggests that part of the reprogramming process follow defined rather than stochastic steps.

## 1.17 TRUE REPROGRAMMED IPSCS VS. REPROGRAMMING INTERMEDIATES

Since generating iPSCs involves forced expression of transcription factors it is not surprising that there is variation among the developmental and growth potential of different clonally expanded colonies. This is especially true since each generated iPSC line is genetically unique (depending on the method used to produce the cell line). Through the use of live cell imaging, observations were made that lead to the conclusion that true reprogrammed colonies express: TRA1-60, DNMT3B, and REX1 and are able to silence viral DNA [142]. It was also determined that partially reprogrammed intermediates are alkaline phosphatase positive as well as express SSEA-4, GDF4, hTERT, and NANOG [142]. This information is very important because iPSCs that

have the greatest developmental potential need to be identified early in the generation process so inferior colonies are not expanded and used for differentiation.

## 1.18 IPSC DEVELOPMENTAL POTENTIALS – DEMONSTRATING IPSCS ARE PLURIPOTENT

True pluripotency is determined by the ability of a stem cell to be differentiated to all 3 germ layers. Therefore in order to claim iPSCs are pluripotent investigators needed to describe *in vitro* and *in vivo* methods to differentiate iPSCs to cell types of all 3 germ layers. Teratoma formation is the "gold standard" *in vivo* method researchers use to determine pluripotency. If injected iPSC lines form tumors consisting of cell types from all 3 germ layers then the cell line is fully reprogrammed and pluripotent. As for *in vitro* methods, investigators used prior studies performed on hESCs as a basis for differentiation. Researchers began to determine chemically defined culture conditions that model the signaling events known to occur in tissue specification during embryogenesis. Reports have been generated where iPSCs were differentiated back toward ventral midbrain dopaminergic neurons [143], neurospheres [144], hematopoietic and endothelial cells [145], cardiomyocytes [146-148], and insulin secreting islet-like clusters [149]. These reports of *in vitro* differentiation of iPSCs represent lineages from all 3 germ layers. In each instance iPSCs were derived from fibroblasts and differentiation to desired cell type was achieved, however phenotype observed in culture is not always 100% comparable to the level of a primary cell. Thus *in vitro* differentiation needs to be optimized, either by a change in iPSC source or differentiation protocol.

## 1.19 IPSC IN VITRO DIFFERENTIATION TO HEPATOCYTES

Many investigators are interested in using iPSCs for drug discovery and regenerative medicine applications to treat liver disease. Therefore, researchers are interested in determining the best *in vitro* protocol for the derivation of high quality hiPSC-derived hepatocytes that is efficient and reproducible. There have been many reports claiming differentiation of iPSCs to hepatocytes using chemically defined culture conditions that include using various growth factors and cytokines, plating techniques and transduction of key liver-specific transcription factors [126-128, 150-155]. All of these reports use different gene expression characteristics and hepatocyte-specific function tests to characterize their iPS-derived hepatocytes. The take home message from all of these reports is that in most function tests, these stem-cell derived hepatocytes were never on the same level as compared to a primary hepatocyte. In fact, iPS-derived hepatocytes showed only 0.3-10% activity when compared to primary hepatocytes in certain hepatocyte specific function tests [154]. Therefore, as mentioned earlier, there needs to be a change in iPSC source or differentiation protocol to produce iPSC-derived hepatocytes that can be used for drug discovery or regenerative medicine applications. Table 12 summarizes the various reports of iPSC differentiation to hepatocytes in various species' cells with a brief summary of their novel differentiation protocol.

**Table 12.** Various Reports of iPSC Differentiation to Hepatocytes
This table summarizes the various reports within the literature on the generation of iPSC-derived hepatocytes from different species' cells. Table includes the reference, species of cell used and a brief summary of the growth factors, cytokines and transduction techniques used within each reference's differentiation protocol. The forward slashes in the "summary of protocol" column denote a change in culture conditions consisting of a basal medium or growth factor change.

| Reference | Species | Summary of Protocol |
|---|---|---|
| Asgari et al. [150] | Human | ActA / FGF4, HGF / OSM, DEX |
| Chen et al. [151] | Human | ActA, Wnt3a, HGF / DMSO / OSM, DEX, ITS |
| Sancho-Bru et al. [152] | Mouse | ActA, Wnt3a / BMP4, FGF2 / FGF1, FGF4, FGF8 / HGF, Follistatin |
| Si-Tayeb et al. [153] | Human | ActA / BMP4, FGF2 / HGF / OSM |
| Song et al. [154] | Human | ActA / BMP2, FGF4 / HGF, KGF / OSM, DEX / OSM, DEX, N2, B27 |
| Takayama et al. [155] | Human | ActA, FGF2, transduction w. HNF3β / BMP4, FGF4, transduction w. HNF3β and HNF1α / HGF, FGF1, FGF4, FGF10, transduction w. HNF3β and HNF1α / HGF, OSM, DEX |
| Rashid et al. [126] | Human | ActA, FGF2, BMP4, LY294002 / ActA / HGF, OSM |
| Ghodsizadeh et al. [127] | Human | EB Formation / ActA / DMSO HGF / DEX |
| Zhang et al. [128] | Human | ActA / FGF4, BMP2 / HGF, KGF / OSM, DEX / OSM, DEX, N2, B27 |

## 1.20 IN VITRO AND IN VIVO DISEASE MODELING WITH IPSCS – AND GENETIC CORRECTION

*In vitro* disease models currently rely on tumor cells lines and transformed derivatives of native tissue, which may not be the best approaches to model disease. If disease specific hiPSCs can be created and subsequently differentiated back to the cell type affected by the disease then these

cells would represent a better *in vitro* disease model than currently available. A recent study generated disease specific iPSCs from patients with familial dysautonomia and differentiated the cells back to neural crest precursor cells that maintain disease phenotype [121]. These neural crest precursor cells were then treated with a candidate drug and shown to respond to the drug as expected, demonstrating iPSC technology's utility in drug development. There was a report of a complete genetic correction of hiPSCs from a muscular dystrophy patient using human artificial chromosome technology to deliver a correct copy of the dystrophin gene [118]. These corrected hiPSCs were differentiated to muscle-like tissues that expressed dystrophin. hiPSCs have been generated from a patient with ALS and were successfully directed to differentiate into motor neurons, the cell type destroyed in ALS [125]. Patients suffering from sickle cell anemia have had hiPSCs generated from their cells and then had their genetic mutation corrected for using zinc finger nuclease technology [115]. ZFNs are a class of engineered DNA-binding proteins that facilitate targeted genome editing my creating a double-stranded break in DNA. This allows for homologous recombination and non-homologous end joining to allow for user specific gene edits. Fanconi anemia disease specific hiPSCs have been generated [124]. These disease specific hiPSCs were shown to be corrected for their defect and could give rise to hematopoietic progenitors of the myeloid and erythroid lineages that are phenotypically normal. Patients afflicted with Timothy syndrome have had hiPSCs generated from their somatic cells [123]. Neurons derived from these hiPSCs have given new insights into the cellular phenotypes associated with Timothy syndrome that would not have been possible with primary cells from these patients. This observation demonstrates the true power of disease modeling with hiPSC technology in how it can provide new targets to treat underlying disease pathologies. Other reports using iPSCs to model disease like the above examples include disease specific cells from

patients with: lysosomal storage disorders [122], spinal muscular atrophy [120] and Parkinson's disease [119].

In terms of liver specific diseases, hiPSCs have been shown to be a powerful tool to model liver-based inborn errors in metabolism *in vitro.* The following diseases have been modeled *in vitro* using hiPSCs: CN-1, tyrosinemia, A1AT, FH, glycogen storage diseases, progressive familial hereditary cholestasis and Wilson's disease [116, 126-128]. The resulting hiPSCs from A1AT, FH and glycogen storage diseases were found to recapitulate key pathological features of the diseases affecting the patients from with they were derived, such as aggregation of misfolded $\alpha_1$-antitrypsin in the endoplasmic reticulum, deficient LDL receptor-mediated cholesterol uptake and glycogen accumulation [126]. Although this report demonstrates the differentiated disease cells recapitulate key disease phenotypes, investigators failed to show hiPSC-derived hepatocytes derived from normal patients have normal protein folding, cholesterol uptake and glycogen accumulation. Therefore an alternative explanation for the findings in this report is that the disease phenotypes are from a failure of the hiPSCs to differentiate properly to hepatocytes.

The hiPSCs derived from a patient with A1AT were shown to be corrected for the point mutation in the A1AT gene that was responsible for the deficiency [116]. This genetic correction restored the structure and function of A1AT in subsequently derived liver cells *in vitro.* The Wilson's disease specific hiPSCs were shown to be differentiated into hepatocyte-like cells that displayed abnormal cytoplasmic localization of mutated ATP7B and defective copper transport, as would be expected in Wilson's disease primary hepatocytes [128]. More importantly, gene correction using a lentiviral vector that expresses the corrected ATP7B gene or treatment with the chaperone drug curcumin could reverse the functional defect within the hepatocyte *in vitro.*

These observations demonstrate the power of disease specific hiPSCs to model and treat liver diseases with gene therapy and potential autologous cell replacement or new drug discovery by treatment of disease specific hiPSC-derived hepatocytes with new small molecules to reverse pathologies.

Many *in vivo* disease models utilize transgenic or pharmaceutical/chemical based approaches that do not control for off target effects and some diseases do not have a relevant model. A Fah(-/-)Rag2(-/-)Il2rg(-/-) mouse has been developed that allows for the robust expansion of human hepatocytes within the host liver [32]. This specialized knockout mouse can be repopulated with human hepatocytes up to 90%. Primary cells have a limited life span in culture and metabolic disease hepatocytes are not readily available. If iPSCs can be generated from patients with genetic metabolic diseases and differentiated back to a hepatocyte-like state, these cells could serve as an unlimited source of cells for research. If these genetically defective hepatocytes can highly repopulate the mouse liver it is hypothesized that the animal would recapitulate a phenotype similar to what is seen in the human. These mice that contain "humanized" livers would be superior animal models for metabolic disorders because the basis for disease in the animal is human cell-derived. These mice could provide the microenvironment milieu to support the tissue's physiological function within the context of the whole organism, enabling greater understanding of disease pathogenesis and providing a platform for preclinical testing of drug candidates.

## 1.21 IPSCS USED TO TREAT DISEASE – POTENTIAL AUTOLOGOUS AND ALLOGENIC CELL REPLACEMENT THERAPIES

Using patient specific iPSCs, correcting them for their genetic defect, and differentiating them back to hepatocytes could be the answer to the immunosuppression and cell shortage problems associated with primary cell replacement therapies for liver-based diseases. If patient specific hiPSCs cannot be corrected for their defect, cell replacement therapies can utilize hiPSCs from a genetically competent donor and the OLT immunosuppression protocol can be used. This would solve the cell shortage issue. The current issue with making hiPSCs relevant for cell replacement therapy involves getting hiPSC-derived hepatocytes to engraft into various liver disease mouse models. This engraftment issue of hiPSC-derived hepatocytes needs to be solved. However, it does appear engraftment of hESC-derived somatic cells is possible given the recent report of hESC-derived neural progenitors elicit a distinct neurogenesis and functional recovery in a rat spinal cord contusion injury model [156].

Importantly, canine iPSCs were differentiated to endothelial cells and transplanted into a mouse hindlimb ischemia model and a mouse myocardial injury model [157]. These investigators reported engraftment of iPSC-derived endothelial cells 14 days following ligation of the femoral artery in the ischemia model along with significantly improved revascularization in mice receiving cells compared to mice receiving controls. iPSC-derived endothelial cells were also transplanted into the border zone of infracted mouse hearts. Engraftment was monitored and detectable at 42 days after infarction. Measurement of systolic function by echocardiography revealed improvement in cardiac contractility for animals receiving cells compared with animals receiving controls at 14 and 28 days post infarction. However their imaging technique revealed

progressive death of the donor cells following transplantation in the heart, hence there were no differences in systolic function between the transplanted animals and control animals at day 42.

A third group has differentiated iPSCs derived from hepatocytes, fibroblasts and keratinocytes into iPSC-derived hepatocytes and shown that all 3 types of hiPSCs have ability to engraft into a mouse model of chemical-induced acute liver failure [158]. They infused the mice with 2 million hiPSC-derived hepatocytes intravenously. They found that these cells could engraft in the mouse liver with efficiency ranging from 8 to 15%, which was comparable to adult human primary hepatocytes (~11%). Furthermore their engrafted cells were deemed functional because they detected albumin, transferrin, +/-1-antritrypsin and fibrinogen in the serum of transplanted animals. These concentrations ranged from 8.1 to 1100 ng/ml. These results suggest that hiPSC-derived hepatocytes may be useful to treat patients with end-stage liver disease and also potentially patients suffering from liver-based inborn errors in metabolism.

These observations show the promise of iPSC-derived cells to treat diseases but at the same time also display the current issue still holding back cellular therapies: long-term engraftment. Taken together, these three reports show promise for hiPSC-derived cellular therapies in the future, especially if long term engraftment issues can be solved.

## 1.22 PATIENT SPECIFIC IPSCS – WOULD THEY BE IMMUNOGENIC?

One of the big promises from iPSC technology is that it would allow for the creation of patient specific cells that can be corrected for their defect and transplanted back to the patient autologously, without the need for immunosuppression. However it has recently been shown that syngeneic mouse iPSCs are immunogenic [159]. The following observations were made: ESCs

derived from C57BL/6 mice can efficiently form teratomas in C57BL/6 mice without any evidence of immune rejection and allogenic ESCs from 129/SvJ mice fail to form teratomas in C57BL/6 mice due to rapid rejection by recipients. C57BL/6 mouse embryonic fibroblasts were reprogrammed into iPSCs. In contrast to C57BL/6 ESCs, teratomas formed by C57BL/6 iPSCs were mostly immune-rejected by C57BL/6 recipients. The majority of teratomas formed by C57BL/6 iPSCs in C57BL/6 mice displayed T cell infiltration, and in a small fraction of recipients there was apparent tissue damage and regression. A global gene analysis was performed to find that C57BL/6 iPSC-derived teratomas had over expression of certain genes compared to C57BL/6 ESC-derived teratomas. These data indicate that in contrast to ESC derivatives, abnormal gene expression in cells differentiated from iPSCs can induce an immune response in syngeneic recipients. Therefore the immunogenicity of patient specific iPSCs should be evaluated before any clinical application for autologous cell therapy. The one downfall from this study was that the investigators did not take skin cells from a mouse, make iPSCs from them, and transplant them into the same mouse to see if the resulting teratoma had evidence of an immune response. This would be the definitive experiment to determine if patient-specific iPSCs will elicit and immune response.

### 1.23 MAKING TRANSPLANTATION OF IPSCS CLINICALLY RELEVANT

One of the biggest issues holding back the usage of hiPSCs (and hESCs) for cellular therapies is the fact that any undifferentiated cells within the differentiated cell population can lead to formation of teratomas and potential cancer metastasis. Therefore technology has to be developed to remove teratoma-forming cells from differentiated cultures. A group has recently

discovered a monoclonal antibody against SSEA-5, and separation based on SSEA-5 expression through FACS greatly reduced teratoma-formation potential of poorly differentiated hESC cultures [160]. To ensure complete removal of potential cancer causing cells, they identified other pluripotency surface makers such as CD9, CD30, CD50, CD90 and CD200. Moreover, when subjecting hESCs to the SSEA-5 antibody and antibodies to 2 of the above mentioned other markers, they completely removed teratoma-formation potential from the poorly differentiated hESC cultures. Thus demonstrating experimentally it is possible to deplete undifferentiated teratoma-forming cells from cells that have been differentiated from hESCs. This report shows promise for hiPSC-derived cellular therapies in the future.

Finally, in order to make hiPSC-derived cellular therapies clinically relevant, investigations on what happens to iPSCs upon transplant into a recipient need to be determined in a large animal model. A recent report discussed the first autologous transplantation of undifferentiated iPSCs in a large animal model through the generation of canine iPSCs and subsequent transplantation into the canines from which the iPSCs were made [157]. Fate of the undifferentiated iPSCs autologously transplanted to the canine heart and flank was tracked *in vivo* using clinical positron emission tomography, computed tomography, and magnetic resonance imagining. Transplanted cells were tracked using these methods after induction with a reporter gene. Although canine hearts were immediately explanted after transplant, they were confirmed to have cells in them by ex vivo microPET imaging and histological analysis. This report shows that iPSCs have the ability to be tracked once transplanted, further helping to provide evidence that one day they may be proven to be useful in a clinical setting.

## 1.24 EPIGENETIC MEMORY, DONOR DIFFERENCES AND CELL SOURCE INFLUENCING IPSC DIFFERENTIATION POTENTIALS

It has recently been discovered that many iPSC lines show ability to differentiate to one lineage more efficiently and reproducibly than other lines. This experimental observation prompted researchers to begin to determine why there are these differentiation differences among cell lines. This information will allow researchers to determine the best characteristics of iPSC lines to differentiate to the hepatocyte lineage to use for drug discovery or regenerative medicine applications.

It has been determined that background mutations in parental cells account for most of the genetic heterogeneity of iPSCs [161]. This was determined by sequencing the genome of ten mouse iPSC clones derived from 3 independent experiments and comparing them to the parental cell genomes. This group detected hundreds of single nucleotide variants in every clone, with an average of 11 in coding regions. In two of the experiments all variants were unique for each clone and did not cluster in pathways, but in the third all four clones contained 157 shared genetic variants, which could also be detected in rare cells within the parental cell pool. In short, these researchers concluded that most of the genetic variation in iPSC lines is not caused by reprogramming, but is rather a consequence of cloning individual cells within the parental pool and “capturing” that single reprogrammed cells unique genetic mutational history. These genetically unique iPSC lines could be the reason why some lines will differentiate to one lineage more efficiently and reproducibly than others. This is especially true if a line has a unique mutation in a coding region of a protein essential for specification to a certain lineage. For example lines that have mutations in the HNF4α gene could potentially have difficulty

differentiating to hepatocytes, since HNF4α is an important transcription factor that controls expression of many key hepatocyte specific genes.

Another group has determined that aberrant silencing of the Dlk1-Dio3 gene cluster on chromosome 12qF1 in mouse iPSCs leads to poor developmental potential of the clones when comparing genetically identical iPSCs [162]. This is consistent with the developmental role that this gene cluster is known to be involved in. More specifically, it was shown that when this gene cluster is silenced the iPSC clones contributed poorly to chimeras and failed to support the development of entirely iPSC-derived animals. In comparison, iPSCs that had normal expression of this locus had the ability to form high-grade chimeras and generated viable all-iPSC mice. Furthermore, treatment of a clone that had silencing of the locus with a histone deacetylase inhibitor, such as valproic acid, reactivated the locus and rescued the lines developmental capabilities. This report is important because it can allow investigators to screen each developed iPSC line for silencing of this gene locus. If the line has silencing then it can be discarded due to its lack of developmental potential.

Evidence has recently been generated that show mouse iPSCs contain epigenetic memory of the donor cell from which they were made from [163] and cell type of origin influences *in vitro* differentiation potentials [111]. It was shown that iPSC lines have DNA methylation signatures that are characteristic of their somatic tissue of origin, which in turn favors the differentiation of that cell line to lineages related to the original cell source. Therefore, according to this data, if hepatocytes were the target cell, iPSCs generated from hepatocytes would represent the best cell source to generate stem cell-derived hepatocytes that most closely resemble their primary cell counterparts. However it was shown that continuous passing of iPSC

lines largely attenuated the differentiation differences, suggesting that early passage iPSCs have superior re-differentiation capacity.

Recently, a group has looked into donor-dependent variations in hepatic differentiation of hiPSCs to see if they could determine the best hiPSC to differentiate to a hepatocyte [164]. They differentiated and compared 28 hiPSC lines originating from various somatic cells. The hiPSCs were also derived from various methods including retroviruses, Sendai viruses or episomal plasmids. This comparison concluded that the origins, not the derivation methods, may be a major determinant of variation in hepatic differentiation. Specifically it was determined that clones derived from peripheral blood cells consistently showed efficient differentiation, whereas many clones from adult dermal fibroblasts showed poor differentiation. When they compared lines from peripheral blood and dermal fibroblasts from the same individual they found that variations in hepatic differentiation were attributable to donor differences, rather than to the cell type of origin of the iPSC line.

A second group has experimental evidence, like the previously discussed report, that regardless of cell origin the different human iPSC lines all showed the same ability to differentiate into hepatic cells *in vitro* [158]. They differentiated iPSCs derived from hepatocytes, fibroblasts and keratinocytes, all of which showed the same ability to differentiate into hepatic cells.

In short, it has been shown experimentally that *both* donor differences as well as the cell type of origin of the created iPSC contribute to the developmental potential and differentiation capacities of clonally expanded iPSCs. This means both factors must be taken into account when determining the best iPSC to be differentiated to a hepatocyte.

## 1.25 MOUSE FETAL LIVER-DERIVED IPSCS – INCREASED REPROGRAMMING CAPACITY AND SUPERIOR RE-DIFFERENTIATION?

Recently, a group has determined that there is an increased reprogramming capacity of mouse liver progenitor cells, compared with differentiated liver cells [112]. Specifically, adult and fetal liver cells from mice were enriched for their progenitor cells and it was determined that enriching for these cells dramatically increased reprogramming efficiencies. Enriching fetal liver cells for progenitor cells resulted in a 275-fold higher reprogramming efficiency compared with unsorted fetal cells. It was determined that this reprogramming efficiency increase was associated with endogenous expression of reprogramming factors and members of the BAF-complex, which mediate epigenetic changes during reprogramming.

Also, a group demonstrated that there is a contribution of hepatic lineage stage-specific donor memory to the differentiation potential of mouse iPSCs [113]. Specifically, iPSCs derived from fetal liver cells retain superior hepatic re-differentiation compared to iPSCs derived from adult liver cells. However once again, continuous passaging negated the differentiation differences.

Taken together, and if translated to humans, human fetal hepatocytes should have a superior reprogramming capacity and re-differentiation potential compared to adult hepatocytes. Therefore fetal hepatocyte-derived iPSCs may represent the easiest, most efficient and cost effective source to generate iPSC-derived hepatocytes that can be used for drug discovery, molecular biology disease research and regenerative medicine for liver-based diseases.

## 1.26 PROPOSAL SUMMARY

Here, we propose to isolate human adult and fetal hepatocytes, with and without various liver-based metabolic diseases, and examine their ability to become fully reprogrammed hiPSCs under entirely feeder-free conditions. Furthermore, to investigate if there are reprogramming efficiency differences between adult and fetal hepatocytes and perform preliminary differentiation experiments to see if the resulting hiPSC lines have the ability to become hepatocyte-like *in vitro* and *in vivo*.

## 1.27 MAJOR SIGNIFICANCE

This research, once completed, will serve as proof of principle that iPSCs derived from primary human adult and fetal hepatocytes represent the best cell source for making unlimited amounts of stem cell-derived hepatocytes that most closely resemble primary cells. Furthermore a bank of human hepatocytes with various liver-based metabolic diseases will be established that can be used for countless numbers of reprogramming experiments in order to generate disease-specific hiPSCs that represent the best source for generating disease-specific hiPSC-derived hepatocytes. Fetal hepatocytes will be shown to represent a source of hiPSCs that can be generated in a highly efficient and cost effective manner. This will be in direct contrast to human adult hepatocytes, where iPSC generation will be low efficiency and labor intensive. In either case, hiPSCs derived from adult and fetal hepatocytes can be an unlimited source for countless numbers of *in vitro* experiments to understand liver cell biology, if an efficient and reproducible hepatic differentiation protocol can be established. Transplantation medicine can utilize these

differentiated cells to stabilize a patient or even potentially correct a defect. Moreover, these stem-cell derived hepatocytes can be generated from disease patients, corrected for their genetic defect, and then transplanted back into the patient to correct the disorder without the need for immunosuppression. Investigators utilizing a pharmaceutical approach to therapy can use these cells for *in* vitro drug screens or use disease-specific cells for *in vitro* or *in vivo* disease modeling. These iPSC-derived hepatocytes theoretically have countless real-life applications in basic and clinical research; the data just needs to be generated to make the theory a reality.

## 2.0 INCREASED REPROGRAMMING OF HUMAN FETAL HEPATOCYTES COMPARED WITH ADULT HEPATOCYTES IN FEEDER-FREE CONDITIONS*

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## 2.1 ABSTRACT

Hepatocyte transplantation has been used to treat liver disease. The availability of cells for these procedures is quite limited. hESCs and hiPSCs may be a useful source of hepatocytes for basic research and transplantation if efficient and effective differentiation protocols were developed and problems with tumorigenicity could be overcome. Recent evidence suggests that the cell of origin may affect hiPSC differentiation. Thus, hiPSCs generated from hepatocytes may differentiate back to hepatocytes more efficiently than hiPSCs from other cell types. We examined the efficiency of reprogramming adult and fetal human hepatocytes. The present studies report the generation of 40 hiPSC lines from primary human hepatocytes under feeder-free conditions. Of these, 37 hiPSC lines were generated from fetal hepatocytes, 2 hiPSC lines from normal hepatocytes and 1 hiPSC line from hepatocytes of a patient with Crigler-Najjar Syndrome, Type-1. All lines were confirmed reprogrammed and expressed markers of pluripotency by gene expression, flow cytometry, immunocytochemistry, and teratoma formation. Fetal hepatocytes were reprogrammed at a frequency over 50-fold higher than adult hepatocytes. Adult hepatocytes were only reprogrammed with 6 factors, while fetal hepatocytes could be reprogrammed with 3 (OCT4, SOX2, NANOG) or 4 factors (OCT4, SOX2, NANOG, LIN28 or OCT4, SOX2, KLF4, C-MYC). The increased reprogramming efficiency of fetal cells was not due to increased transduction efficiency or vector toxicity. These studies confirm that hiPSCs can be generated from adult and fetal hepatocytes including those with genetic diseases. Fetal hepatocytes reprogram much more efficiently than adult, although both could serve as useful sources of hiPSC-derived hepatocytes for basic research or transplantation.

## 2.2 INTRODUCTION

Hepatocyte transplantation has been proposed as a cellular therapy for metabolic liver disease and acute liver failure. Primary hepatocyte transplantation has been used to partially correct patients with Crigler-Najjar, urea cycle, glycogen storage disorders and other metabolic liver diseases [40, 43, 53] and this therapy shows promise despite continuing issues with cell engraftment and proper immunosuppression. The availability of transplantable hepatocytes remains an ongoing problem.

Stem cell-derived hepatocytes from human embryonic stem cell (hESC) [67] and induced pluripotent stem cell (hiPSC) [88, 89] technology has the potential to provide a nearly unlimited source of hepatocytes for basic research, or *in vitro* drug discovery if efficient protocols were developed to produce mature hepatocytes; however issues concerning tumorigenicity still limit the clinical application of hESCs or hiPSCs. Furthermore, hiPSC generation is limited by low reprogramming efficiency as well as long duration from induction to cell line establishment. hiPSCs have been derived from multiple cell types including fetal lung and neonatal foreskin fibroblasts, mesenchymal stem cells [105], mouse adult and fetal hepatocytes [90, 112, 113], amnion epithelial [103, 108] and mesenchymal cells from the umbilical cord matrix or amnion [109] and even one report with human adult hepatocytes [107]. Furthermore, hiPSCs have also been derived from fibroblasts from patients with different genetic disorders, including inherited metabolic disorders of the liver [126]. These disease-specific cell lines potentially serve as an unlimited source of disease-specific cells if they could be differentiated back to a phenotype that faithfully recreates the disease. Theoretically, disease-specific cells could be genetically corrected and used for autologous cell transplantation to correct the phenotype. The idea of

patient specific therapy has recently been put into question with a report that differentiated mouse iPSCs induce T-cell-dependent immune responses in syngeneic recipients [159].

Several groups have tried to increase reprogramming efficiencies by using all six transcription factors [131], by combination of transcription factors and chromatin modulation [165, 166] or by addition of chemical compounds [167]. However, if populations of cells are more easily reprogrammed, methods to increase reprogramming may not be necessary, and these cell populations could be useful for translational research and could also be good models for understanding the mechanisms of the reprogramming process.

Recently, an increased reprogramming capacity of mouse liver progenitor cells compared with differentiated liver cells was reported [112]. There is evidence that mouse iPSCs retain epigenetic markings providing “memory” of the cell from which they were made [163] and that cell type of origin influences *in vitro* differentiation potential [111]. A recent report demonstrated hepatic lineage stage-specific donor memory in mouse iPSCs, and interestingly, fetal hepatocyte-derived iPSCs demonstrated superior capacity for hepatic re-differentiation [113]. Therefore, iPSCs from fetal human hepatocytes might be a particularly useful source of stem cell-derived hepatocytes.

There are no reports describing derivation of hiPSCs from human fetal hepatocytes or from hepatocytes from patients with an inborn error in metabolism. Here, we provide a method to generate hiPSCs from adult and fetal human hepatocytes under entirely feeder-free conditions and report that reprogramming of fetal hepatocytes was over 50-fold more efficient than adult hepatocytes and can be accomplished with only 3 reprogramming factors.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Human Adult Hepatocyte Isolation

All human tissues were collected with informed consent following ethical and institutional guidelines. Liver tissues were obtained either from liver resections or organ donors whose livers were not used for orthotopic liver transplantation. Residual tissue not needed for diagnostic purposes was transported to the laboratory from the operating rooms in cold Eagle's Minimum Essential Medium (EMEM) (Lonza, Walkersville, MD, cat no. 12-136Q) within 30 minutes of removal. Organs not used for orthotopic liver transplantation were obtained 3-24 hours after cross-clamp and *in situ* perfusion. When whole organs were obtained, they were surgically reduced to smaller size for more effective perfusion. The left lateral segment was processed first after anatomical dissection from the right lobe, 2-3 cm from the falciform ligament. If necessary, the right lobe was divided in two parts and processed as separate tissues. From this point forward, the procedure for hepatocyte isolation was the same for organ donor and liver resection tissues. Silicone catheters were inserted into the major portal and/or hepatic vessels and the tissue was perfused with Hank's Balanced Salt Solution (HBSS; without calcium, magnesium and phenol red; Lonza) to determine which vessel(s) provide the most uniform perfusion of the tissue. After the best vessels were identified, purse string sutures were used to encircle each vessel with its accompanying catheter and the sutures were drawn tight to prevent the leakage of buffer around the catheter during the perfusion. Excess suture material was tied to the catheters to further secure them to the liver. Perfusion proceeded once all remaining major vessels on the cut surfaces were closed with sutures or surgical grade super glue. The liver tissue was then placed in a sterile plastic bag and connected to a peristaltic pump with flow rate

dependent on the number of catheters and size of tissue (35 to 240 ml/min). The bag containing the tissue was placed in a circulating water bath at 37°C and the tissue was perfused with HBSS supplemented with 0.5 mmol/L ethylene glycol tetraacetic acid (EGTA) (Sigma Chemical Co., St. Louis, MO) without recirculation. Chelation of calcium by EGTA aids in the dissolution of intercellular junctions between hepatocytes and in the washing of hematopoietic cells. A second, non-recirculating perfusion with HBSS without EGTA was performed to flush residual EGTA from the tissue since calcium is essential for tissue dissociation enzyme activity. Finally, tissues were perfused with a third buffer composed of EMEM supplemented with VitaCyte's Clzyme™ Collagenase MA (MA) and recirculated as long as needed to complete the digestion. Enzyme was reconstituted per manufacturerer's recommendations, filtered, and added to the EMEM just prior to perfusion. Clzyme™ collagenase MA (VitaCyte) concentration was 100 mg/L with 24 mg Clzyme™ BP Protease (VitaCyte) in 1 L of EMEM immediately before perfusion. In general, hepatocyte isolations from organ donor required 1L of each buffer solution for each lobe, while those from liver resections, utilized half this volume. The duration that the tissue was exposed to the different buffers was dependent upon flow rate of the peristaltic pump, number of catheters and quality of the tissue; but perfusion of the first two buffers usually resulted in sufficient rinsing and warming of the tissue within 20-30 minutes. Perfusion times for the EMEM-collagenase solution varied among the tissue specimens and were determined subjectively by continual monitoring of the tissue integrity. Perfusion was stopped when the liver tissue beneath the capsule surface was visibly digested and separated from the capsule, which normally took about 25-35 minutes, thus, the total time of perfusion needed for cell isolation ranged from 40-65 minutes. The tissue was then transferred to a sterile plastic beaker that contained ice-cold EMEM and was gently chopped with sterile scissors to release hepatocytes.

The cell suspension was filtered through sterile gauze-covered funnels to remove cellular debris and clumps of undigested tissue. Hepatocytes were enriched by three consecutive centrifugation steps at 80g for 6 min each at 4°C. After three washes in EMEM, hepatocytes were suspended in cold Hepatocyte Maintenance Medium® (HMM, Lonza). Cell viability was assessed by mixing an aliquot of the final suspension with an equal volume of 0.4% (w/v) trypan blue in phosphate buffered saline and counting the number of viable (unstained) and dead (blue) hepatocytes with a hemacytometer. Viabilities were expressed as a percentage of the total cell number. Cultures were changed out with fresh media 2 hours after plating and washed extensively 18-24 hours post plating to remove dead and unattached cells. Primary human hepatocytes were cultured in HMM (Lonza) and supplemented with 1X penstrep (Invitrogen, Carlsbad, CA) and $10^{-7}$M insulin and $10^{-7}$M dexamethasone (Lonza). 5% bovine calf serum (Atlanta Biologicals, Norcross, GA) was supplemented to the above mentioned media for the first 2 hours of plating to help facilitate hepatocyte attachment. Table 13 summarizes the various liver-based metabolic disorders where human hepatocytes have been isolated and cryopreserved to establish a cell bank where disease-specific hiPSCs can be derived from.

**Table 13.** Human Hepatocyte Bank of Various Liver-based Metabolic Disorders
This table summarizes the various liver-based metabolic disorders where human hepatocytes have been isolated and cryopreserved to establish a cell bank where disease-specific hiPSCs can be derived from. Table outlines the metabolic disease and the number of donors where disease-specific cells have been isolated from.

| **Metabolic Disease** | **# of Donors** |
|---|---|
| Crigler-Najjar Syndrome Type-1 | 2 |
| Ornithine Transcarbamylase Deficiency | 2 |
| Carbamoyl Phosphate Synthetase-1 Deficiency | 2 |
| Alpha 1-antitrypsin Deficiency | 2 |
| Maple Syrup Urine Disease | 3 |
| Familial Hypercholesterolemia | 1 |
| Oxalosis | 1 |
| Biliary Atresia | 1 |
| Progressive Familial Intrahepatic Cholestasis Type-2 | 3 |
| Alagille Syndrome | 1 |

### 2.3.2 Human Fetal Hepatocyte Isolation

HBSS supplemented with 1 mM EGTA was warmed to 37°C and 10 ml was added to the fetal liver tissue in a petri dish. The tissue was held with sterile forceps and gently chopped with a sterile cell scraper. After the tissue was chopped the biliary tree was removed and discarded. 10 ml of warm HBSS with EGTA was added to the chopped tissue. This solution was pipetted into a new 50 ml conical tube(s) using a 25 ml pipette and the volume in the tube was brought up to 50 ml using the warmed HBSS with EGTA. The tube(s) were gently inverted several times to mix the tissue inside well and centrifuged at 80g for 5 minutes at 4°C. The subsequent supernatant was poured off and a pellet formed containing a band of red cells and a band of buffy colored fibroblasts on top. The red cells and fibroblasts were aspirated carefully by holding the tube in the near horizontal position and rotating the tube while using an aspirating pipette along the edge of the pellet. The resulting pellet was washed by inversion using 50 ml of warmed HBSS

(without EGTA) and centrifuged at 80g for 5 minutes at 4°C. If red cells were present in the pellet they were carefully aspirated again. The resulting pellet was resuspended using warmed EMEM supplemented with 0.5 mg/ml collagenase-Type XI (Sigma Chemical Co.) and 0.2 mg/ml DNase (Sigma Chemical Co.) to a final volume of 50 ml. The tubes were placed on a lab quake shaker/rotisserie (Barnstead Intl., Dubuque, IA) in a 37°C incubator (5% $CO_2$/ambient $O_2$) for 40 minutes. After the incubation the tube(s) were inverted several times and centrifuged at 80g for 5 minutes at 4°C. The subsequent supernatant was poured off and the pellet was resuspended in 20 ml of warm EMEM. The pellet was resuspended by pipetting up and down several times using a 25 ml pipette and shaking the tube vigorously to break up the remaining tissue pieces. An aliquot of this cell suspension was used for total cell count and viability. This was assessed by mixing the aliquot with an equal volume of 0.4% (w/v) trypan blue in phosphate buffered saline and counting the number of viable (unstained) and dead (blue) hepatocytes with a hemacytometer. Viabilities were expressed as a percentage of the total cell number. The remaining cells were spun at 80g for 5 minutes at 4°C. The subsequent supernatant was poured off and the cells were resuspended in warm DMEM supplemented with 10% FBS (PAA Laboratories, Pasching, Austria), $10^{-7}$M insulin, 1X penstrep, and 10 ng/ml EGF (BD Biosciences). Cultures were changed out with fresh media 2 hours after plating and washed extensively 18-24 hours post plating to remove dead and unattached cells as well as red blood cells.

### 2.3.3 hESC and hiPSC Cell Culture

The H1 hESC and all hiPSC lines were maintained on hESC-qualified matrigel (BD Biosciences, San Jose, CA) and cultured in mTeSR™1 medium (Stem Cell Technologies,

Vancouver, BC, Canada) in a HERAcell150 incubator at 37°C and ambient $O_2$ / 5% $CO_2$. Cells were passaged using 1 mg/ml dispase (Gibco, Carlsbad, CA) using conventional stem cell culture techniques. All cell lines used for data collection were passage 30 or under. The H1 hESC line was obtained from the WiCell Research Institute, Inc. The hiPSC inductions and cell line establishment section describes the cell culture process in detail.

### 2.3.4 Matrigel Coating of 6-well Plates

6-well plates were coated with hESC-qualified matrigel by diluting an aliquot of cold matrigel in 25 ml of cold DMEM/F12. The volume of the matrigel aliquot was noted on the specification sheet that came with the matrigel. 1 mL of matrigel diluted in DMEM/F12 was placed in each well of a 6-well plate. The plates were left undisturbed in the tissue culture hood for at least 1 hour. After an hour the plates were washed once with DMEM/F12. Plates were used immediately or could be stored in the 4°C refrigerator for up to 14 days. Stored plates contained 2 mL of DMEM/F12 per well and were wrapped in plastic wrap to ensure sterility.

### 2.3.5 hiPSC Inductions and Cell Line Establishment

Primary human adult and fetal hepatocytes were added to matrigel-coated 6-well plates at 100,000 cells/well in their respective media and allowed to attach overnight. The following day transduction media was given to the cells that consisted of each cell type's respective culture medium supplemented with 6 µg/ml polybrene and lentiviruses delivering up to six reprogramming factors OCT4, SOX2, KLF4, C-MYC, NANOG, and LIN28, (Thermo Fisher Scientific) each at a targeted MOI of up to 10 (Day 0). After 24 hours the cells were washed with

DMEM/F12 (Thermo Fisher Scientific) 3X. Cells were cultured in their respective media and media was changed to mTeSR™1 on days 1-7. Media was refreshed every 2-3 days. Large hiPSC colonies appeared between days 16 and 21 and individual colonies were selected for expansion between days 25 and 35.

The following describes the colony selection process and resulting hiPSC line culture techniques: Colonies for selection have well defined borders, a high nucleus to cytoplasm ratio and were morphologically indistinguishable from hESCs. Once these colonies were seen, the inverted microscope was placed into the tissue culture hood and the colonies were picked up manually using a P20 pipetman. hiPSCs were scratched gently off the culture dish and aspirated into the P20 pipet tip. Each aspirated colony was plated into one well of a 6-well plate on hESC-qualified matrigel in mTeSR. hiPSC clones attached 24 hours after initial selection and retained the morphology observed prior to the selection. Once the one well of a 6-well plate became about 80% confluent with hiPSCs the cells were passaged. Briefly, the hiPSCs were washed 2X with DMEM/F12 and given 1 mg/ml dispase (Gibco) for about 5 minutes, or until the edges of the colonies folded up. The cells were washed 2X with DMEM/F12 and then given mTeSR. The hiPSCs were gently scraped with a cell scraper to remove all the colonies off the bottom of the well. The floating colonies were aspirated 2 to 3 times with a pipet in order to break the colonies up into smaller ones more suitable for passage. Colonies for passaging were smaller than those used for freezing, however not smaller than 20 cells. The colonies in the one well of a 6-well plate were then transferred to another well of a 6-well plate coated with hESC-qualified matrigel in mTeSR. The splitting ratio was based on how confluent the well was prior to dispase treatment. This splitting procedure was repeated until each individually picked clonally expanded colony could be established in a completely undifferentiated state with a normal

proliferation rate (passaging every 7-10 days). Figure 2 outlines the hiPSC induction protocol with examples of developing and selected colonies harvested into hiPSC lines.

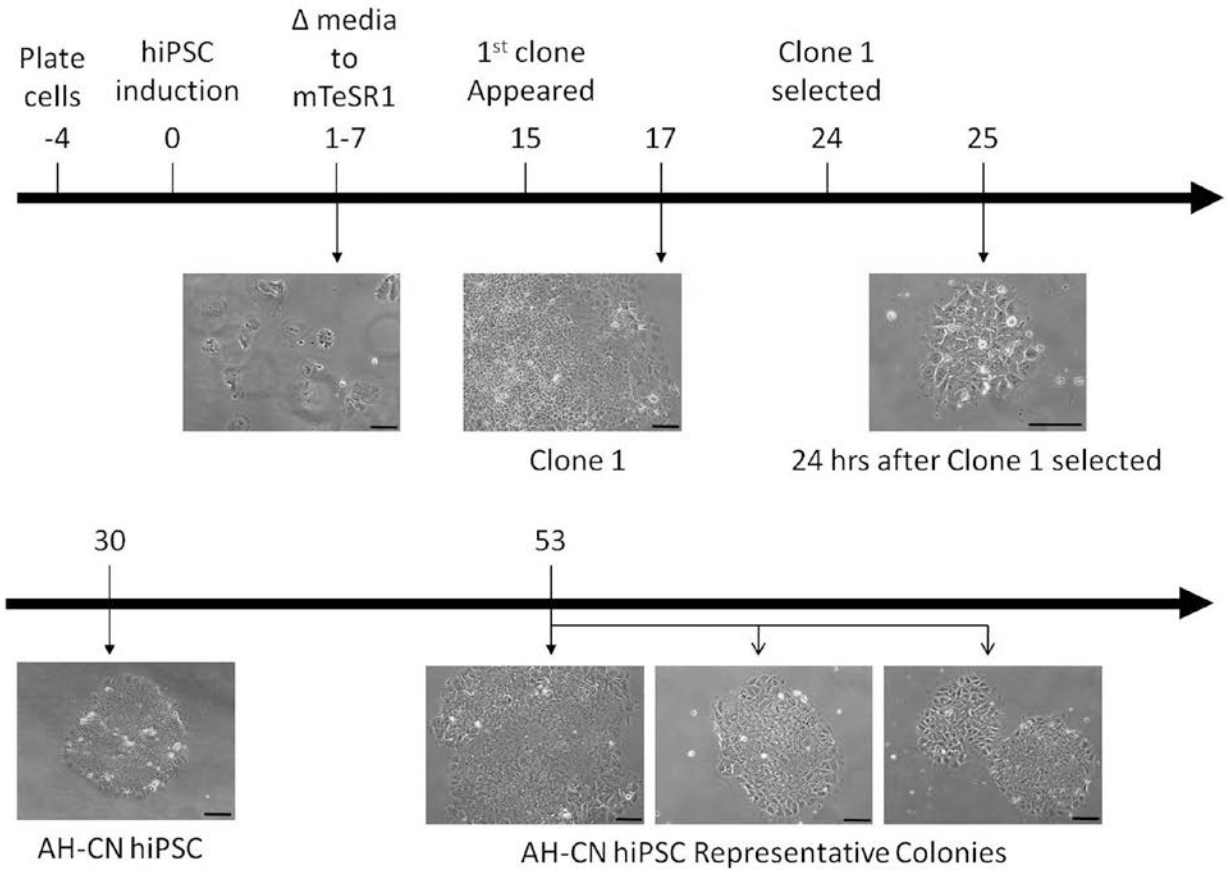


**Figure 2.** Protocol for hiPSC Inductions
This figure summarizes the hiPSC induction protocol to establish hiPSCs derived from adult and fetal hepatocytes in entirely feeder-free conditions. Accompanied photographs show a timeline for the hiPSC induction experiment that lead to the formation of the AH-CN hiPSC line. Scale bars are 100 μM.

Multiple vials of each established cell line were cryopreserved in mFreSR (Stem Cell Technologies) to establish a bank of human adult and fetal hepatocyte-derived hiPSC lines. Table 14 summarizes all of the established hiPSC lines, their associated Strom lab donor numbers and how many vials have been cryopreserved in mFreSR.

**Table 14.** Adult and Fetal Hepatocyte-derived hiPSC Bank
This table summarizes the various hiPSC lines that have been derived from both adult and fetal hepatocytes, the associated Strom lab donor numbers and the number of vials that have been cryopreserved.

| Cell Line Name | Strom Lab Donor # | # Vials Cryopreserved |
|---|---|---|
| AH-CN | HH1655 | 80 |
| AH1 | HH1591 | 26 |
| AH2 | HH1591 | NA |
| FH1 | HH1781f | 70 |
| FH2 | HH1795f | 60 |
| FH3 | HH1795f | 6 |
| FH4 | HH1791f | 1 |
| FH5 | HH1791f | 4 |
| FH6 | HH1791f | 3 |
| FH7 | HH1791f | 3 |
| FH8 | HH1791f | 3 |
| FH9 | HH1791f | 3 |
| FH10 | HH1902f | 3 |
| FH11 | HH1902f | 4 |
| FH12 | HH1902f | 18 |
| FH13 | HH1902f | 4 |
| FH14 | HH1902f | 18 |
| FH15 | HH1902f | 12 |
| FH16 | HH1902f | 4 |
| FH17 | HH1902f | 4 |
| FH18 | HH1902f | 4 |
| FH19 | HH1902f | 5 |
| FH20 | HH1902f | 3 |
| FH21 | HH1902f | 3 |
| FH22 | HH1902f | 3 |
| FH23 | HH1902f | 4 |
| FH24 | HH1902f | 5 |
| FH25 | HH1902f | 4 |
| FH26 | HH1902f | 4 |
| FH27 | HH1902f | 4 |
| FH28 | HH1902f | 3 |
| FH29 | HH1902f | 16 |
| FH30 | HH1902f | 3 |
| FH31 | HH1902f | 4 |
| FH32 | HH1986f | 1 |
| FH33 | HH1986f | 1 |
| FH34 | HH1986f | 3 |
| FH35 | HH1986f | 2 |
| FH36 | HH1986f | 2 |
| FH37 | HH1986f | 1 |

Table 15 outlines each individually performed hiPSC induction experiment. It outlines the donor #, age, sex, viability, days in culture prior to induction, days into induction mTeSR™1 was started, viruses used, MOI of viruses and what hiPSC lines were formed (if any).

**Table 15.** Details of hiPSC Induction Experiments on Adult and Fetal Hepatocytes
Information includes donor #, age, sex, viability, days in culture prior to induction, days into induction mTeSR™1 was started, viruses used, MOI of viruses and what hiPSC lines were formed (if any).

| Donor # | Age | Sex | Viability (%) | hiPSC lines formed | Days in culture prior to induction | Days into induction mTeSR™1 started | Viruses used | MOI (of each virus) |
|---|---|---|---|---|---|---|---|---|
| HH1655 | 3 mos | F | 83.4 | AH-CN | 4 | 7 | OSKMNL | 10 |
| HH1705 | 9 yrs | M | 96.0 | No | 3 | 1 | OSKMNL | 10 |
| HH1758 | 6 yrs | F | 78.0 | No | 1 | 7 | OSKMNL | 5 |
| HH1758 | 6 yrs | F | 78.0 | No | 1 | 7 | OSKMNL | 10 |
| HH1764 | 10 yrs | M | 76.0 | No | 2 | 7 | OSKMNL | 10 |
| HH1806 | 1 mos | M | 70.5 | No | 1 | 7 | OSKMNL | 10 |
| HH1813 | 1 yrs | M | 91.0 | No | 2 | 7 | OSKMNL | 10 |
| HH1815 | 13 mos | F | 90.0 | No | 1 | 7 | OSKMNL | 10 |
| HH1815 (thawed) | 13 mos | F | UND | No | 1 | 2 | OSKMNL | 10 |
| HH1843 | 12 yrs | F | 94.0 | No | 2 | 1 | OSKMNL | 10 |
| HH1892 | 1 yrs | M | 88.0 | No | 1 | 3 | OSKMNL | 10 |
| HH1901 | 4 mos | M | 89.0 | No | 2 | 3 | OSKMNL | 10 |
| HH1904 | 8 mos | M | 84.0 | No | 1 | 3 | OSNLM | 10 |
| HH1591 | 12 yrs | F | 76.0 | AH1-2 | 3 | 6 | OSKMNL | 1.67 |
| HH1995 (thawed) | 3 yrs | F | 70.0 | No | 1 | 1 | OSNL | 10 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HH1995 (thawed) | 3 yrs | F | 70.0 | No | 1 | 1 | OSNL | 5 |
| HH1996 (thawed) | 14 yrs | M | 70.0 | No | 1 | 1 | OSNL | 10 |
| HH1996 (thawed) | 14 yrs | M | 70.0 | No | 1 | 1 | OSNL | 5 |
| HH1781f | 20 wks | UND | 95.0 | FH1 | 3 | 2 | OSN | 10 |
| HH1782f | 15 wks | M | 98.5 | No | 2 | 1 | OSNL | 10 |
| HH1791f | 12.7 wks | M | 91.0 | FH4-9 | 1 | 1 | OSNL | 10 |
| HH1794f | 18 wks | UND | 91.0 | No | 1 | 1 | OSNL | 10 |
| HH1795f | 21 wks | M | 91.0 | FH2-3 | 1 | 1 | OSNL | 10 |
| HH1902f | 22 wks | M | 95.0 | FH10 | 1 | 3 | OSKM | 10 |
| HH1902f | 22 wks | M | 95.0 | FH11-31 | 1 | 3 | OSNL | 10 |
| HH1986f | 24 wks | M | 96.0 | FH32-37 | 1 | 1 | OSNL | 10 |

Colony formation was determined by counting total number of individual hESC-like colonies formed in all wells. Reprogramming efficiency was determined by the following equation: (total number of hESC-like colonies/total number of cells plated) X 100. Details of each individual experiment (n=18 adult and n=8 fetal) can be found in Table 15.

### 2.3.6 Freezing of hESCs and hiPSCs

The freezing procedure was the same procedure for splitting the cells, however instead of splitting the cells to a new matrigel coated plate the cells were centrifuged in mTeSR at 1000 RPM for 5 minutes. The resulting cell pellet was resuspended in mFreSR, placed into a CoolCell® alcohol-free cryopreservation container (BioCision) and placed in the -80°C freezer

overnight. After 24 hours the frozen cell vials were permanently stored in liquid nitrogen. The ratio of mFreSR for each cell pellet was generally 2 80%-confluent wells of a 6-well plate per 1 mL of mFreSR.

### 2.3.7 Thawing of hESCs and hiPSCs

A vial of cells was quickly taken out of liquid nitrogen and placed on dry ice and transported to a 37°C water bath for rapid thawing. Once the vial was completely thawed the solution of cells was gently aspirated using a pipet, put into mTeSR and centrifuged at 1000 RPM for 5 minutes. The resulting cell pellet was resuspended in mTeSR supplemented with 10 µM rock inhibitor (Sigma-Aldrich), being careful not to break up the colonies too much. Larger colonies generally plated better after thawing than smaller ones. Cells were plated onto 6-well matrigel coated plates according to how they were frozen. For example, if 2-wells of a 6-well plate were frozen in one vial, the thawed colonies were plated back to 2-wells of a 6-well plate. 24-hours after thawing the attached colonies were similar in size to what the colonies looked like 24-hours after normal passaging.

### 2.3.8 Gene Expression

RNA was isolated using TRIZOL® reagent (Life Technologies, Carlsbad CA) according to the manufacturer's protocol. RNA integrity and purity were confirmed. DNase-I treated RNA of each sample were reverse-transcribed by PCR with RT-PCR reagents (Promega, Fitchburg, WI) according to manufacturer's protocol. The resulting cDNA was analyzed by quantitative real-time PCR using specific TaqMan® assays and TaqMan® Gene Expression Master Mix

(Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. Ct values were entered into the following equation to determine the arbitrary unit value: $1 \times 10^9 \times e(-0.6931 \times Ct)$ [168] then normalized to cyclophilin A. All samples were run and analyzed on a ABI Prism 7000 Sequence Detection System. Table 16 summarizes the TaqMan® assays that were used:

**Table 16.** TaqMan® Assays Used for Chapter 2 Experiments of this Dissertation
Table summarizes the TaqMan® assays used for Chapter 2 experiments and the associated gene symbols, gene name and assay ID numbers.

| Gene Symbol | Gene Name | Assay ID # |
|---|---|---|
| CYCA (PP1A) | Cyclophilin A (peptidylprolyl isomerase A) | Hs99999904_m1 |
| OCT4 | Octamer 4 | Hs03005111_g1 |
| SOX2 | SRY (sex determining region Y)-box 2 | Hs01053049_s1 |
| NANOG | Nanog homeobox | Hs02287400_g1 |
| GDF3 | Growth differentiation factor | Hs00220998_m1 |
| hTERT | Human telomerase reverse transcriptase | Hs00972656_m1 |
| DNMT3B | DNA (cytosine-5-)-methyltransferase 3 beta | Hs00171876_m1 |
| c-MYC | Myelocytomatosis viral oncogene homolog | Hs00905030_m1 |
| KLF4 | Kruppel-like factor 4 | Hs00358836_m1 |
| BAF155 (SMARCC1) | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 | Hs00268265_m1 |
| BRG1 (SMARCA4) | SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 | Hs00231324_m1 |

### 2.3.9 Flow Cytometry

After a 3-5 minute exposure to accutase (Gibco), cells were counted and viability was determined. The cells were resuspended at a concentration of $10^7$/ml in PBS enriched with human albumin and EGTA. We characterized the cells according their expression of nuclear and surface markers: NANOG, OCT3/4, SOX2, SSEA-3, SSEA-4, TRA1-60 and TRA 1-81 (all from

BD Biosciences). The staining procedure was the same for both type of markers: a minimum number of 0.5 x $10^6$ viable cells per tube were incubated for 45 minutes at 4°C with appropriate amount of monoclonal antibodies directly conjugated with six fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), cyanine chrome 5 (Cy5), allophycocyanine (APC), Alexa 466 (Al.466) and Alexa 667 (Al.667) (according to manufacturer dilutions). After staining, the samples were fixed with 2% paraformaldehyde for 10 min at room temperature. The evaluation of nuclear markers required a fixation-permeabilization procedure (Perm/Wash Buffer), as described in the manufacture protocol (BD Biosciences, San Jose, CA), before the staining procedure. Appropriate non-specific fluorescence-conjugated antibodies of identical isotype were used as negative controls in order to evaluate positive cells for each specific antibody used. Four color flow-cytometry acquisition was performed by dual laser FACSCalibur® equipped with CellQuest® software (BD Biosciences). To analyze and immunophenotype different cell subpopulations, at least 0.3 x $10^6$ cells per tube were acquired. Sequential gating was implemented based on negative-control staining profiles. All samples were analyzed by FlowJo® software (Tree Star Inc., OR).

### 2.3.10 Immunofluorescence

hiPSCs were plated on matrigel-coated coverslips in 24-well plates. Cells were washed 3X with PBS and fixed using 4% PFA for 20 minutes. Cells were permeabilized with 0.1% Triton X made in PBS for 15 minutes for Oct4, Sox2, and Nanog. Cells were not permeabilized for SSEA4. Cells were rehydrated with 3 washes of PBS and 5 washes of 0.5% BSA in PBS. Blocking was performed in 2% BSA for 45 minutes followed by 5 washes of 0.5% BSA. Primary antibody was diluted in 0.5% BSA and incubated for 60 minutes followed by 5 washes

of 0.5% BSA. Secondary antibody was diluted in 0.5% BSA and incubated for 60 minutes followed by 5 washes of 0.5% BSA and 5 washes of PBS. Nuclei were stained using Hoechst for 1 minutes followed by 2 washes with PBS. Cells were mounted using gelvatol mounting media consisting of PVA (Sigma-Aldrich, Cat. # P-8136), glycerol (Sigma-Aldrich, Cat. # G-9012) and sodium azide (Thermo Fisher Scientific, Cat. # S227-100) and refrigerated until images were captured on an Olympus Provis AX70 fluorescent microscope with Magnafire 2.1B software. The following primary antibodies were used: Rabbit anti-Oct3/4 (Santa Cruz sc-9081) 1:250, Rabbit anti-Sox2 (Millipore AB5603) 1:300, Rabbit anti-Nanog (Abcam ab-80892) 1:100, SSEA-4-Alexa Fluor 555 (BD Bioscience 560218) 1:100. The following secondary antibodies were used: Goat anti-rabbit Alex Fluor 488 (Invitrogen) 1:500 and Donkey anti-rabbit TR (Santa Cruz sc-2784) 1:250.

### 2.3.11 Alkaline Phosphatase Staining

hiPSCs were plated on matrigel in 24-well plates. Cells were fixed with 4% PFA in PBS and stained using the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories) using manufacturer's protocol.

### 2.3.12 Teratoma Formation

hiPSCs were grown in 6-well plates to about 85% confluence. The entire 6-well plate was treated with 1 mg/ml dispase and cells were centrifuged at 200 x g for 5 minutes in a 50 mL conical tube. Cells were resuspended in 1 mL of mTeSR™1 and 1 mL of undiluted matrigel and equal volumes were transplanted subcutaneously between the scapulas of 3 NOD/SCID IL2γ-

chain receptor knockout mice (dorsal injection). All animals were housed and euthanized according to University of Pittsburgh Animal Husbandry protocols. Tumors were excised from euthanized animals and fixed with 10% formalin overnight at 4°C. Tumor sections were processed and sections were made and stained for hematoxylin and eosin y. A pathologist from Children's Hospital of Pittsburgh confirmed cell types from all 3 germ layers were present.

### 2.3.13 Transduction Efficiency

Primary human adult and fetal hepatocytes were plated on matrigel in 6-well plates at 100,000 cells/well in their respective media and allowed to attach overnight. The following day transduction media was added to the cells that consisted of each cell type's respective culture medium supplemented with 6 μg/ml polybrene (Millipore, Billerica, MA) and GFP lentivirus (Thermo Fisher Scientific, Waltham, MA) at an MOI of 5, 10, 25, and 50 in triplicate wells (Day 0). After 24 hours the cells were washed with DMEM/F12 (Thermo Fisher Scientific) 3X and cells were given new cell culture media (Day 1). On Day 3 fluorescent images of the cells were taken using a Nikon Eclipse Ti inverted fluorescent microscope and Elements software (Nikon, Melville, NY). Ten random areas of each well from each MOI were taken for a total of 10 pictures per MOI. Transduction efficiency was determined by the following equation: (GFP+ cells/total cells) X 100.

### 2.3.14 Toxicity Assays

Adult and fetal hepatocytes were isolated as described earlier. Ten thousand cells/well were plated on 96-well plates coated with matrigel in their respective cell culture media and

allowed to attach overnight. The next day each respective cell type's media was supplemented with 6 µg/ml polybrene and a lentivirus delivering GFP (Thermo Fisher Scientific) at an MOI of 0-60. The next day the cells were washed and given their respective cell culture media. Cells for the 24 and 72 hour time points were analyzed for total double strand DNA using the Quanti-iT™ assay (Invitrogen), caspase activity by Caspase-Glo® 3/7 assay (Promega) and CellTiter-Glo® cell viability assay (Promega) according to manufacturer's instructions.

### 2.3.15 Statistical Analysis

Reprogramming efficiency and gene expression analysis were analyzed using a two-tailed student's t-test. The incidence of hESC-like colony formation was analyzed using a Fisher's Exact Test where colony formation in an individual experiment was scored either as a yes or a no. All toxicity assays were analyzed using a two way ANOVA with a Bonferroni posttest. A p-value of $<0.05$ was determined as significant.

### 2.3.16 Use of Animal- and Human-derived Tissue

All animal experiments were performed in accordance with the University of Pittsburgh's Institutional Animal Care and Use Committee. Furthermore all human tissue was obtained and approved by the University of Pittsburgh's Institutional Review Board and appropriate measures were taken to insure patient privacy. Informed consent was given for the human material.

## 2.4 RESULTS

### 2.4.1 Establishing a Bank of Human Adult and Fetal Hepatocyte-derived hiPSCs in Entirely Feeder-Free Conditions

Donor characteristics for the hepatocyte cases used in these studies are presented in Table 15. For clarity, adult hepatocytes are defined here as any hepatocytes isolated from postnatal livers. Fetal hepatocytes were isolated from tissue ranging from 89 – 168 days of gestation. Adult and fetal hepatocytes were freshly isolated and seeded on matrigel in their respective culture media and allowed to attach overnight (Figure 3). The average viability according to trypan blue exclusion was 83% and 94% for adult and fetal cells, respectively. The cells were transduced with lentiviruses delivering the reprogramming factors for 24 hours and media was changed every 2 to 3 days until hESC-like colonies appeared (see Experimental Procedures). Large hESC-like colonies appeared between days 16 and 21 and individual colonies were selected for expansion between days 25 and 34 and were seeded onto new matrigel-coated plates in mTeSR™1 medium. Fully reprogrammed colonies were easily distinguishable by their similarity to hESC colonies (Figure 3, 5-10). All selected colonies had high nuclei to cytoplasm ratio, well defined borders and were otherwise morphologically indistinguishable from hESCs (Figure 3, 5-10). In contrast, transformed / not fully reprogrammed colonies did not grow in well defined borders and the individual cells growing within the colonies were distinguishable from each other (Figure 4). Colonies, like the one pointed out by the green arrow (Figure 4), were selected for and expanded into cell lines when the original colony was big enough to be harvested with a pipet under a microscope placed in the tissue culture hood to ensure sterility. Three hiPSC lines were isolated from 2 different adult hepatocyte cases; one of which was from

a 13 month old patient diagnosed with CN-1 syndrome and 2 lines were derived from a 12 year old female organ donor. Attempts at reprogramming 12 other adult and pediatric cases failed to generate hiPSC colonies (n=18 total experiments with adult hepatocytes). These cases ranged from 4 months to 39 years. In contrast, 37 hiPSC lines were isolated from 5 different fetal hepatocytes cases. Importantly, one line was reprogrammed with only 3 factors (OCT4, SOX2, and NANOG; FH1). Attempts at reprogramming 2 other fetal hepatocyte cases failed to generate hiPSC colonies (n=8 total experiments with fetal hepatocytes).

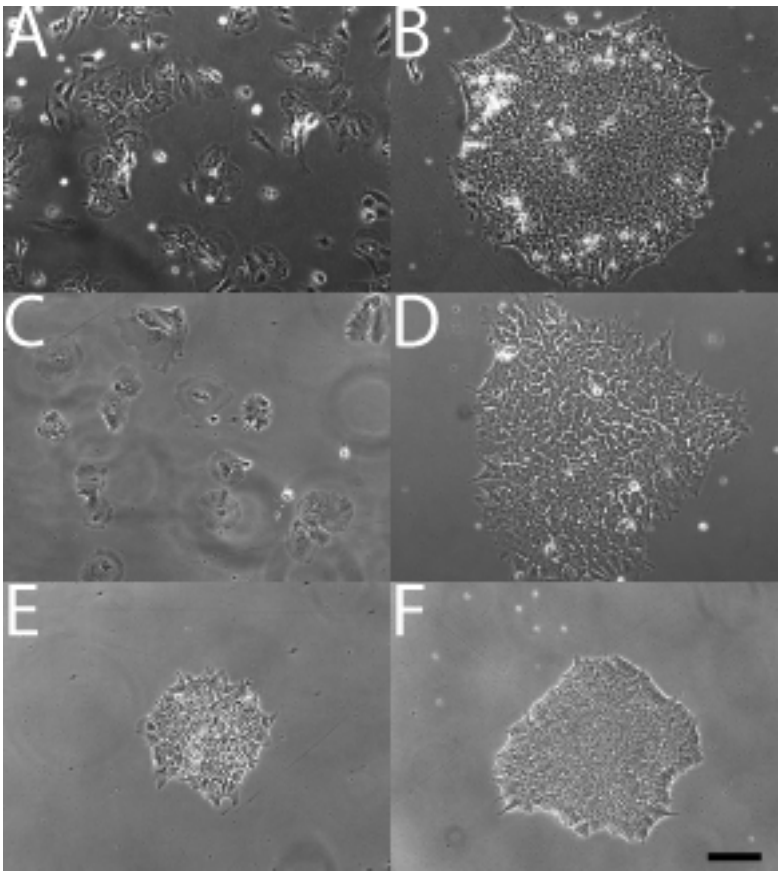


**Figure 3.** Generation of hiPSCs from Adult and Fetal Hepatocytes in Entirely Feeder-Free Conditions
Adult or fetal hepatocytes were exposed to lentiviruses delivering the reprogramming factors in their respective culture media overnight (Day 0). The culture medium was changed to mTeSR1 on Days 1-7. The pictures are representative images of starting cells and hiPSC lines grown on matrigel in mTeSR1. The hiPSC lines are morphologically indistinguishable from the H1 hESC line and grow in colonies with well defined borders and high nuclei to cytoplasm ratio. Scale bar is 100 µM. (A) Fetal hepatocytes in culture on Day 0 of hiPSC induction. (B) Representative colony of fetal hepatocyte-derived hiPSC clone FH1. FH1 was derived using only OCT4, SOX2 and NANOG. (C) Adult hepatocytes in culture on Day 0 of hiPSC induction. (D) Representative colony of adult hepatocyte-derived hiPSC clone AH1. (E) Representative colony of adult hepatocyte-derived hiPSC clone made from cells of a patient diagnosed with CN-1. Clone AH-CN (F) Representative colony of H1 hESC.

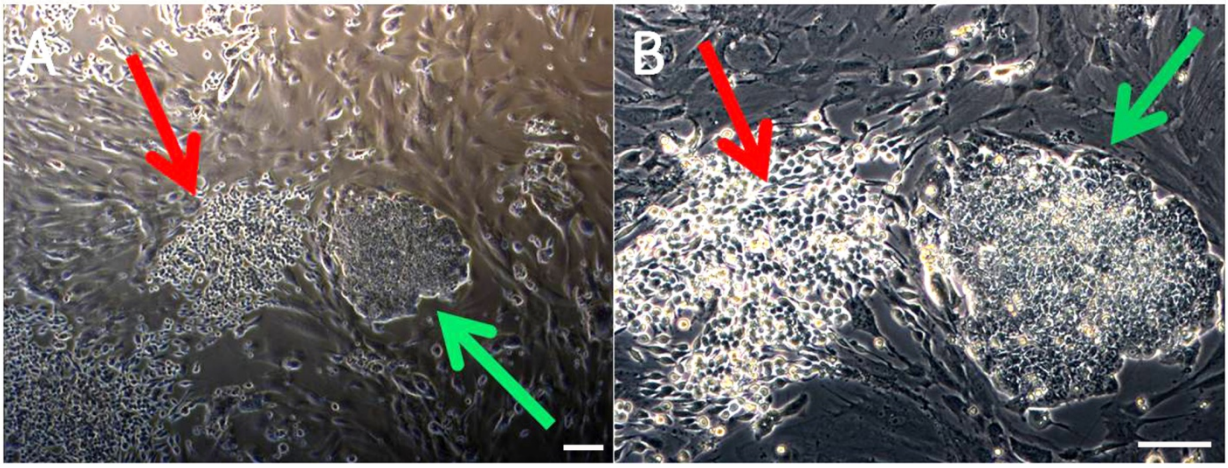


**Figure 4.** Distinguishing Between Transformed vs. Fully Reprogrammed Colonies
This figure denotes the morphology of transformed (red arrows) and fully reprogrammed (green arrows) colonies. Fully reprogrammed colonies grow with well defined borders and individual cells are not distinguishable from each other. In contrast, transformed colonies do not have well defined borders and individual cells within the growing colony are distinguishable from each other. Scale bars are 100 µM. Colonies like the one pointed out by the green arrow were selected for and expanded into cell lines when the original colony was big enough to be harvest with a pipet under a microscope placed in the tissue culture hood to ensure sterility.

Of the 40 lines generated, 8 were selected and examined for characteristics of fully reprogrammed cells. Two adult (AH1 and AH-CN) and 6 fetal (FH1, FH2, FH12, FH14, FH15 and FH29) hepatocyte-derived hiPSC lines were further characterized for pluripotency for this report. All 8 of these hiPSC lines were characterized by morphology (Figure 3, 5-7, 9), gene expression, flow cytometry and ICC of pluripotency markers and teratoma formation. All other hiPSC lines established (both adult and fetal-derived) were expanded, cryopreserved and banked for later use. All of the characterized cell lines were carried out to at least passage 35 and maintained characteristic hESC-like morphology and a consistent proliferation rate. Please refer to Table 14 above for the established bank of hiPSCs derived from adult and fetal hepatocytes. Figures 5-10 shows the morphology of all other established hiPSC lines (AH2 line not shown).

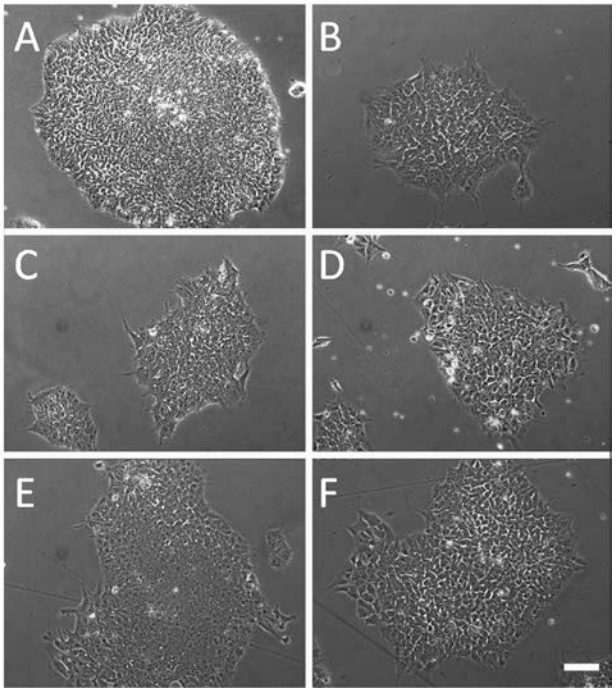


**Figure 5.** Morphology of hiPSCs FH2-7 (A-F)
Established hiPSC lines FH2-7 are morphologically indistinguishable from hESCs in culture. Scale bar is 100 µM.

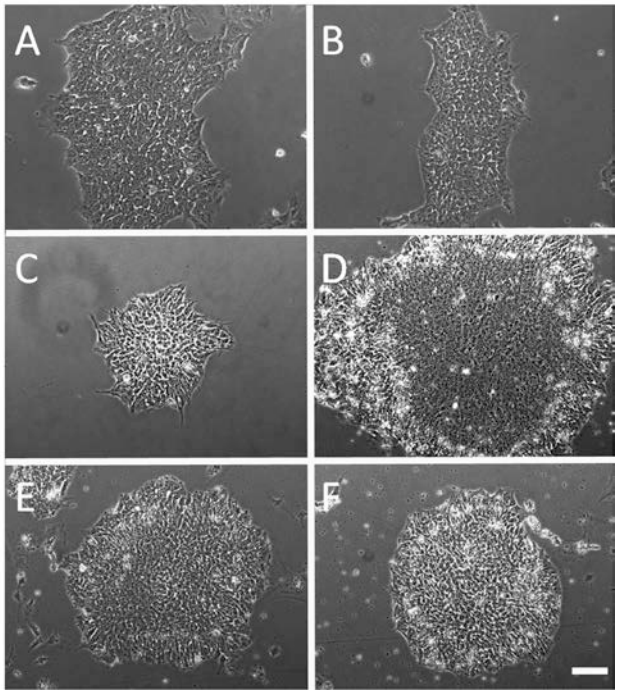


**Figure 6.** Morphology of hiPSCs FH8-13 (A-F)
Established hiPSC lines FH8-13 are morphologically indistinguishable from hESCs in culture. Scale bar is 100 µM.

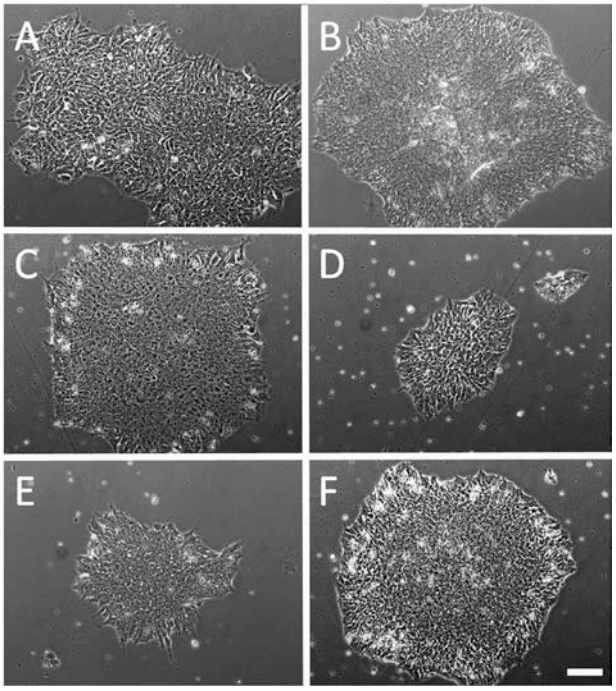


**Figure 7.** Morphology of hiPSCs FH14-19 (A-F)
Established hiPSC lines FH14-19 are morphologically indistinguishable from hESCs in culture. Scale bar is 100 µM.

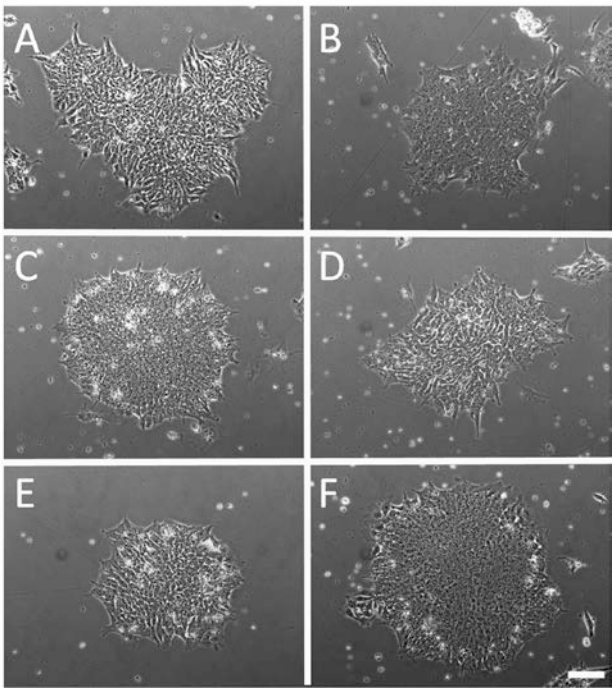


**Figure 8.** Morphology of hiPSCs FH20-25 (A-F)
Established hiPSC lines FH20-25 are morphologically indistinguishable from hESCs in culture. Scale bar is 100 µM.

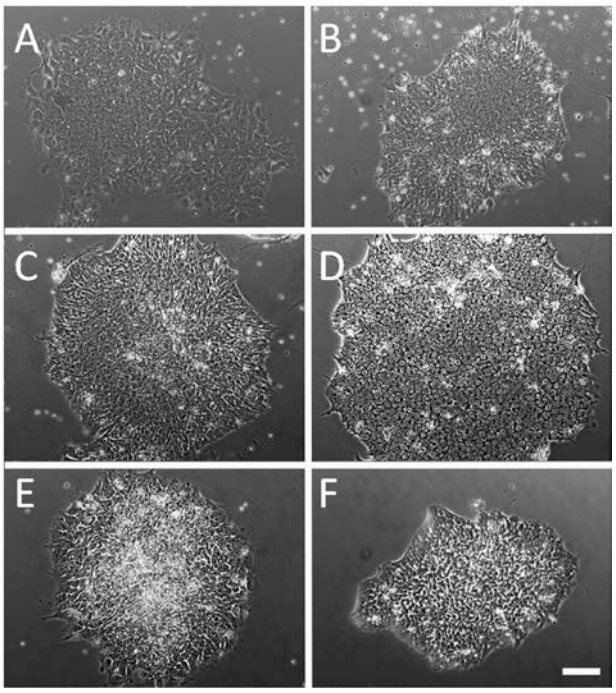


**Figure 9.** Morphology of hiPSCs FH26-31 (A-F)
Established hiPSC lines FH26-31 are morphologically indistinguishable from hESCs in culture. Scale bar is 100 µM.

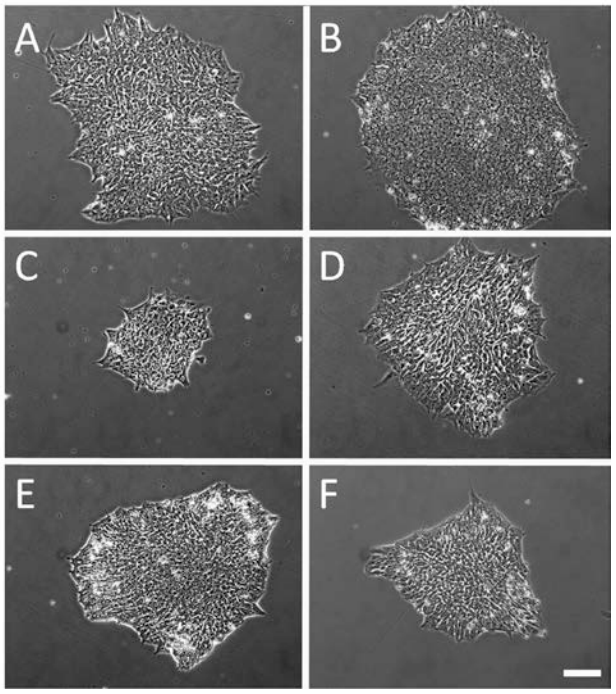


**Figure 10.** Morphology of hiPSCs FH32-37 (A-F)
Established hiPSC lines FH32-37 are morphologically indistinguishable from hESCs in culture. Scale bar is 100 µM.

### 2.4.2 Gene Expression Analysis of Pluripotency Genes of hiPSC Lines from Adult and Fetal Hepatocytes

Quantitative RT-PCR was performed using human specific TaqMan® assays. The hESC line, H1, was used as a positive control and the IMR90 fibroblast line was used as a negative control. All hiPSC lines expressed OCT4, SOX2, NANOG, GDF3, hTERT and DNMT3B at levels comparable to H1 hESCs, although the latter 2 were the most variable between different hiPSC clones (Figure 11). All analyzed cell lines were passage 35 or under.

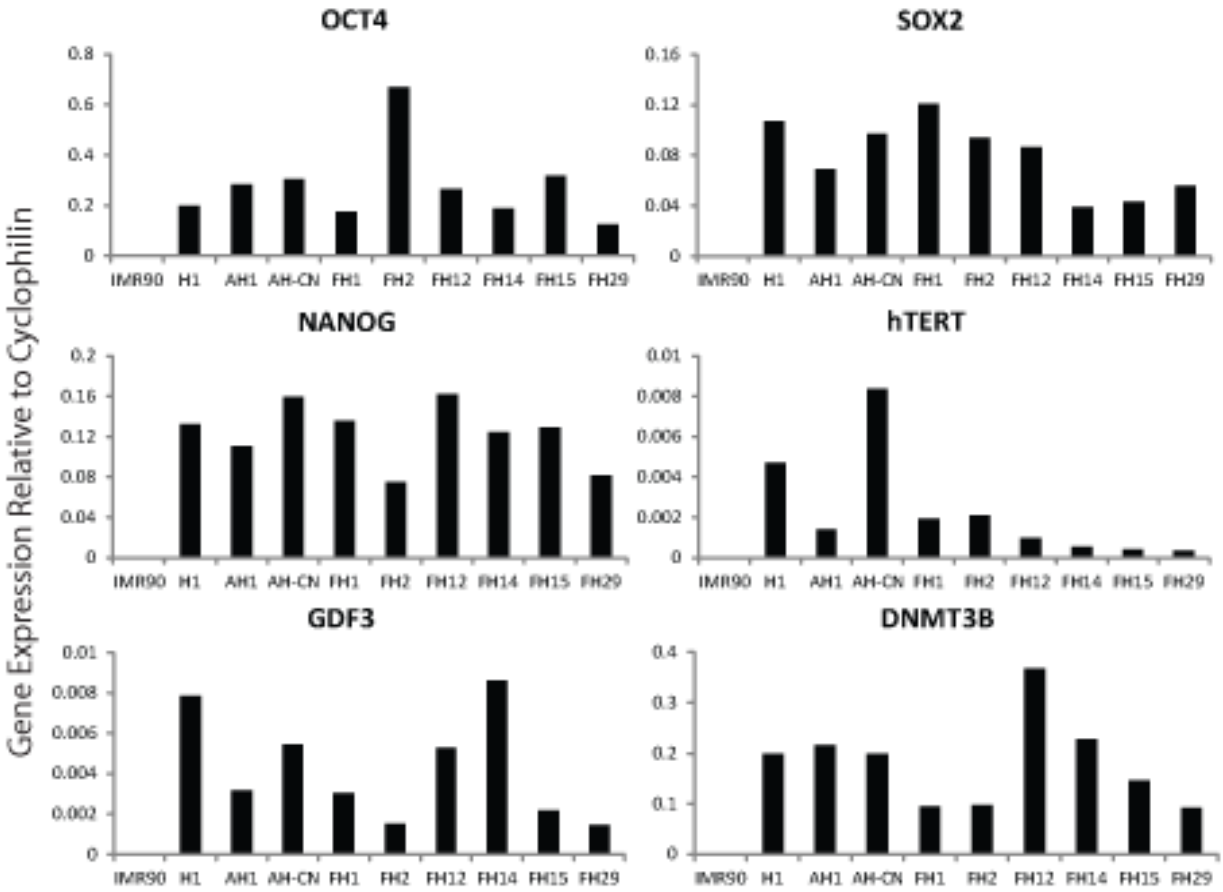


**Figure 11.** Gene Expression Analysis of Pluripotency Marker Expression of Selected Clonally Expanded hiPSC Lines from Both Adult and Fetal Hepatocytes
This figure displays the characterized hiPSC line gene expression. The H1 hESC line is used for a positive control an the IMR90 fibroblasts line is used for a negative control of expression levels. All expression level values are normalized to the cyclophilin housekeeping gene. All characterized cell lines express the pluripotency markers OCT4, SOX2, NANOG, hTERT, GDF3 and DNMT3B. All analyzed cell lines were passage 35 or under.

### 2.4.3 Flow Cytometry Analysis of Pluripotency Marker Expression of hiPSC Lines From Adult and Fetal Hepatocytes

Flow cytometry analysis indicated that every hiPSC line analyzed expressed the pluripotency surface markers SSEA-3, SSEA-4, TRA1-60, and TRA1-81 and nuclear markers OCT4, SOX2, and NANOG at levels comparable to H1 hESCs (Figure 3). IMR90 fibroblasts

were completely negative for all markers analyzed. The expression of TRA1-60 and 1-81 were the most variable between the different lines examined. All analyzed cell lines were passage 35 or under.

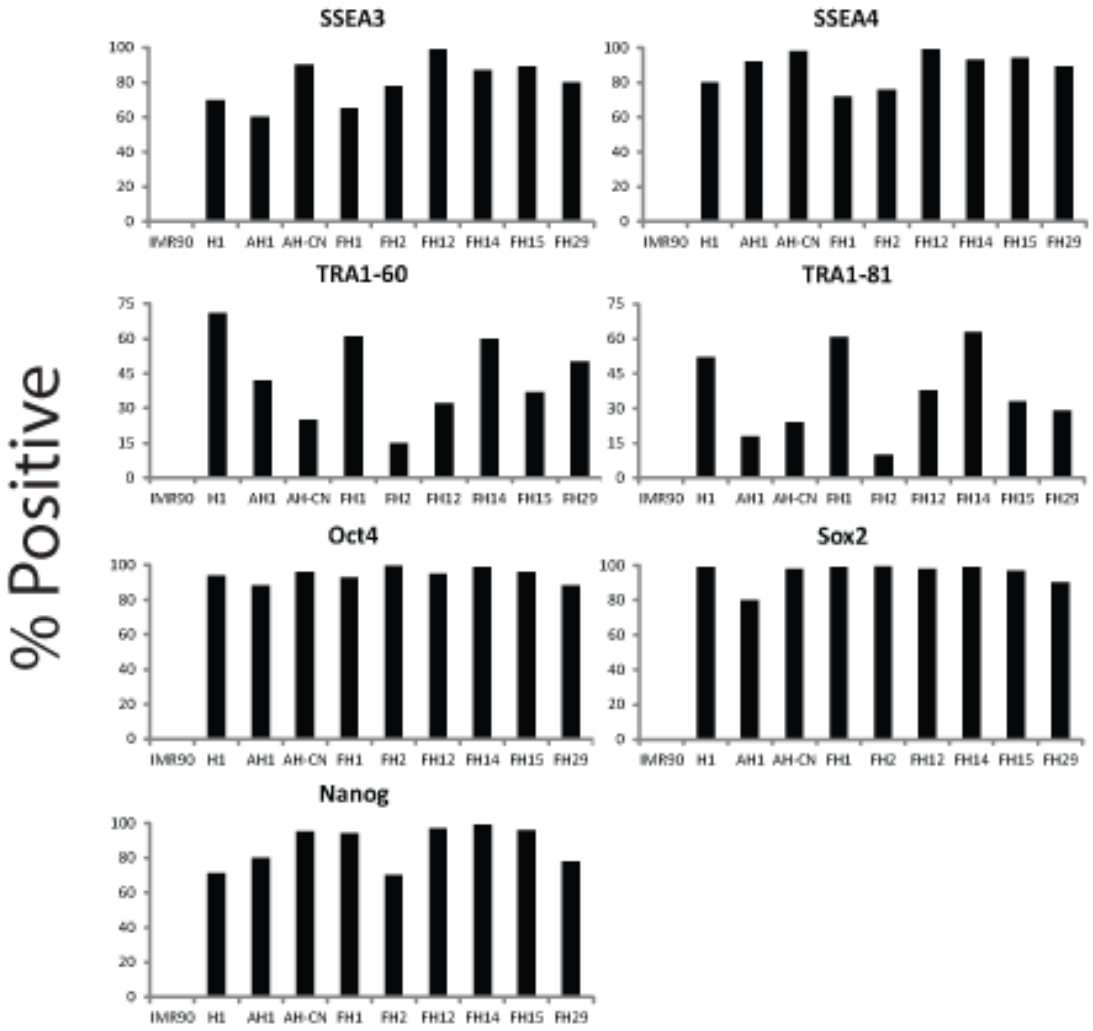


**Figure 12.** Flow Cytometry Analysis of Pluripotency Marker Expression of Selected Clonally Expande4d hiPSC Lines from Both Adult and Fetal Hepatocytes

This figure displays the characterized hiPSC line protein expression. The H1 hESC line is used for a positive control and the IMR90 fibroblasts line is used for a negative control of expression levels. All characterized cell lines express the pluripotency markers SSEA3, SSEA4, TRA1-60, TRA1-81, OCT4, SOX2 and NANOG. All analyzed cell lines were passage 35 or under.

### 2.4.4 Alkaline Phosphatase Activity of hiPSC Lines from Adult and Fetal Hepatocytes

Alkaline phosphatase activity, a property of pluripotent cells, was detected in adult and fetal-derived hiPSCs and H1 hESC; IMR90 fibroblasts were negative (Figure 13-14). A blue staining denotes a positive alkaline phosphatase signal. All analyzed cell lines were passage 35 or under.

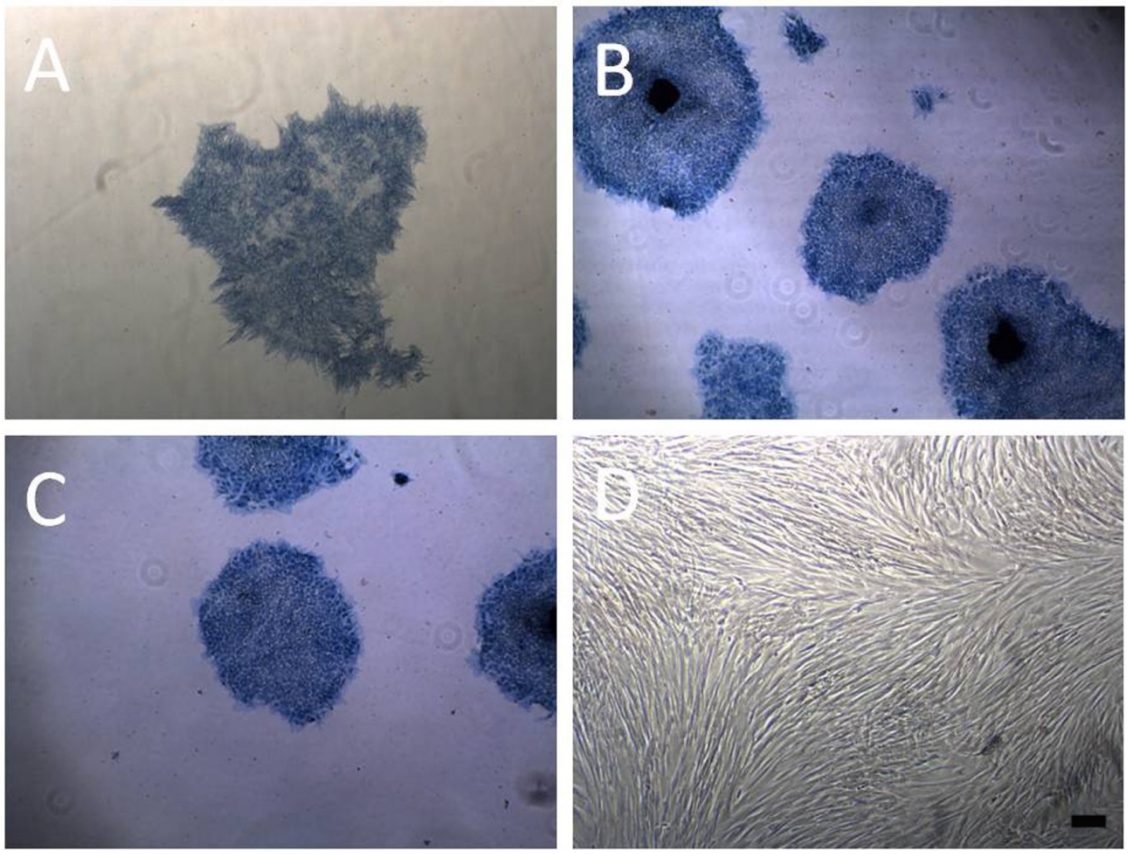


**Figure 13.** Alkaline Phosphatase Activity of Adult Hepatocyte-derived hiPSCs
Established hiPSC lines have alkaline phosphatase activity denoted by the blue stain. (A) Both AH1 and (B) AH-CN are alkaline phosphatase positive. (C) H1 hESC and (D) IMR90 fibroblasts were used as positive and negative controls respectively. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

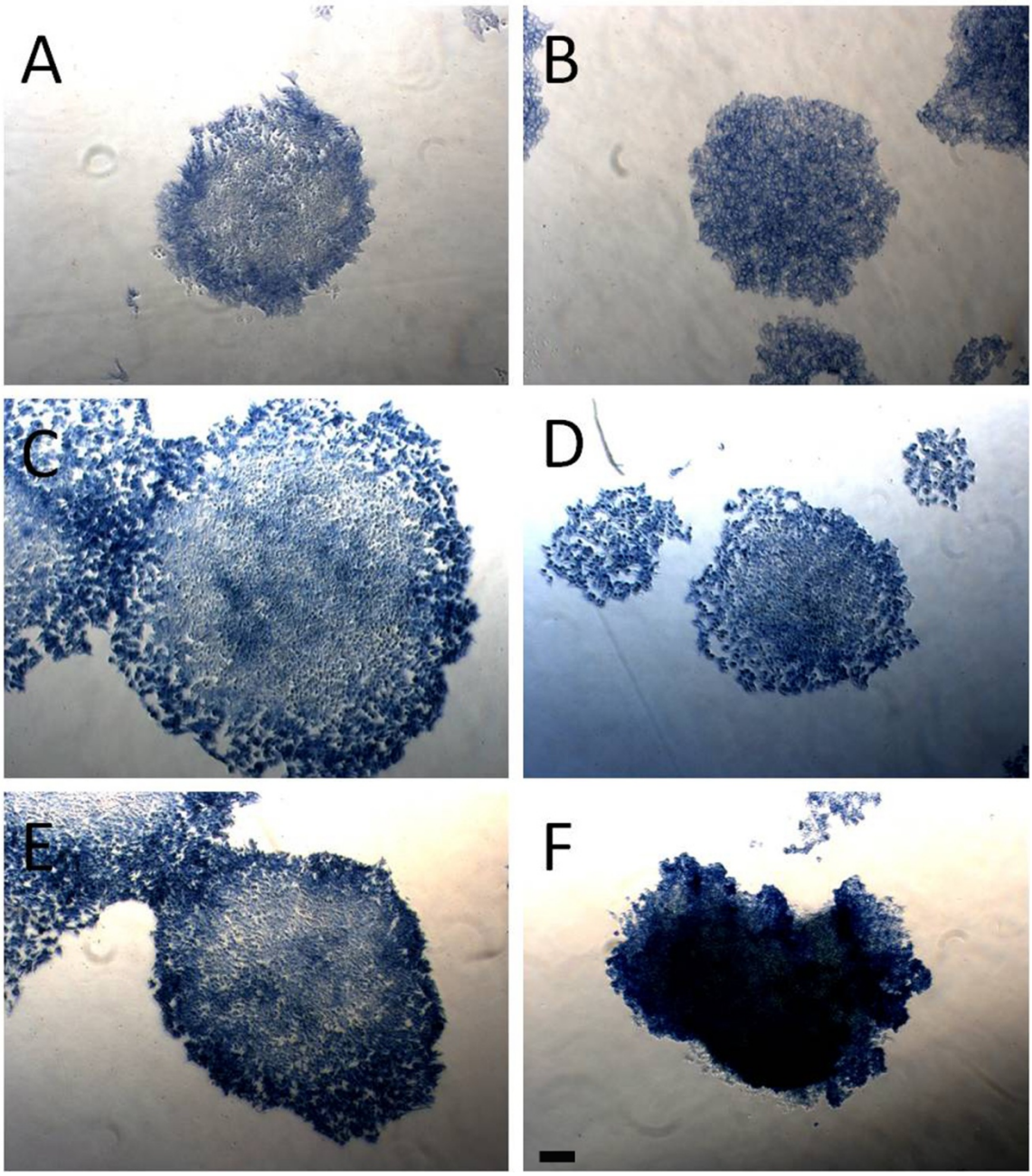


**Figure 14.** Alkaline Phosphatase Activity of Fetal Hepatocyte-derived hiPSCs
Established hiPSC lines have alkaline phosphatase activity denoted by the blue stain. (A) FH1, (B) FH2, (C) FH12, (D) FH14, (E) FH15 and (F) FH29 are alkaline phosphatase positive. H1 hESC and IMR90 fibroblasts were used as positive and negative controls respectively and can be seen in Figure 13. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

### 2.4.5 Immunofluorescence Staining of hiPSC Lines from Adult and Fetal Hepatocytes

Nuclear OCT4 and SOX2 were observed in AH-CN and FH1 shown by the green staining (Figure 15). IMR90 fibroblasts were negative and H1 hESCs were positive (data not shown). Nuclear NANOG and cell surface SSEA4 were also observed in AH-CN and FH1 shown by the red staining (Figures 16 and 17). Again, IMR90 fibroblasts were negative and H1 hESCs were positive (data not shown). IF staining for OCT4, SOX2, NANOG and SSEA-4 were also performed on all other characterized adult and fetal-derived hiPSCs (AH1, FH2, FH12, FH14, FH15 and FH29). All lines were positive denoted by the red staining. OCT4, SOX2 and NANOG were nuclear and SSEA4 was found on the surface (Figures 18-29). All analyzed cell lines were passage 35 or under.

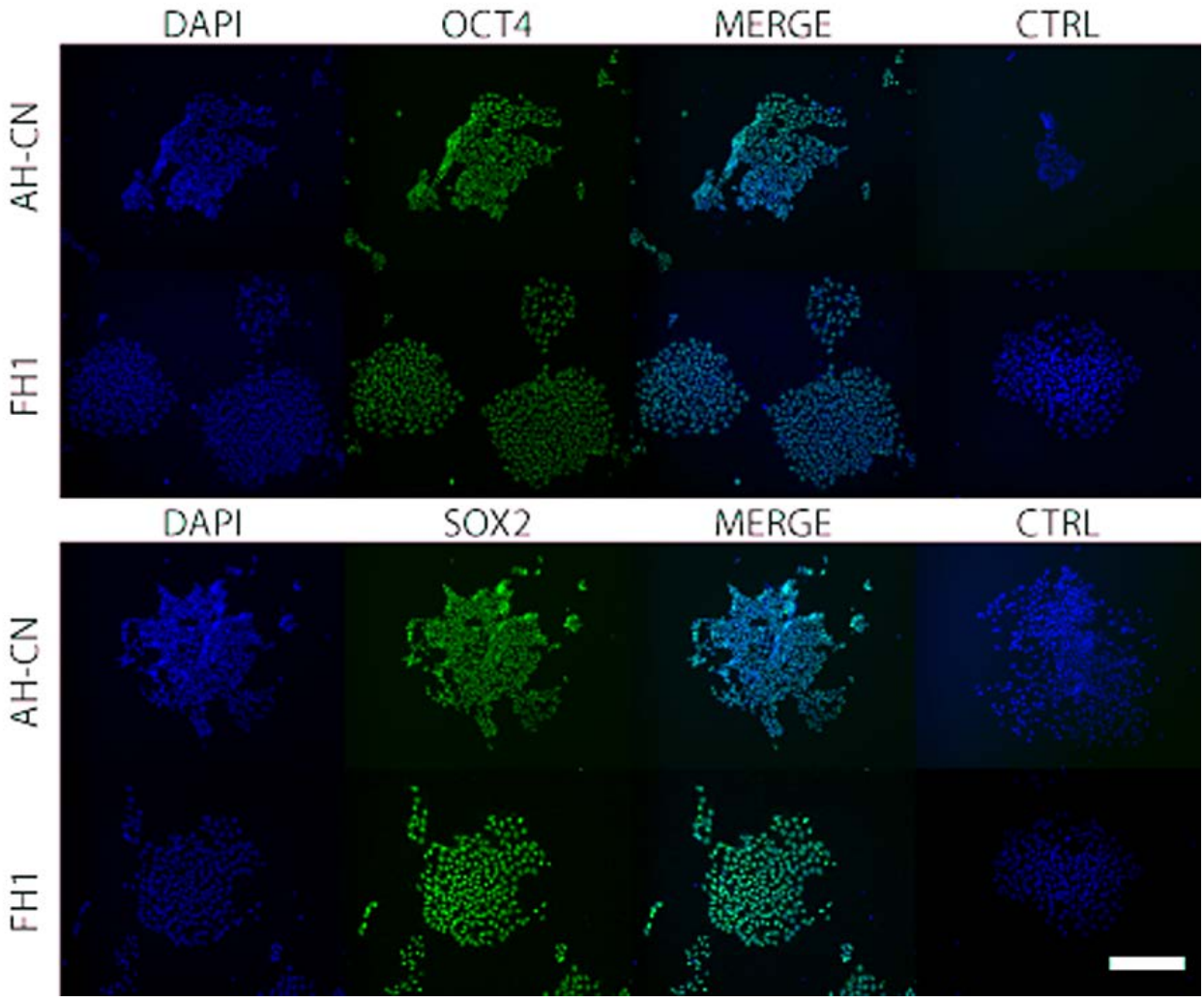


**Figure 15.** OCT4 and SOX2 Immunofluorescence Staining of AH-CN and FH1
The adult hepatocyte line depicted is AH-CN and the fetal hepatocyte line depicted is FH1. The H1 hESC line is used for a positive control and the IMR90 fibroblast line is used for a negative control of expression (data not shown). Scale bar is 100 μM. Positive protein staining is found in green and nuclei are stained with DAPI, seen in blue. The merge panel shows that both AH-CN and FH1 colonies express OCT4 and SOX2 entirely in the nucleus. CTRL panels depict colonies that did not receive primary antibody. As expected these colonies did not stain for OCT4 and SOX2 protein. All analyzed cell lines were passage 35 or under.

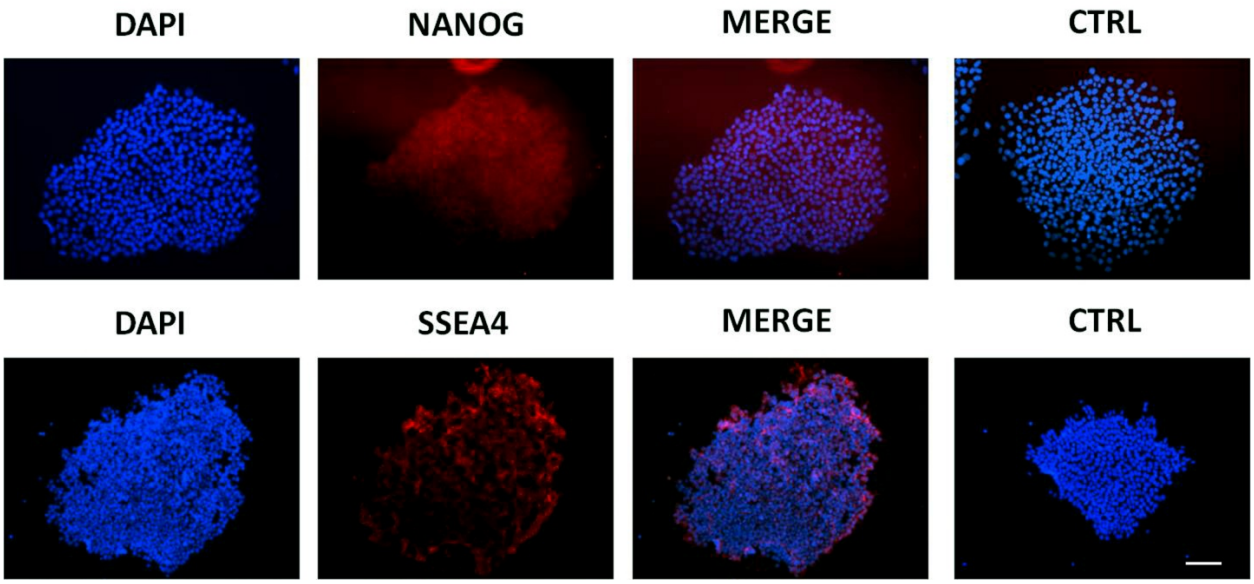


**Figure 16.** NANOG and SSEA4 Immunofluorescence Staining of AH-CN
The AH-CN line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

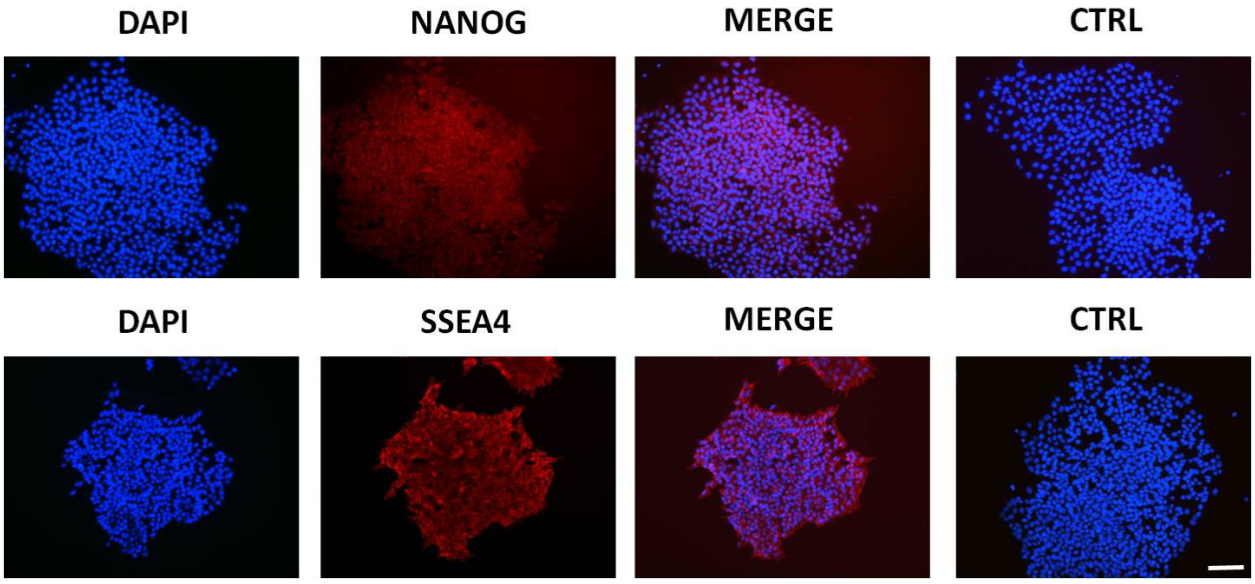


**Figure 17.** NANOG and SSEA4 Immunofluorescence Staining of FH1
The FH1 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

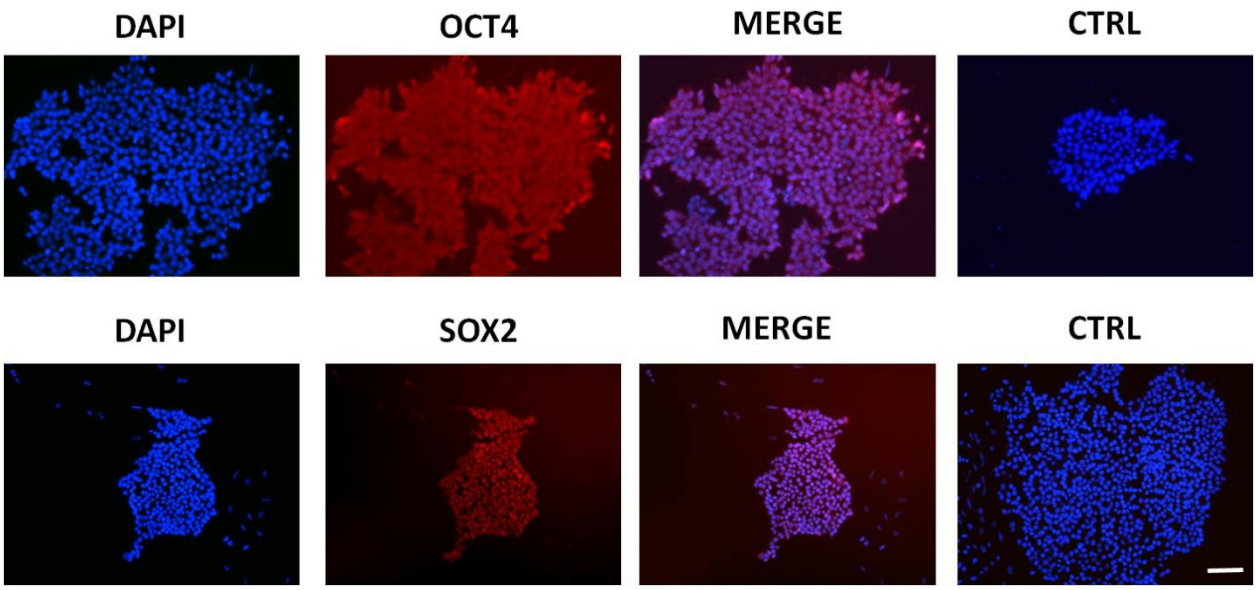


**Figure 18.** OCT4 and SOX2 Immunofluorescence Staining of AH1
The AH1 line is positive for OCT4 and SOX2 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

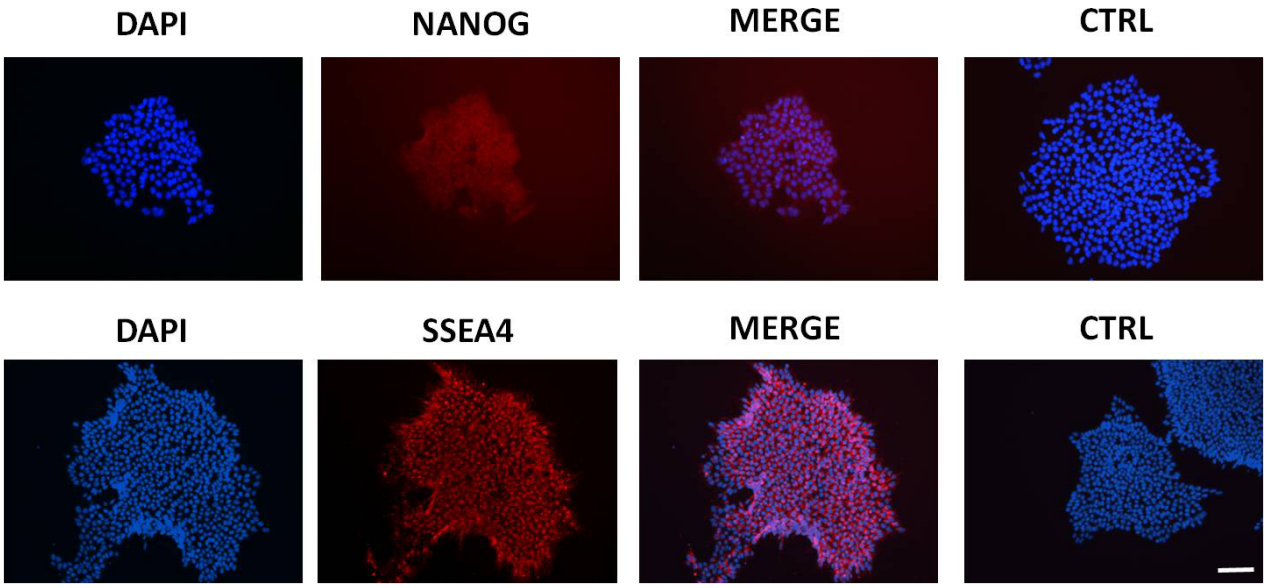


**Figure 19.** NANOG and SSEA4 Immunofluorescence Staining of AH1
The AH1 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

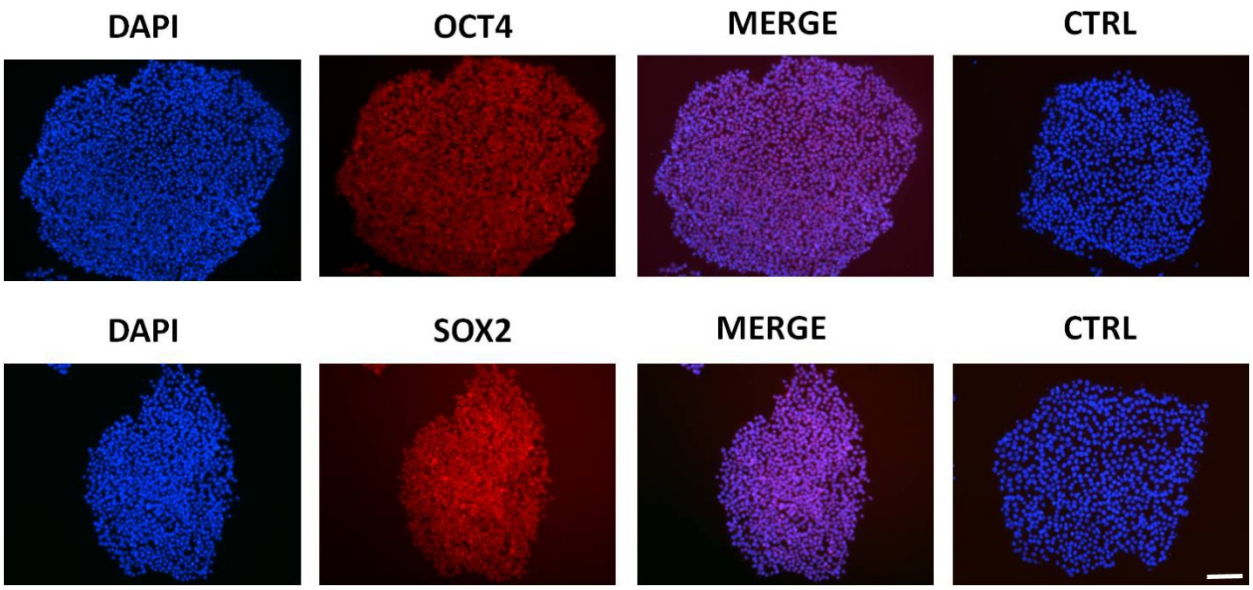


**Figure 20.** OCT4 and SOX2 Immunofluorescence Staining of FH2
The FH2 line is positive for OCT4 and SOX2 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

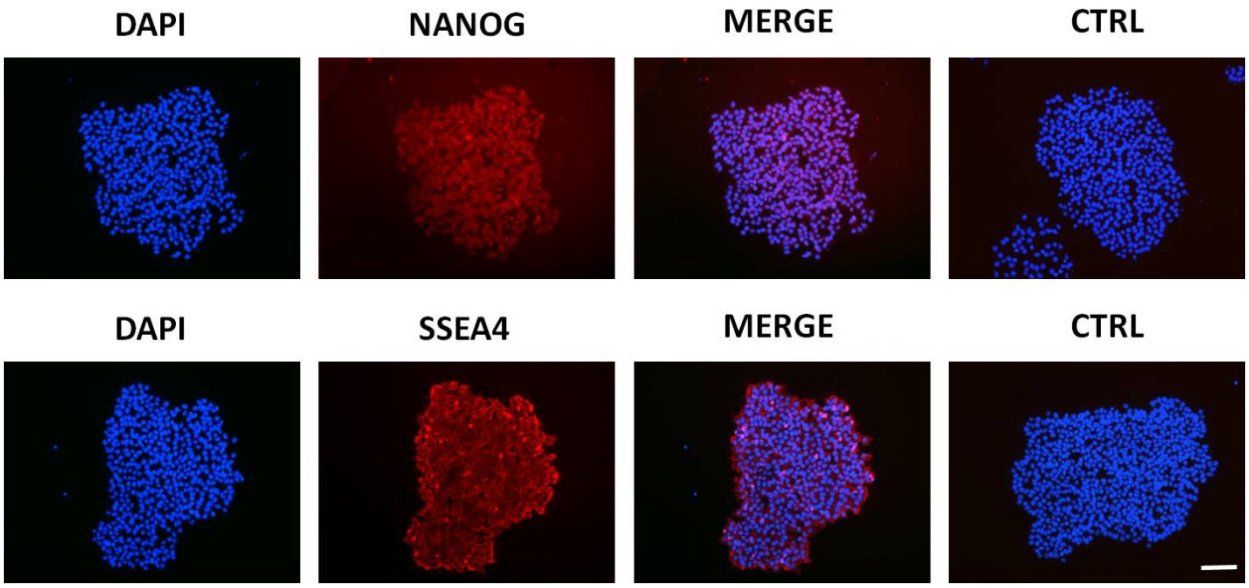


**Figure 21.** NANOG and SSEA4 Immunofluorescence Staining of FH2
The FH2 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

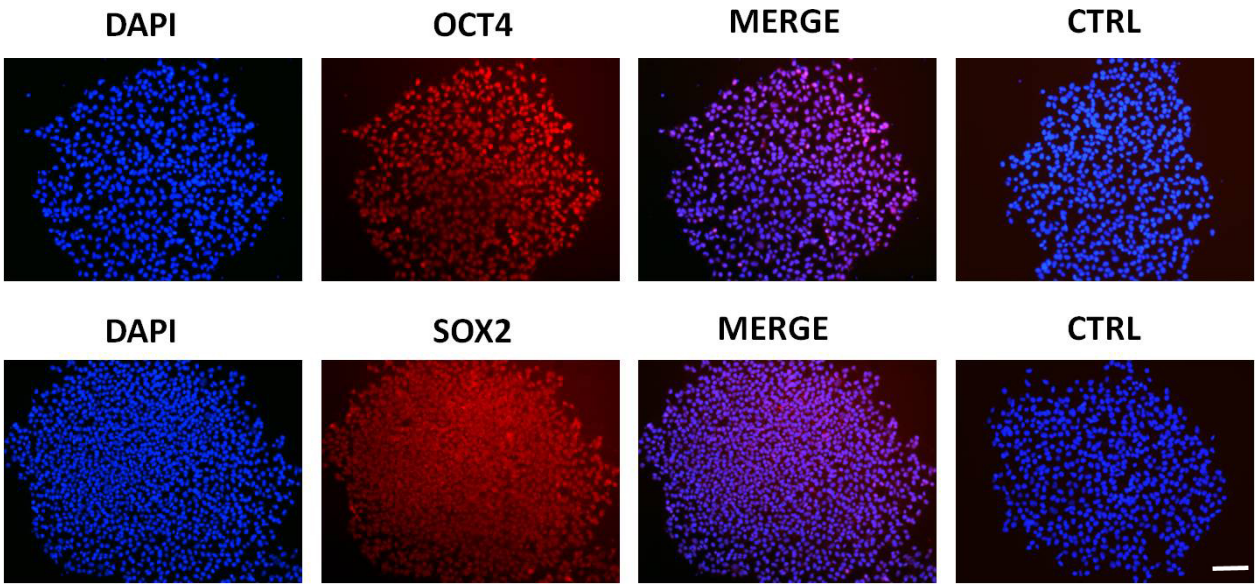


**Figure 22.** OCT4 and SOX2 Immunofluorescence Staining of FH12
The FH12 line is positive for OCT4 and SOX2 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

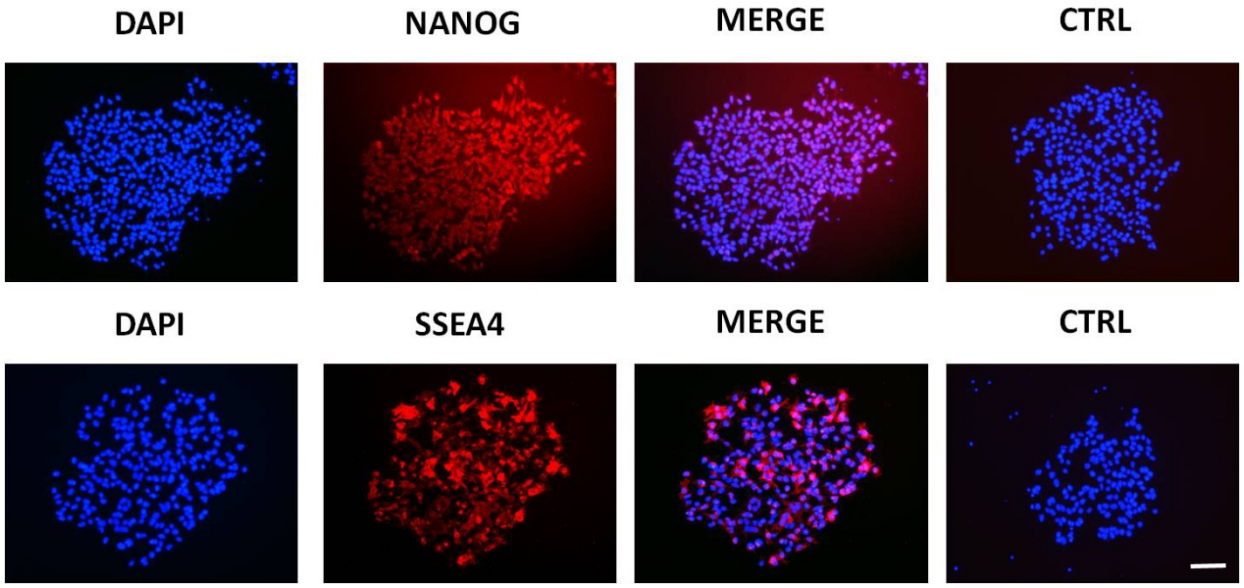


**Figure 23.** NANOG and SSEA4 Immunofluorescence Staining of FH12
The FH12 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

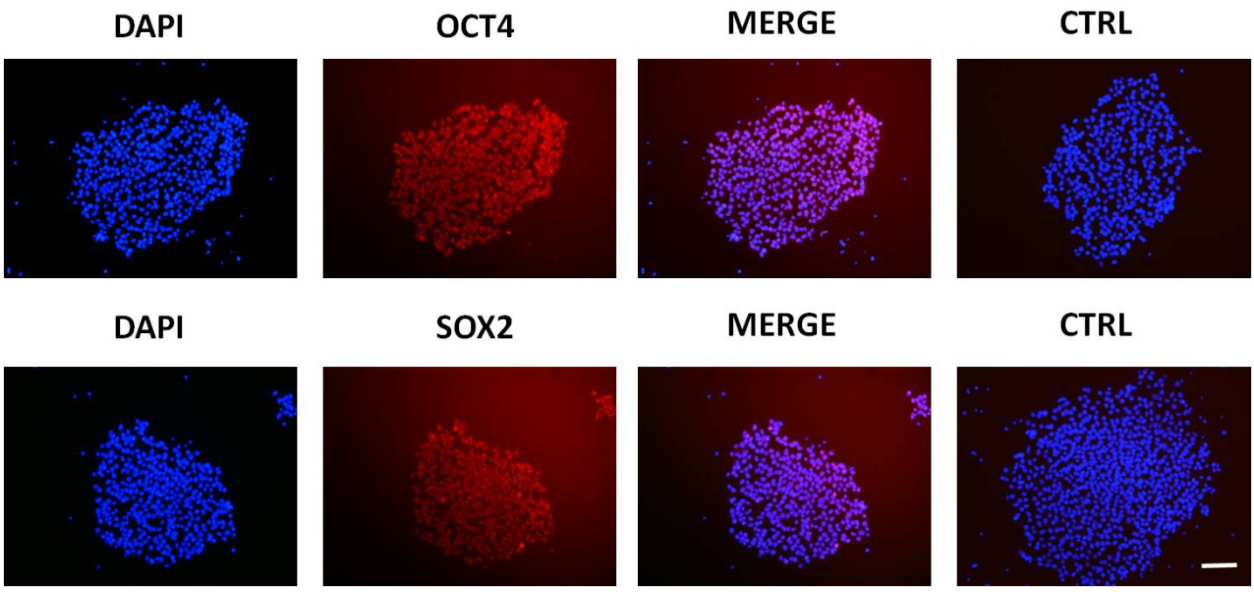


**Figure 24.** OCT4 and SOX2 Immunofluorescence Staining of FH14
The FH14 line is positive for OCT4 and SOX2 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

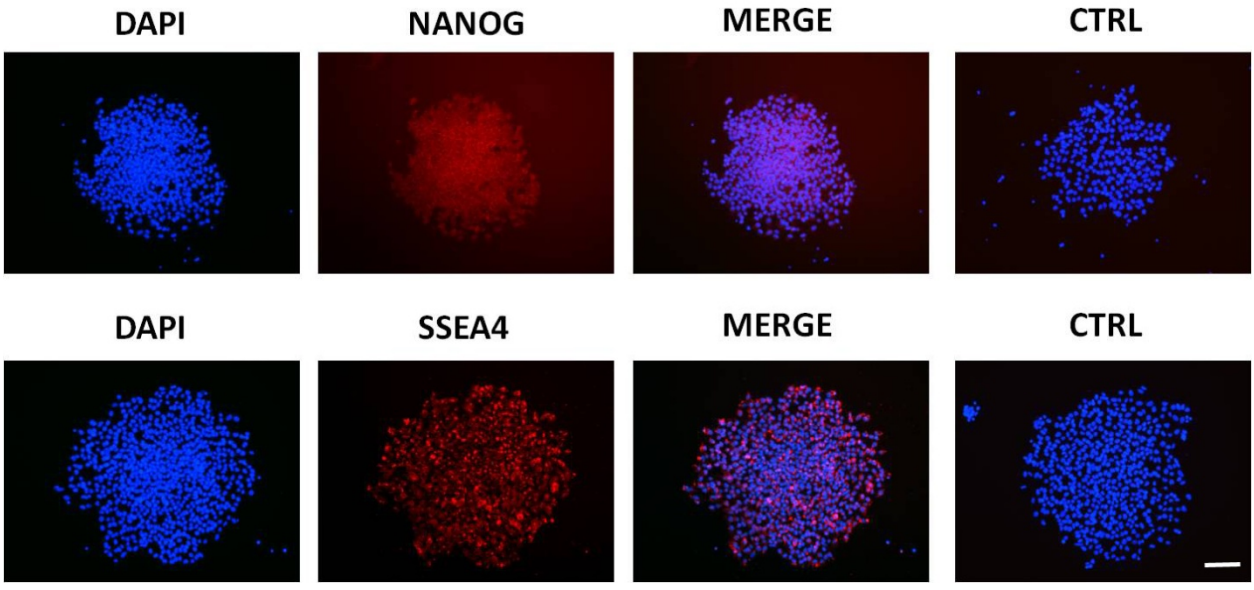


**Figure 25.** NANOG and SSEA4 Immunofluorescence Staining of FH14
The FH14 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

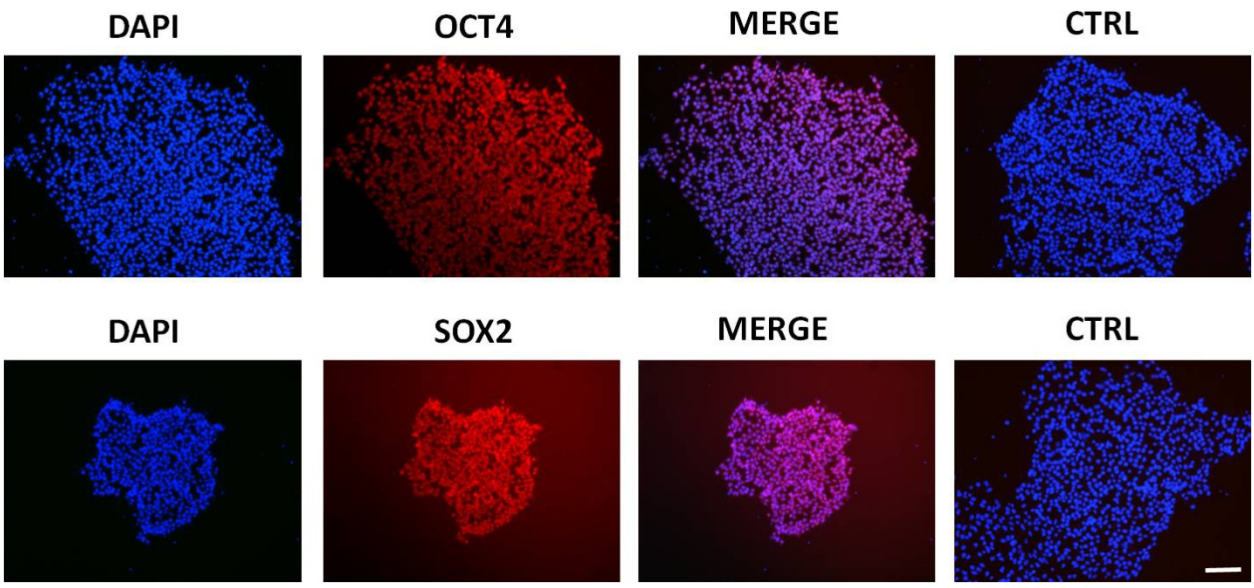


**Figure 26.** OCT4 and SOX2 Immunofluorescence Staining of FH15
The FH15 line is positive for OCT4 and SOX2 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

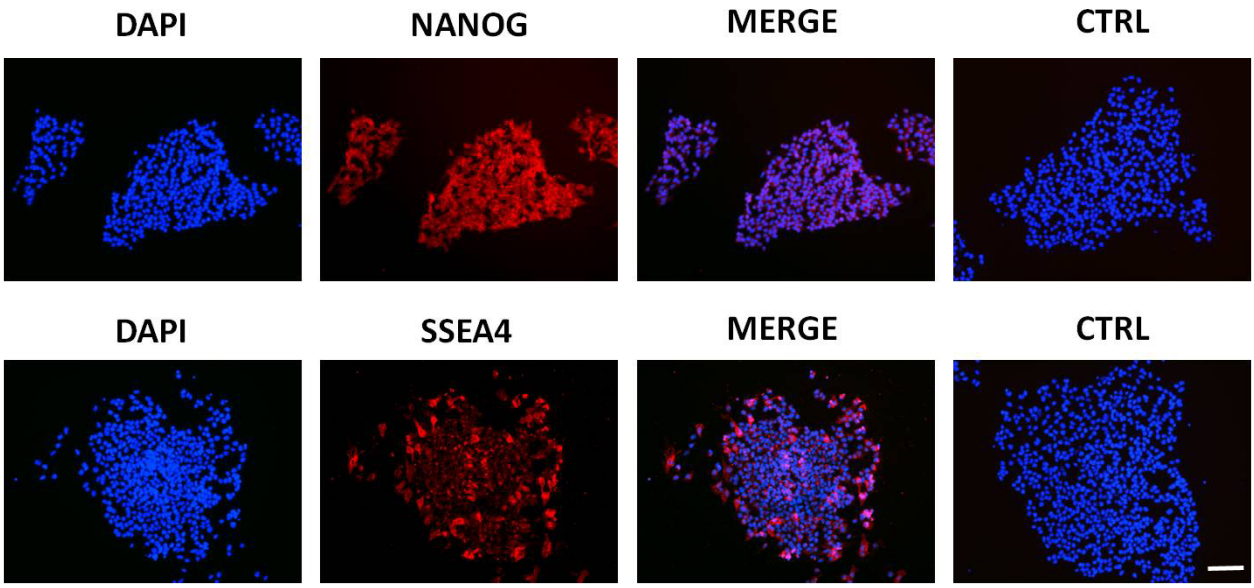


**Figure 27.** NANOG and SSEA4 Immunofluorescence Staining of FH15
The FH15 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

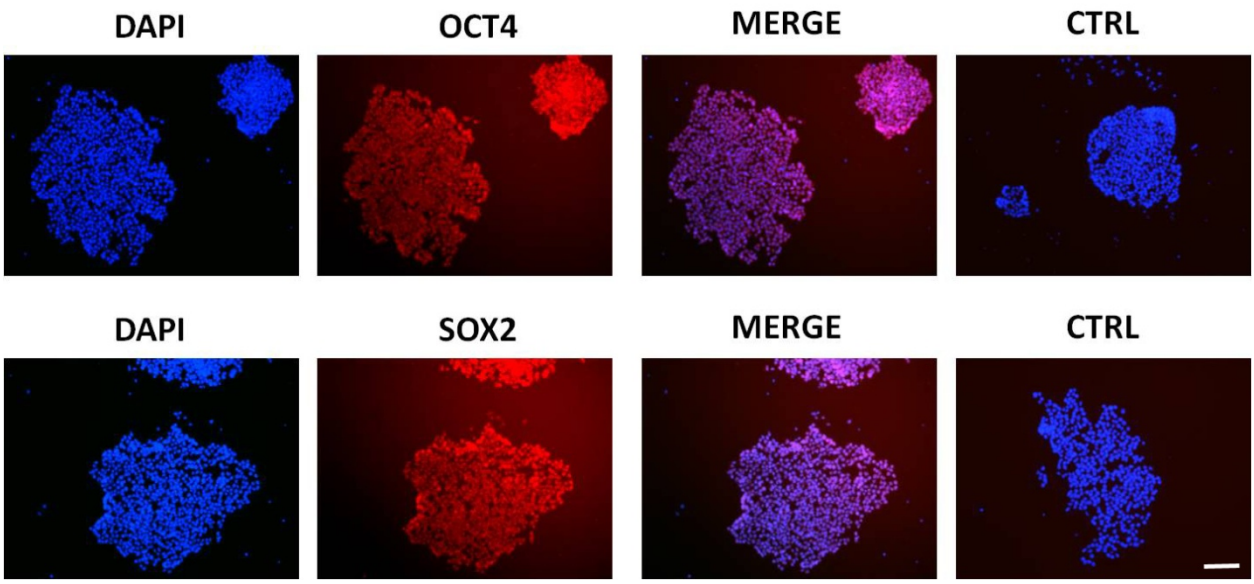


**Figure 28.** OCT4 and SOX2 Immunofluorescence Staining of FH29
The FH29 line is positive for OCT4 and SOX2 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

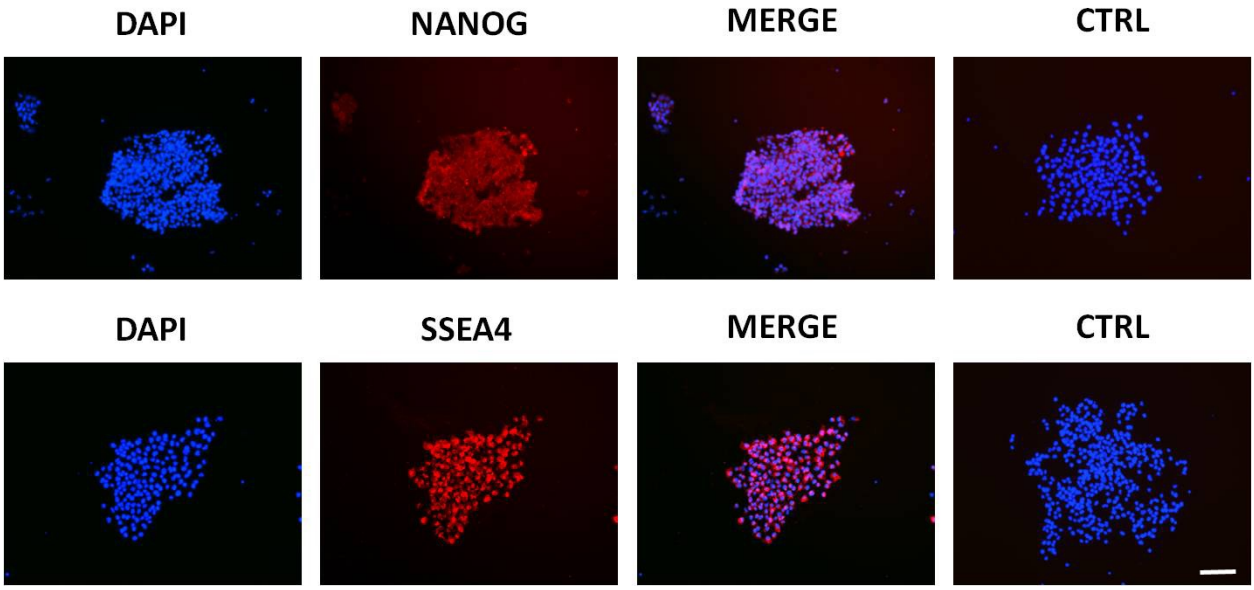


**Figure 29.** NANOG and SSEA4 Immunofluorescence Staining of FH29
The FH29 line is positive for NANOG and SSEA4 denoted by the red stain. CTRL panels depict colonies that did not receive primary antibody. All analyzed cell lines were passage 35 or under. Scale bar is 100 µM.

### 2.4.6 Teratoma Formation of hiPSC Lines from Adult and Fetal Hepatocytes

Teratoma formation is considered the definitive test of complete reprogramming to pluripotency. Each hiPSC line derived from either adult or fetal hepatocytes generated complex teratomas within 8-12 weeks in NOD/SCID IL2γ-chain receptor knockout mice. Representative pictures of structures resembling all three germ layer tissues, such as neural tissue and neural rosettes (ectoderm), chondroid tissue (mesoderm), and intestinal epithelium (endoderm) are shown in Figure 30. Figures 30-36 show teratoma formation capabilities of the rest of the characterized hiPSC lines. All analyzed cell lines were passage 35 or under.

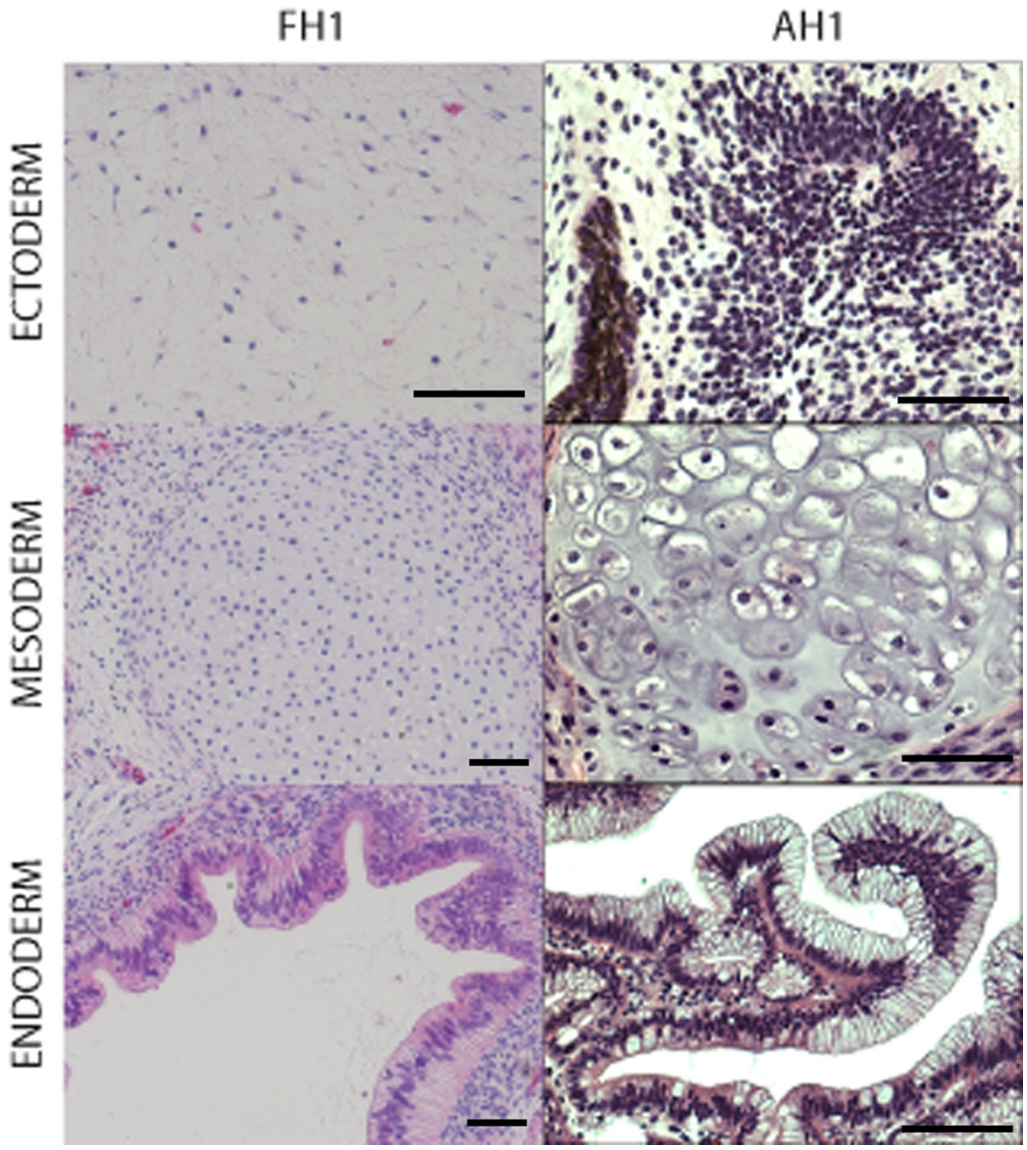


**Figure 30.** Teratoma Formation of Selected Clonally Expanded hiPSC Lines from Both Adult and Fetal Hepatocytes

The adult hepatocyte line depicted is AH1 and the fetal hepatocyte line depicted is FH1. The H1 hESC line is used for a positive control and the IMR90 fibroblast line is used for a negative control (data not shown). Cell lines were subcutaneously injected into the backs of NOD/SCID IL2γ-chain receptor knockout mice. Once tumors were large enough they were excised and processed for hematoxylin and eosin y staining. A pathologist then examined cross sections of tumors from different cell lines for evidence of cell types from all 3 germ layers. All characterized adult and fetal-derived hiPSC lines formed complex teratomas *in vivo*. FH1 and AH1 hiPSC lines display cell types ranging from neural tissue (ectoderm), chondroid tissue (mesoderm) and intestinal epithelium (endoderm). All analyzed cell lines were passage 35 or under. Scale bars are 50 µM.

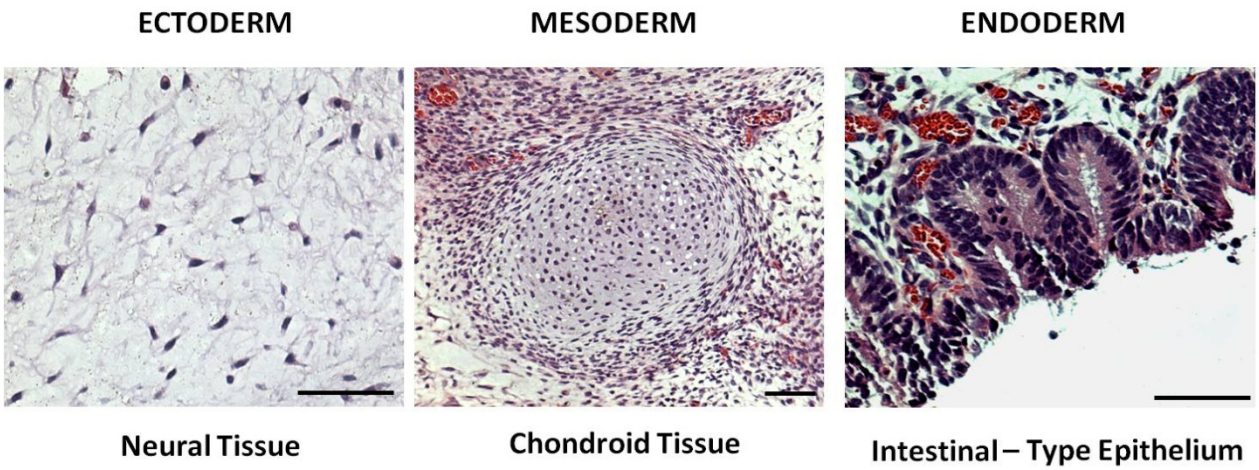


**Figure 31.** Teratoma Formation of AH-CN
Scale bars are 50 µM.

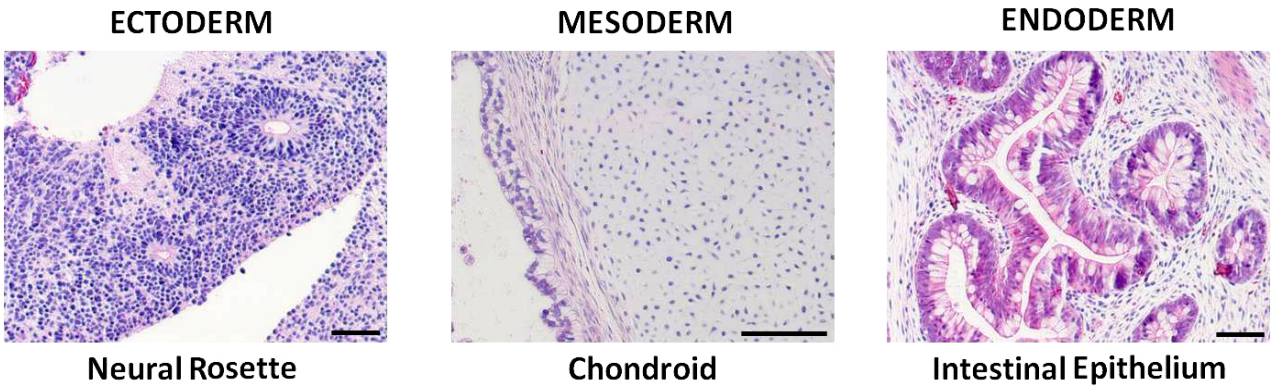


**Figure 32.** Teratoma Formation of FH2
Scale bars are 50 µM.

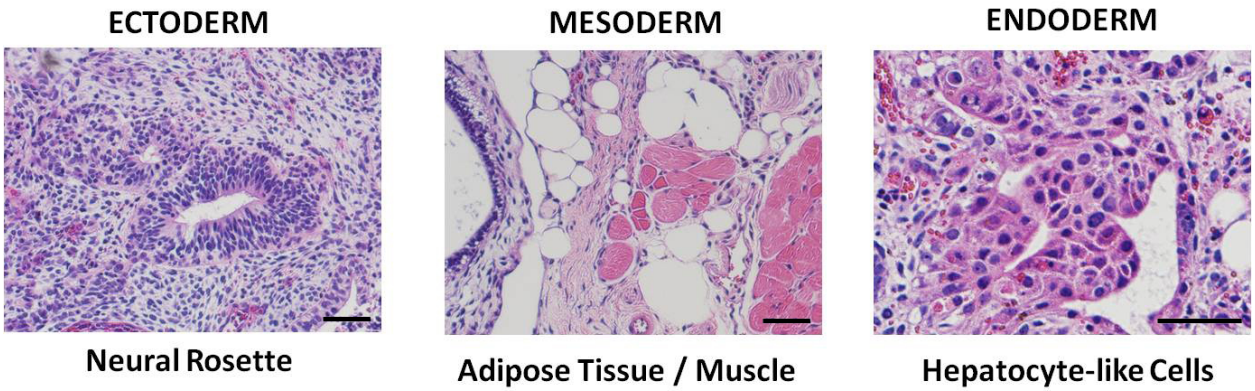


**Figure 33.** Teratoma Formation of FH12
Scale bars are 50 µM.

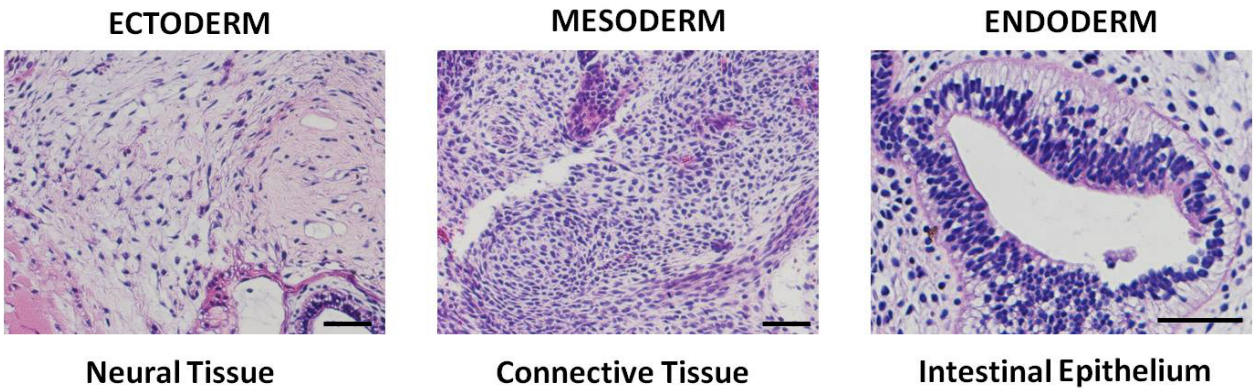


**Figure 34.** Teratoma Formation of FH14
Scale bars are 50 µM.

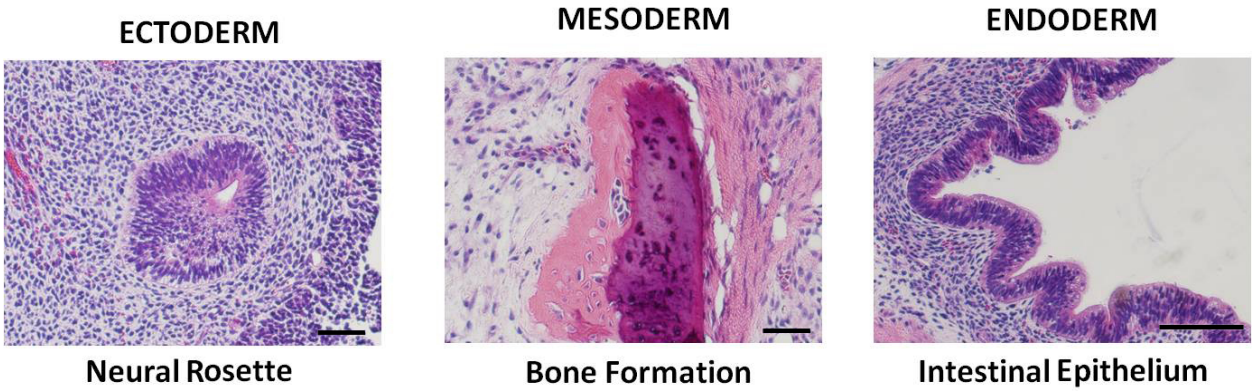


**Figure 35.** Teratoma Formation of FH15
Scale bars are 50 µM.

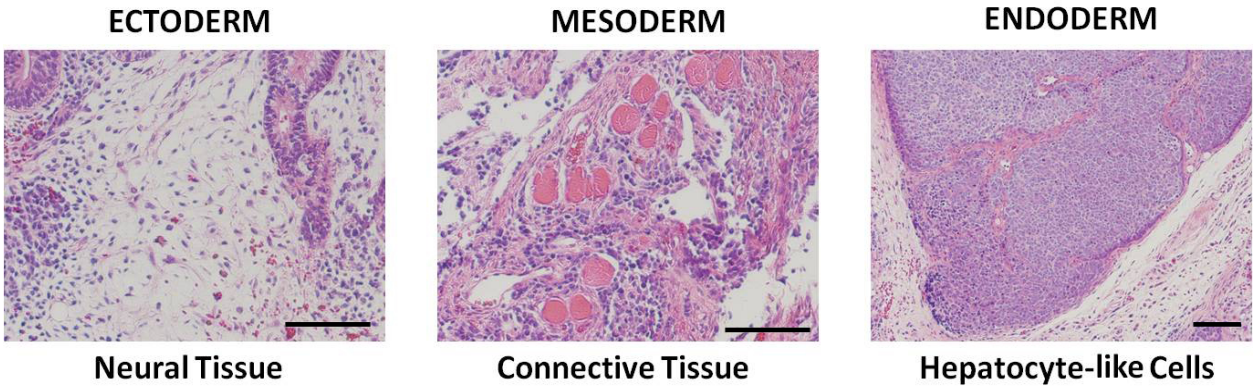


**Figure 36.** Teratoma Formation of FH29
Scale bars are 100 µM.

### 2.4.7 Human Fetal Hepatocytes Reprogram More Efficiently than Human Adult Hepatocytes

Only 3 hESC-like colonies were identified from 18 experiments with 14 different donors when adult cells were used for attempted reprogramming. A total of 19,950,000 adult hepatocytes were exposed to reprogramming factors, and that resulted in 3 hiPSC colonies that were identified by their morphology and collected, expanded and cryopreserved. Vastly different results were obtained when reprogramming was attempted with fetal hepatocytes. A total of 80 hESC-like colonies were identified from 8 experiments with 7 different donors when fetal cells were used for attempted reprogramming. A total of 4,900,000 fetal hepatocytes were exposed to reprogramming factors. Of the 80 total hiPSC colonies generated from fetal hepatocytes, 37 hiPSC colonies were selected at random and were collected, expanded and cryopreserved. Reprogramming efficiency, defined as (number of hESC-like colonies formed/total number of cells exposed to the vectors) x 100, are $2.41 \times 10^{-5}$ and $1.23 \times 10^{-3}$, for adult and fetal hepatocytes, respectively (Figure 37A). This represents a highly significant, and over a 50-fold higher reprogramming efficiency of fetal hepatocytes as compared to adults ($p=0.0015$). Furthermore, 75% of the fetal hepatocyte cases yielded hESC-like colonies as compared to only 11% of the adult hepatocyte isolations (Figure 37B). The incidence, or likelihood, of hESC-like colony formation in any given experiment from adult vs. fetal hepatocytes is also highly significant (Fisher's Exact Test with a p-value of 0.0028).

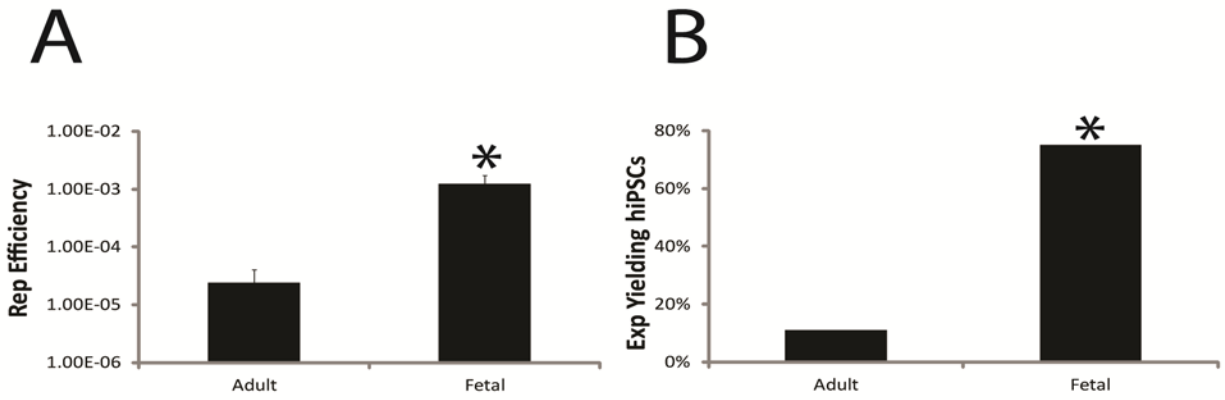


**Figure 37.** Fetal Hepatocytes Reprogram at Over a 50-fold Higher Rate as Compared to Adult Hepatocytes (A) A total of 19,950,000 adult hepatocytes were exposed to reprogramming factors as compared to only 4,900,000 fetal hepatocytes. Reprogramming efficiency, defined as (number of hESC-like colonies formed/total number of cells exposed to the vectors) X 100, was more than 50-fold higher for fetal hepatocytes as compared to adult hepatocytes. *p = 0.0015 versus adult hepatocytes and data are SEM of n=18 adult experiments and n=8 fetal experiments. (B) When looking at individual hiPSC induction experiments of adult and fetal hepatocytes and scoring each experiment as a yes, hESC-like colonies formed, or no, hESC-like colonies did not form; fetal hepatocytes form hESC-like colonies at a higher incidence or likelihood than adult hepatocytes. This was highly statistically significant by Fisher's Exact Test. *p = 0.0028 versus adult hepatocytes and data are SEM of n=18 adult experiments and n=8 fetal experiments.

Because the fetal cells reprogrammed with a higher efficiency than the adult cells we examined the donor characteristics of the adult cases to determine if there was a consistent trend towards more efficient reprogramming in younger donors. As shown in Table 15, reprogramming was achieved with 2 donors, one 12 years and the other 13 months of age. Reprogramming efficiency does not appear to be related to donor age in the adult cells, as the youngest "adult" donor, 4 months of age, and 2 cases from 1 year old donor, also failed to reprogram. Many younger donors were examined and in total 11 of the 14 adult cases tested were from donors 12 years or under. Thus, from these 14 cases, there is no clear connection between donor age and reprogramming efficiency post birth. However, we did not specifically design the experiments to examine reprogramming efficiency in different age groups.

### 2.4.8 Human Fetal Hepatocytes' Higher Reprogramming Efficiency is Not Due to Transduction Efficiency or Toxicity to Reprogramming Factor Viruses

A trivial explanation for such large differences in reprogramming efficiency could be related to the infectivity of the cells with the lentiviruses. To determine if there were differences between adult and fetal cells with respect to transduction efficiency, a lentivirus carrying GFP was used in place of the reprogramming vectors at an MOI ranging from 0-50 and the percentage of GFP-positive cells was determined 72 hours after viral exposure (Figure 38A). The percentage of GFP-positive cells increased with increasing MOI in both fetal and adult cells. The data from adult cells was consistently higher than that observed with fetal cells, however there were no significant differences between adult and fetal cells at any MOI.

Large differences in reprogramming efficiency could also result if reprogramming viruses were more toxic to adult cells. To test this, a lentivirus carrying GFP was used in place of the reprogramming vectors at MOI ranging from 0 to 60. Apoptosis levels were measured by caspase 3/7 activity and cell viability was measured by ATP content and total double strand DNA content 24 hours after viral exposure. All data was normalized to MOI 0. Adult and fetal hepatocytes showed no statistical differences between caspase 3/7 activity (Figure 38B), cell viability measured by ATP content (Figure 38C) or total double strand DNA levels (Figure 38D) at all tested MOI, however fetal cells trended towards higher caspase activity and lower amounts of ATP. Total double strand DNA levels were consistent between adult and fetal cells at all tested MOIs. Similar data was obtained when toxicity was investigated 72 hours after viral exposure (data not shown).

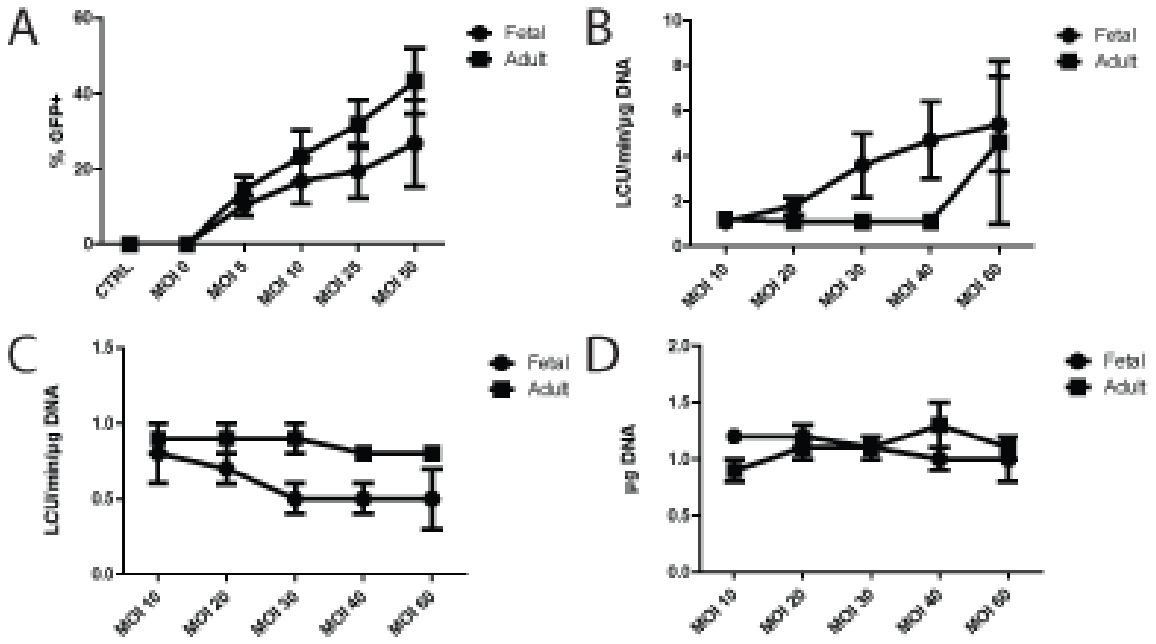


**Figure 38.** Fetal Hepatocytes' Higher Reprogramming Efficiency Not Due to Transduction Efficiency Differences or Toxicity to Reprogramming Factor Viruses

(A) A transduction efficiency experiment was performed on adult and fetal hepatocytes where a lentivirus delivering GFP was given to the cells at increasing MOIs to determine if the fetal cells are infected more efficiently than the adult cells. Both adult and fetal hepatocytes were readily and efficiently infected with the lentivirus, however adult cells were more efficiently infected than fetal cells at all tested MOIs but this was not statistically significant. (B-D) Toxicity to a lentivirus delivering GFP was investigated at increasing MOIs and analyzed 24 hours after infection. Data was normalized to MOI 0. Toxicity was measured by caspase 3/7 activity (B), ATP content (C) and total double strand DNA (D). Data are SEM of n=3 adult and fetal experiments. No statistical differences were seen between adult and fetal cells in the three assays at the different MOIs.

### 2.4.9 Endogenous Gene Expression Analysis of Reprogramming Factors and BAF-complex Members BAF155 and BRG1 in Cultured Adult or Fetal Hepatocytes

It was previously shown in mouse that the increased reprogramming efficiency was associated with the endogenous expression of reprogramming factors (OCT4, SOX2, NANOG, KLF4 and C-MYC) and mediators of epigenetic changes during reprogramming (BAF-complex components BRG1 and BAF155) [112]. Therefore quantitative RT-PCR was performed using human specific TaqMan® assays to determine the endogenous levels of expression of these genes

in adult and fetal hepatocytes. Adult hepatocytes expressed higher levels of endogenous OCT4, SOX2, NANOG, and KLF4; while fetal hepatocytes showed higher expression of the two BAF factor complex members and C-MYC (Figure 39). Again, there were no statistical differences between expression of these genes in adult and fetal hepatocytes.

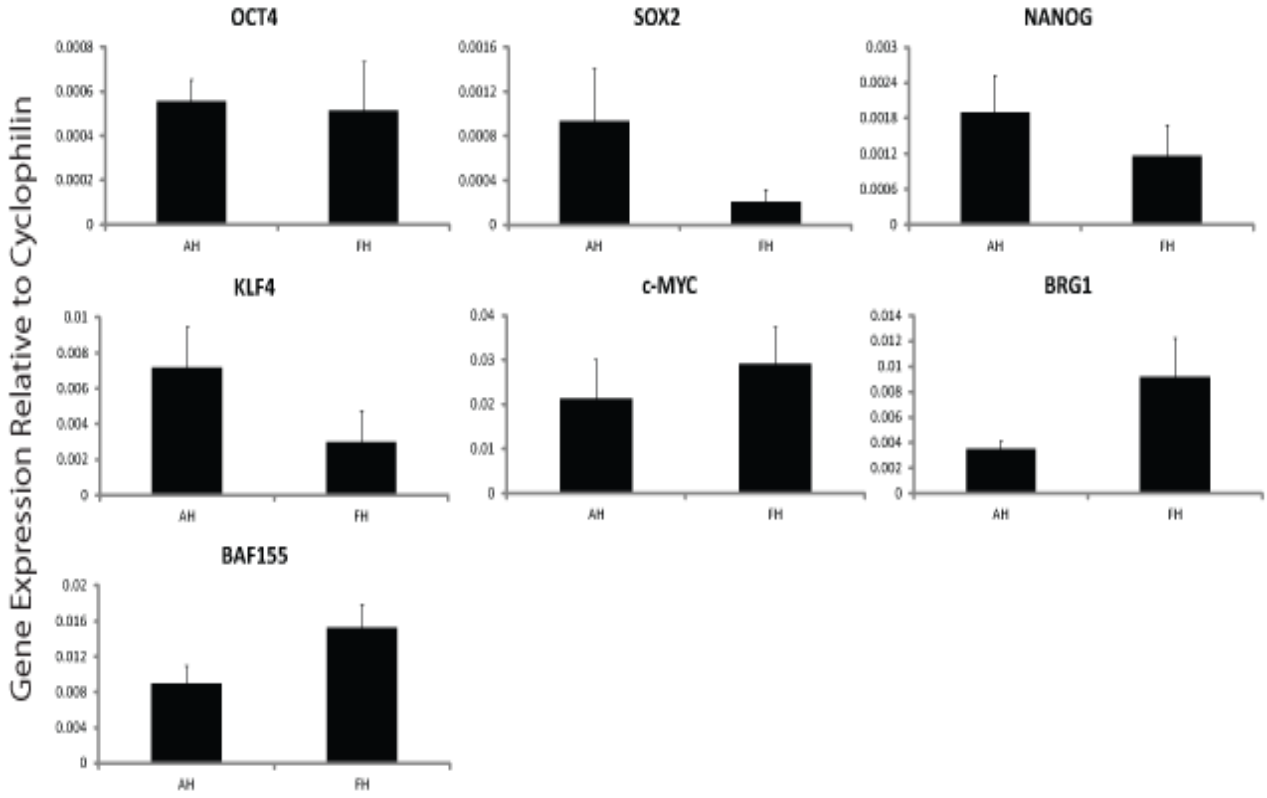


**Figure 39.** Endogenous Gene Expression Analysis of Reprogramming Factors and BAF (Brg1/Brm associated factor)-complex Members BAF155 and BRG1

Expression of these markers was performed in cultured primary adult and fetal hepatocytes. BAF155 and BRG1 are known to mediate epigenetic changes during reprogramming. All expression level values are normalized to the cyclophilin housekeeping gene. Adult and fetal hepatocytes expressed levels of all markers analyzed. Adult hepatocytes had a trend of higher expression of reprogramming factors while fetal hepatocytes had higher expression of the two BAF factor complex members. Data are SEM of n=4 adult and n=3 fetal samples. No statistical differences were seen between adult and fetal cells.

## 2.5 DISCUSSION

Here, we report the generation of 40 new hiPSC lines; derived from adult hepatocytes, and fetal hepatocytes. To the best of our knowledge this is the first report of generation of human fetal hepatocyte-derived hiPSCs, the generation of a metabolic disease specific hiPSC line from an adult hepatocyte (CN-1), and also the generation of hiPSC lines in entirely feeder-free conditions. Furthermore, derivation of fetal-derived hiPSCs was possible without the use of the oncogene C-MYC (using only OCT4, SOX2, and NANOG).

Although initial experiments with adult hepatocytes were conducted with 4 reprogramming factors (n=4) and at 5 and 10 MOI for each factor, none of these experiments yielded reprogrammed colonies. Other investigators have reported that there is an enhanced efficiency of generating hiPSCs by a combination of six factors [131]. Successful reprogramming of adult cells was finally accomplished when six factors were transduced. Fetal hepatocytes were found to reprogram over 50-fold more efficiently than adult hepatocytes even though only 3 or 4 reprogramming vectors were transduced. It was recently reported that increased reprogramming efficiency was obtained with a population of cells enriched for progenitor cells [112]. Even though an enriched progenitor cell population was not used in our experiments, human fetal cells at the hepatoblast stage of development still show over a 50-fold higher reprogramming efficiency as compared to adult, in this feeder-free system.

Additional experiments demonstrated that the higher reprogramming efficiency of fetal cells was not due to a more efficient lentiviral infection of fetal hepatocytes than adult hepatocytes; as adult cells were actually transduced more efficiently than fetal cells (Figure 38A). Likewise, the differences in reprogramming efficiency were not due to the lentivirus being more toxic to the adult hepatocytes because at all tested MOI and time points the adult and fetal

cells showed no statistical difference between levels of apoptosis, ATP content and total double strand DNA (Figure 38B-D). In fact, the trend was the viruses were more toxic to the fetal cells.

It was recently shown that an increased reprogramming efficiency of mouse liver progenitor cells, compared with differentiated liver cells, was associated with endogenous expression of reprogramming factors and BAF-complex members BAF155 and BRG1, which mediate epigenetic changes during reprogramming [112]. The BAF-complex components achieve a euchromatic chromatin state and enhance reprogramming efficiencies by increasing the binding of reprogramming factors onto key pluripotency gene promoters [166]. However, when endogenous levels of expression of these markers in human adult and fetal hepatocytes were examined, adult hepatocytes trended to higher levels of expression of the reprogramming factors (Figure 39), although this was not statistically significant. Interestingly, fetal hepatocytes trended to higher expression levels of the BAF-complex members BAF155 and BRG1, although again, this was not statistically significant. This suggests that the increased reprogramming capacity of human fetal hepatocytes in a feeder-free system is not due to higher levels of expression of the reprogramming factors or known mediators of reprogramming. Future experiments will be needed to determine if there is a molecular basis for increased reprogramming capability of human fetal hepatocytes because it is not conserved from the mouse models currently published. Increased reprogramming efficiency in fetal cells could simply be due to the observation that fetal human hepatocytes replicate robustly in culture even in the absence of added growth factors and are more likely to be simple diploid cells. In contrast, adult cells are mainly polyploid (4, 8 and 16 N) and proliferate poorly, *in vitro*, which together may make it more difficult to reprogram the adult hepatocytes.

Recent evidence suggests that at low passages numbers, mouse iPSCs may retain epigenetic "memory" of the donor cell from which they were made [163] and that cell type of origin influences *in vitro* differentiation potential [111]. This has since been corroborated in liver when a group demonstrated hepatic lineage stage-specific donor memory to mouse iPSCs [113]. More importantly these researchers showed that mouse fetal hepatocyte-derived iPSCs retained a superior capacity for hepatic re-differentiation, however this was lost after continuous passaging. Although, not studied here, future experiments will need to determine if human adult and fetal hepatocyte-derived hiPSCs differentiate efficiently to cells with mature hepatocyte characteristics and functions.

Fetal hepatocytes have some advantages for making new hiPSC lines as compared to adults. They can be generated in far fewer experiments, and at a lower expenditure of time and resources. With more clones to choose from, the best lines in terms of differentiation potential could be selected. Furthermore, fetal hepatocyte tissue serves as a potential source of tissue to generate disease specific hiPSCs; as many elective abortions are due to identification of genetic abnormalities. Moreover the fetal cells can be induced to pluripotency without the use of oncogenes such as C-MYC. Taken together, these results demonstrate that human adult and fetal hepatocytes can be reprogrammed to pluripotency using entirely feeder-free conditions and the efficiency of reprogramming fetal hepatocytes is more than 50-fold higher than that observed with adult cells. Although less frequent, fetal hepatocytes can be reprogrammed using only 3 factors, OCT4, SOX2, and NANOG. Human fetal and adult hepatocyte-derived hiPSCs may be a useful source for stem cell-derived hepatocytes that could be used for basic hepatic biology research, drug metabolism, and toxicology studies and possibly, as a cell source for hepatocyte transplants.

# 3.0 TRANSPLANTATION AND DIFFERENTIATION OF FETAL HEPATOCYTE-DERIVED HIPSCS

## 3.1 ABSTRACT

Since primary human hepatocytes do not proliferate *in vitro,* the cells isolated from a donor are all that one has to work with. To compound issues, primary cells cannot be cultured long term, as hepatocytes lose their activity and function as the time spent in culture increases. Moreover, tissues to isolate hepatocytes from are not readily available and are of marginal quality. It has recently been reported that mouse fetal hepatocyte-derived iPSCs have superior re-differentiation capacity. Here, we report the transplantation and differentiation of human fetal hepatocyte-derived iPSCs. Specifically we show preliminary data that undifferentiated cells can engraft in mouse livers of FRG and NOD/SCID mice. Engraftment was based on human DNA being found in liver tissue. Furthermore we show that fetal hepatocyte-derived hiPSCs can be differentiated to definitive endoderm and this definitive endoderm can be transplanted to FRG mice and human DNA can be found in mouse livers and human albumin can be found in mouse serum. Finally, we differentiate these fetal hepatocyte-derived hiPSCs to hepatocyte-like cells. Although we show limited differentiation in terms of the cells' ability to express liver specific genes and perform liver-specific metabolism, we believe this is due to the lack of definitive endoderm formation in the experiments where the cells were differentiated through the entire hepatic differentiation protocol. Taken together, this report demonstrates that fetal hepatocyte-derived hiPSCs can engraft in mouse livers, can form definitive endoderm in culture and show promise in being able to be fully differentiated to hepatocyte-like cells. Issues with finding the best differentiation protocol and engraftment being associated with tumorigenicity need to be overcome.

## 3.2 INTRODUCTION

Since primary human hepatocytes do not proliferate *in vitro,* the cells isolated from a donor are all that one has to work with. To compound issues, primary cells cannot be cultured long term, as hepatocytes lose their activity and function as the time spent in culture increases. Moreover, tissues to isolate hepatocytes from are not readily available and are of marginal quality. Therefore, if a stem cell source can be identified that readily differentiates to hepatocytes efficiently and reproducibly; we would have a limitless supply of cells for transplantation and research purposes.

If the cell source, techniques and protocols become available, hiPSCs will have countless numbers of real life applications if they can be differentiated to hepatocytes that are indistinguishable from primary cells. These stem cell-derived hepatocytes can be an unlimited source of cells for basic hepatic biology research and drug discovery. They could also be used for transplant to treat liver-based metabolic disorders or liver failure. This cell replacement therapy could be curative or used as a "bridge to transplant" to keep the patient alive until a donor liver becomes available for OLT. More importantly, disease-specific hiPSCs could be made to give researchers a limitless source of disease-specific hepatocytes to study new disease pathologies or to come up with drug interventions to treat underlying disease symptoms. Patient specific cells could be created and corrected for their genetic defect and transplanted back to the patient without the need for immunosuppression since the transplant is autologous.

Three recent publications have suggested that hiPSCs derived from fetal hepatocytes may be a good source of cells to differentiate to hepatocytes. One issue with creating iPSCs is that it is very labor intensive, costly and time consuming. If a source of cells is readily reprogrammable though, this is not an issue. Investigators have determined that there is an increased

reprogramming capacity of mouse liver progenitor cells, compared with differentiated liver cells [112]. Specifically, adult and fetal liver cells from mice were enriched for their progenitor cells and it was determined that enriching for these cells dramatically increased reprogramming efficiencies. Enriching fetal liver cells for progenitor cells resulted in a 275-fold higher reprogramming efficiency compared with unsorted fetal cells. It was determined that this reprogramming efficiency increase was associated with endogenous expression of reprogramming factors and members of the BAF-complex, which mediate epigenetic changes during reprogramming. This finding was reproduced in human fetal hepatocytes in entirely feeder-free conditions (Hansel et al. 2012; IN PRESS September 2012). Although fetal hepatocytes were not enriched for progenitor cells, they still were shown to reprogram at more than a 50-fold rate as compared to adult hepatocytes. Although it was shown that this increase in reprogramming efficiency was not mediated by BAF-complex members, investigators demonstrated that human fetal hepatocytes can be reprogrammed very efficiently and cost effectively compared to their adult cell counterparts.

There is also evidence that there is hepatic lineage stage-specific donor memory to the differentiation potential of mouse iPSCs [113]. Specifically, iPSCs derived from fetal liver cells retain superior hepatic re-differentiation compared to iPSCs derived from adult liver cells.

Taken together, mouse and human fetal hepatocytes are efficiently reprogrammed in a timely and cost efficient manner and mouse fetal hepatocyte-derived iPSCs have superior re-differentiation back to hepatocytes compared to mouse adult hepatocytes. Therefore it needs to be determined if human fetal hepatocyte-derived iPSCs contain this superior re-differentiation capacity.

Many protocols have been established to differentiate ESCs [75-84] and iPSCs [126-128, 150-155] to hepatocytes in culture. Here, we use a modified protocol established by Si-Tayeb and colleagues to differentiate our fetal hepatocyte-derived hiPSCs to the hepatocyte lineage. We characterize our stem cell-derived hepatocytes by liver-specific gene expression and metabolic activity. Furthermore, although very preliminary, we transplant undifferentiated and endoderm differentiated fetal hepatocyte-derived hiPSCs into two different mouse models and demonstrate potential engraftment and differentiation *in vivo*.

## 3.3 MATERIALS AND METHODS

### 3.3.1 Transplantation of Undifferentiated Fetal Hepatocyte-derived hiPSCs in FRG Mice

Cells were transplanted to recipient animals as described previously with the following modifications [32]: Fetal hepatocyte-derived hiPSCs were maintained in a pluripotent state by methods previously described in this thesis. Undifferentiated cells were lifted off the tissue culture plates through a 5 minute accutase (Gibco) digestion at 37°C. Cells were centrifuged at 1000 RPM for 5 minutes, re-suspended in HBSS, counted by trypan blue exclusion and diluted to 10 X $10^6$ viable cells/mL. 43 to 78 days old $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ mice (FRG mice) were injected intrasplenically with 200 µL of the cell suspension (2 X $10^6$ cells/animal). Animals were not pretreated with adenoviral vector expressing human urokinase. 4 FRG mice were transplanted with FH1 and 4 mice were transplanted with FH2.

### 3.3.2 Transplantation of Undifferentiated Fetal Hepatocyte-derived hiPSCs in NOD/SCID Mice

Fetal hepatocyte-derived hiPSCs were maintained in a pluripotent state by methods previously described in this thesis. Undifferentiated cells were lifted off the tissue culture plates through a 5 minute accutase digestion at 37°C. Cells were centrifuged at 1000 RPM for 5 minutes, re-suspended in HBSS, counted by trypan blue exclusion and diluted to 10 X $10^6$ viable cells/mL. 19 day old NOD/SCID Il2rg$^{-/-}$ mice (NOD/SCID mice) were directly injected with 100 µL of the cell suspension into the left and right sides of their liver (1 X $10^6$ cells/side of liver) and 200 µL of cells were delivered by an intraperitoneal (IP) injection (2 X 10 cells/IP). Transplants were performed by puncturing each animal's skin with a 27-gauge needle and administering each side of the liver with 100 µL of cell suspension and 200 µL IP. 3 mice were transplanted with FH1 and 3 mice were transplanted with FH2.

### 3.3.3 Detection and Quantification of Human DNA in Transplanted Mouse Liver Tissue

After transplantation, mouse livers (and tumors) were dissected from animals and snap frozen using liquid nitrogen. Tumor tissue was carefully dissected away from tumor adjacent tissue and snapped frozen separately. Total DNA in tumor and tumor adjacent tissue was isolated with DNeasy blood and tissue kit following manufacturer's instructions (Qiagen). Human DNA was detected and quantified by the human TaqMan® RNase P detection reagents kit according to manufacturer's instructions (Applied Biosystems, Cat. # 4316831). A standard curve was made by spiking known amounts of human DNA in mouse DNA at different concentrations. The

percentage of unknown amounts of human DNA in tumor and tumor adjacent tissue was interpolated using this standard curve.

### 3.3.4 Differentiation of Fetal Hepatocyte-derived hiPSCs to Definitive Endoderm

Fetal hepatocyte-derived hiPSCs were maintained in a pluripotent state by methods previously described in this thesis. Undifferentiated cells were lifted off the tissue culture plates through a 5 minute accutase digestion at 37°C. Cells were counted, plated in 6-well plates on BDESMG and allowed to attach overnight. Differentiation was initiated once the wells were ≈ 85% confluent. Cells were maintained in mTeSR™1 media until differentiation was initiated followed by culturing in RPMI media (Gibco) supplemented with complete B27 (Gibco), Activin A (Peprotech) and LY294002 (Cell Signaling Technology). Figure 40 outlines the definitive endoderm differentiation protocol.

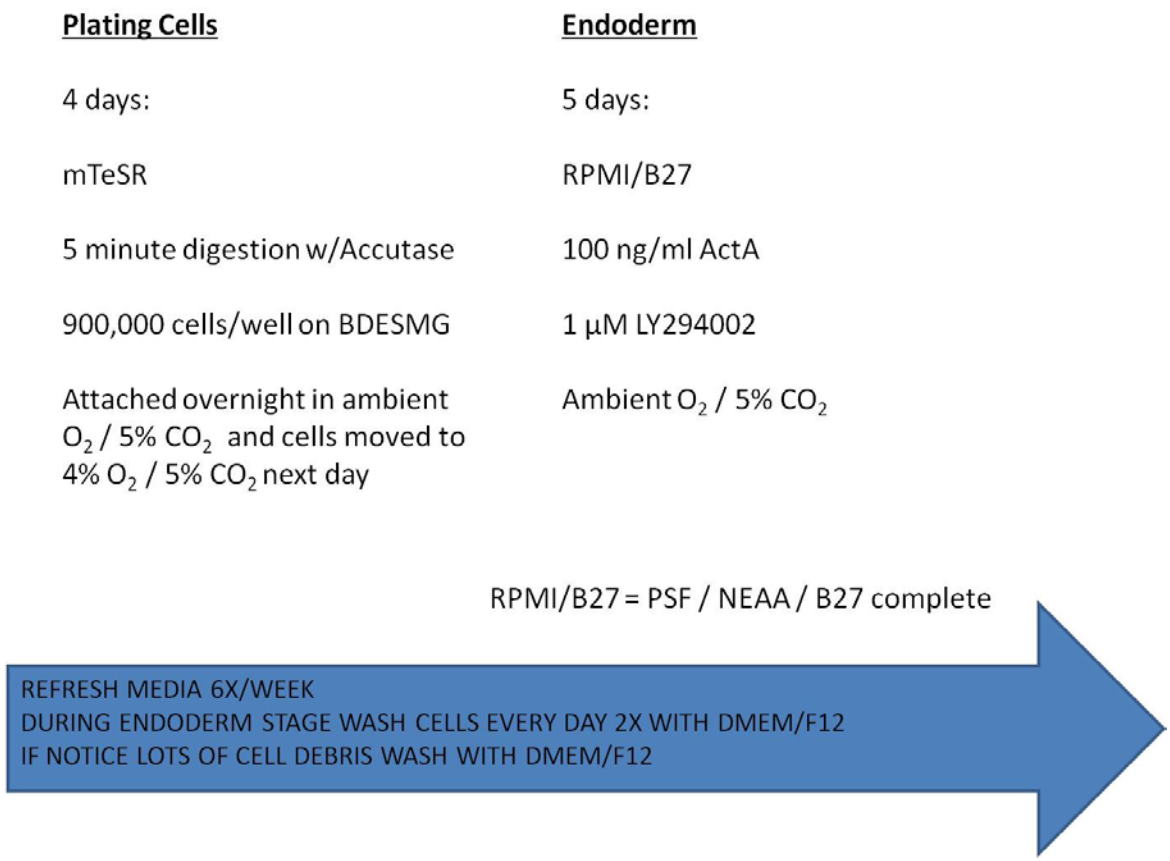


**Figure 40.** Definitive Endoderm Differentiation Protocol
This figure outlines the definitive endoderm differentiation protocol.

### 3.3.5 Transplantation of Definitive Endoderm derived from hiPSCs into FRG Mice

hiPSCs derived from fetal hepatocytes were differentiated to definitive endoderm as previously described. Definitive endoderm cells were lifted off the tissue culture plates through a 5 minute accutase digestion at 37°C. Cells were centrifuged at 1000 RPM for 5 minutes, re-suspended in HBSS, counted by trypan blue exclusion and diluted to 10 X $10^6$ viable cells/mL. FRG mice (17 days old) were directly injected with 100 µL of the cell suspension into the left and right sides of their liver (1 X $10^6$ cells/side of liver). Transplants were performed by

puncturing each animal's skin with a 27-gauge needle and administering each side of the liver with 100 µL of cell suspension. 7 mice were transplanted with endoderm derived from FH2.

### 3.3.6 Detection of Human-specific Albumin in Transplanted FRG Mouse Serum

Blood samples were collected from the mice via the vascular bundle located at the rear of the jaw bone and serum was prepared using a microtainer serum separator tube (BD) according to manufacturer's protocol. Human albumin secretion was determined by ELISA according to manufacturer's protocols (Bethyl Laboratories).

### 3.3.7 Differentiation of Fetal Hepatocyte-derived hiPSCs to Hepatocyte-like Cells

Fetal hepatocyte-derived hiPSCs were maintained in a pluripotent state by methods previously described in this thesis. Differentiation protocols used were modified versions of the protocol published by Si-Tayeb and colleagues [153]. Briefly, undifferentiated cells were lifted off the tissue culture plates through a 5 minute accutase digestion at 37°C. Cells were plated in 6-well plates on BDESMG and allowed to attach overnight. Differentiation was initiated once the wells were ≈ 85% confluent (unless otherwise specified). Cells were maintained in mTeSR™1 media until differentiation was initiated. Six unique differentiation experiments were performed and are outlined in Figures 41-46. These figures denote the cell line that was differentiated along with the growth factors and viruses given to the cells to differentiate them to the hepatocyte lineage. Each differentiation protocol is named by the date on which the undifferentiated cells were lifted off the tissue culture plate by a 5 minute digestion with accutase. Protocol variations included: plating (low seeding and growing to ≈ 85% confluence or

high seeding and initiating differentiation the next day), the use of an adeno-associated virus expressing human HNF4α (on Day 1 of the hepatic specification stage or Day 1 of the hepatic induction stage) and different hepatic maturation media (HMM+ or HBM). Inductions on the last 3 days of the hepatic maturation stage were carried out as follows: Cells were incubated for 72 hours in the presence of specific inducers Rifampicin (RIF; 10 µM; CYP3A4; Sigma-Aldrich), Phenobarbital (PB; 1 µM; CYP3A4; Sigma-Aldrich) or β-naphthoflavone (BNF; 25 µM, CYP1A1/1A2; Sigma-Aldrich) in HMM media used to culture primary human hepatocytes. DMSO was used as vehicle control for all 3 specific inducers (0.1%; Sigma-Aldrich). Media was refreshed every 24 hours.

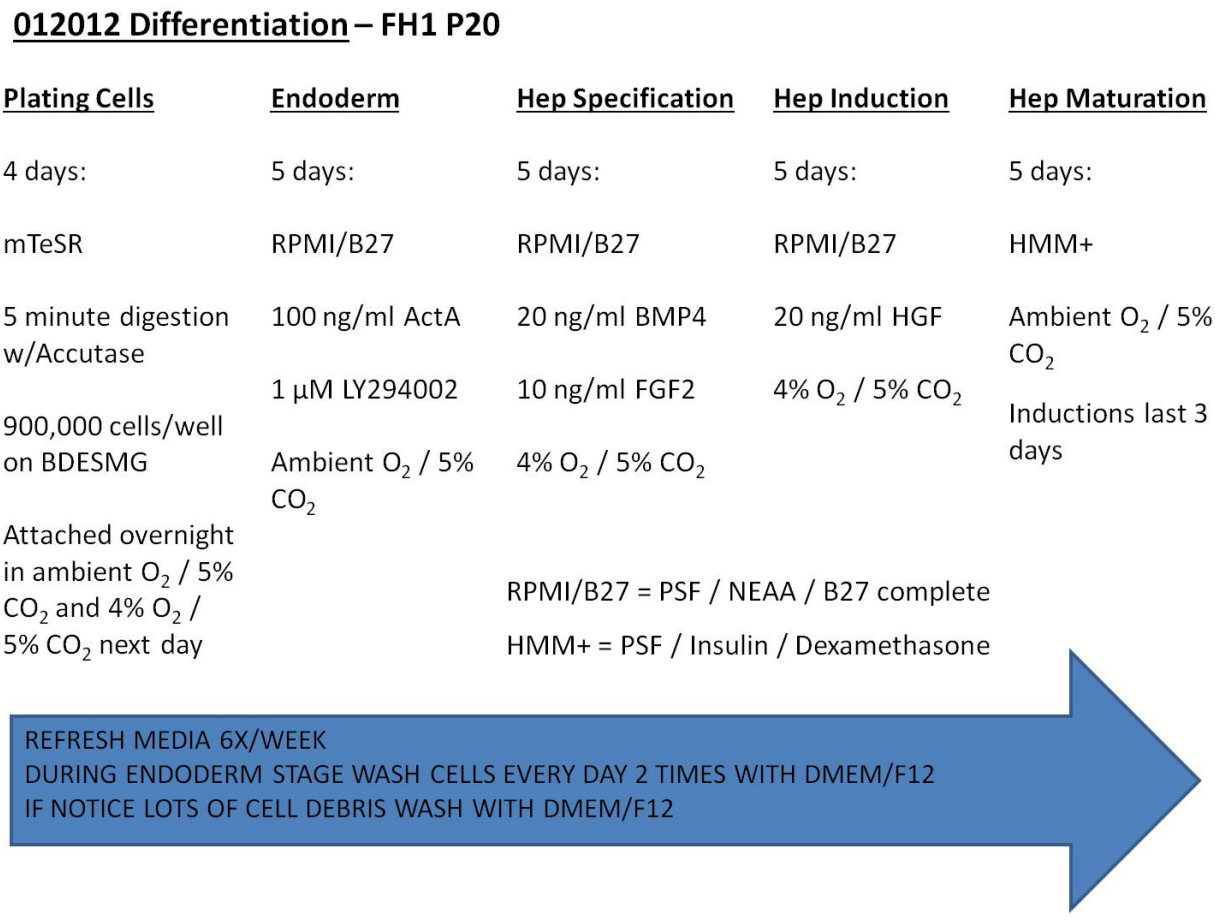


**Figure 41.** 012012 Differentiation of FH1 to Hepatocyte-like Cells
Outline of the 012012 differentiation to stem cell-derived hepatocytes. Figure includes cell line used and its passage number and media components used to differentiate the cells.

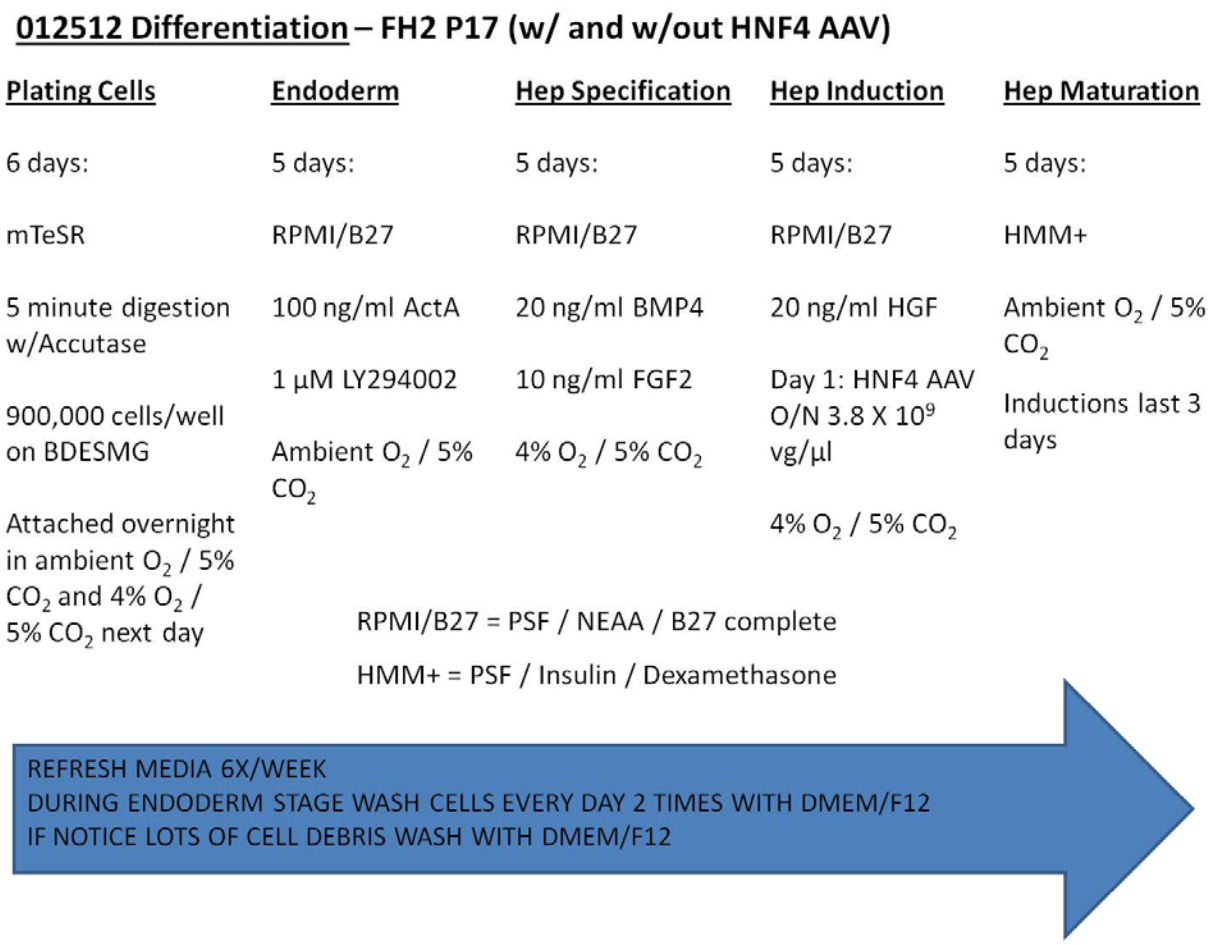


**Figure 42.** 012512 Differentiation of FH2 to Hepatocyte-like Cells
Outline of the 012512 differentiation to stem cell-derived hepatocytes. Figure includes cell line used and its passage number and media components used to differentiate the cells. HNF4 AAV was given overnight (O/N) on Day 1 of the hepatic induction stage in half of the wells being differentiated.

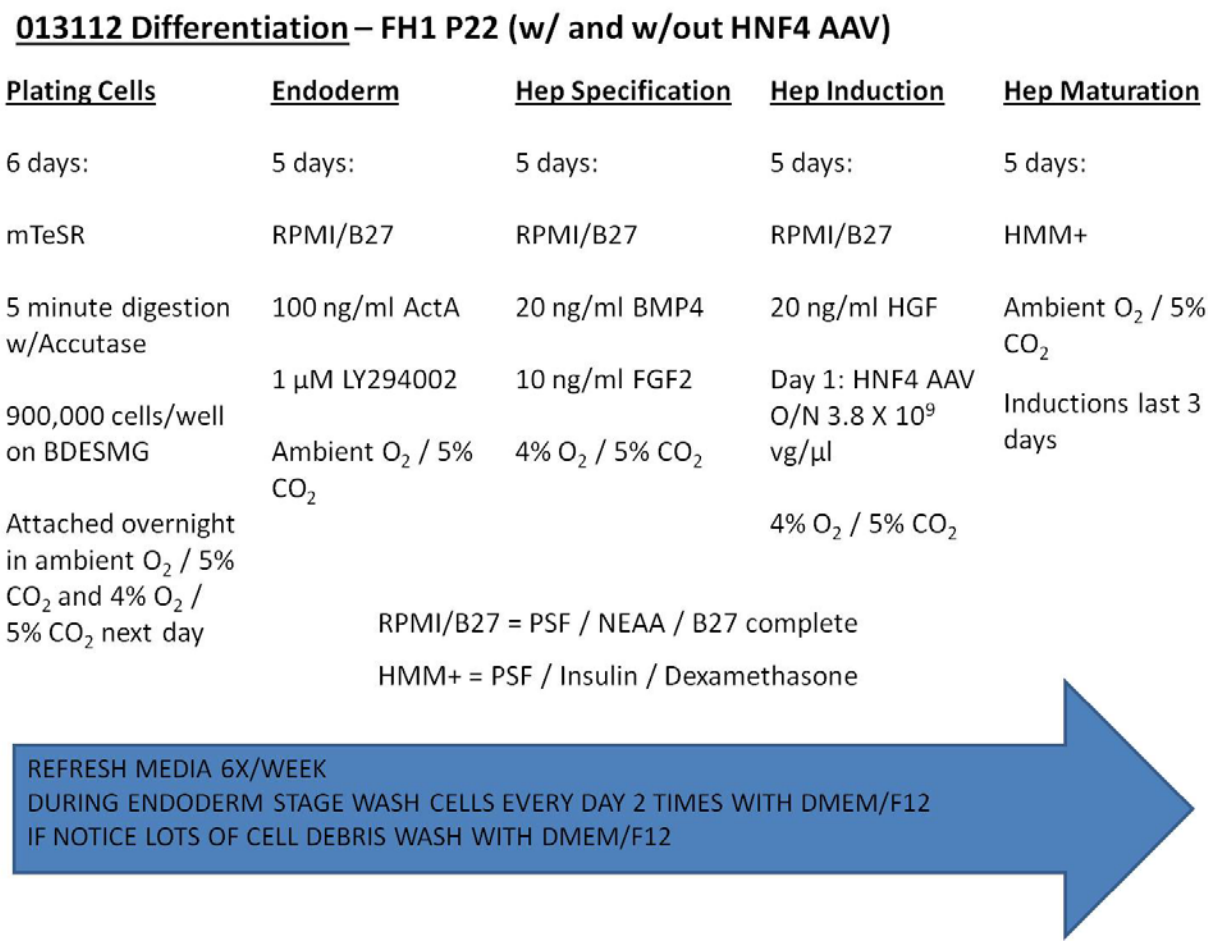


**Figure 43.** 013112 Differentiation of FH1 to Hepatocyte-like Cells
Outline of the 013112 differentiation to stem cell-derived hepatocytes. Figure includes cell line used and its passage number and media components used to differentiate the cells. HNF4 AAV was given overnight (O/N) on Day 1 of the hepatic induction stage in half of the wells being differentiated.

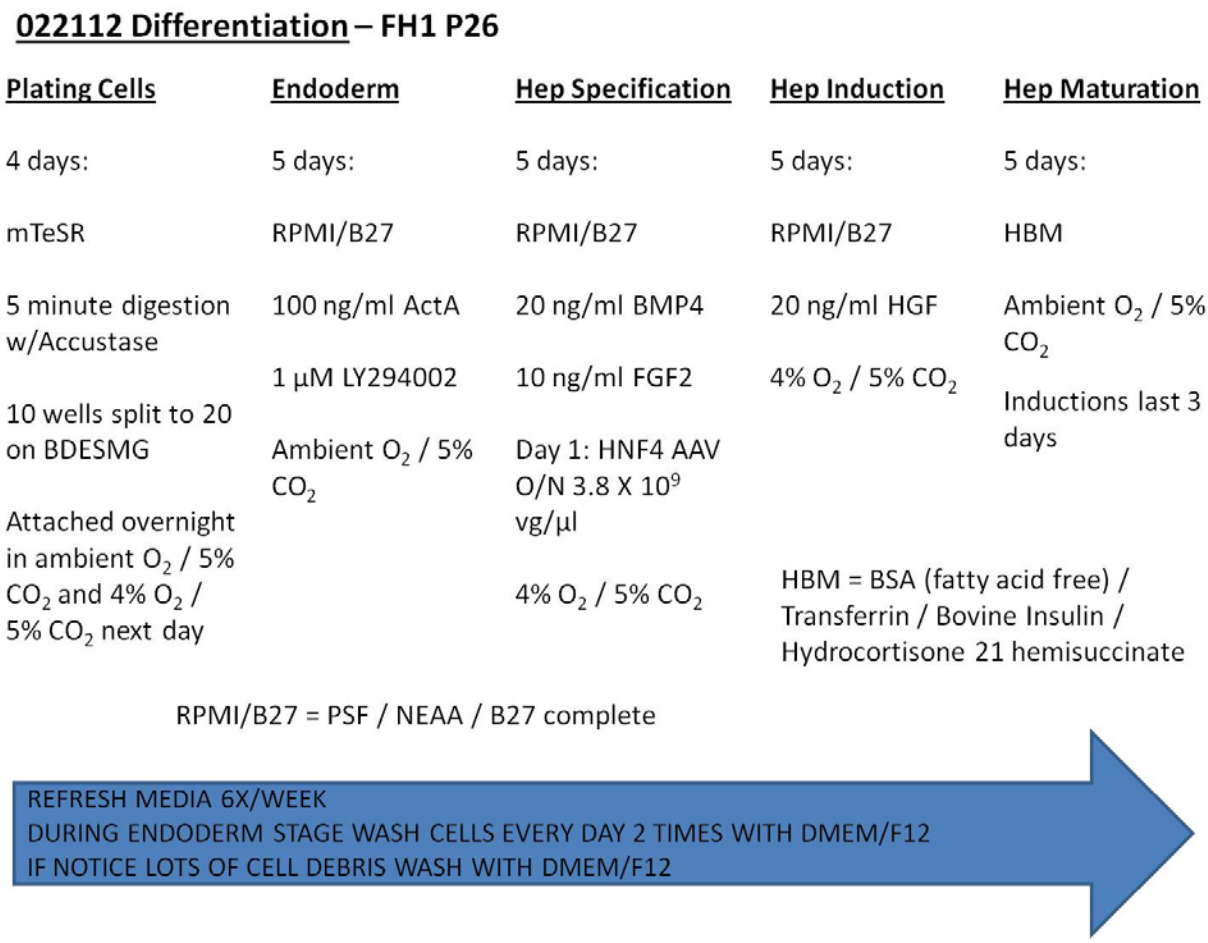


**Figure 44.** 022112 Differentiation of FH1 to Hepatocyte-like Cells
Outline of the 022112 differentiation to stem cell-derived hepatocytes. Figure includes cell line used and its passage number and media components used to differentiate the cells. HNF4 AAV was given overnight (O/N) on Day 1 of the hepatic specification stage.

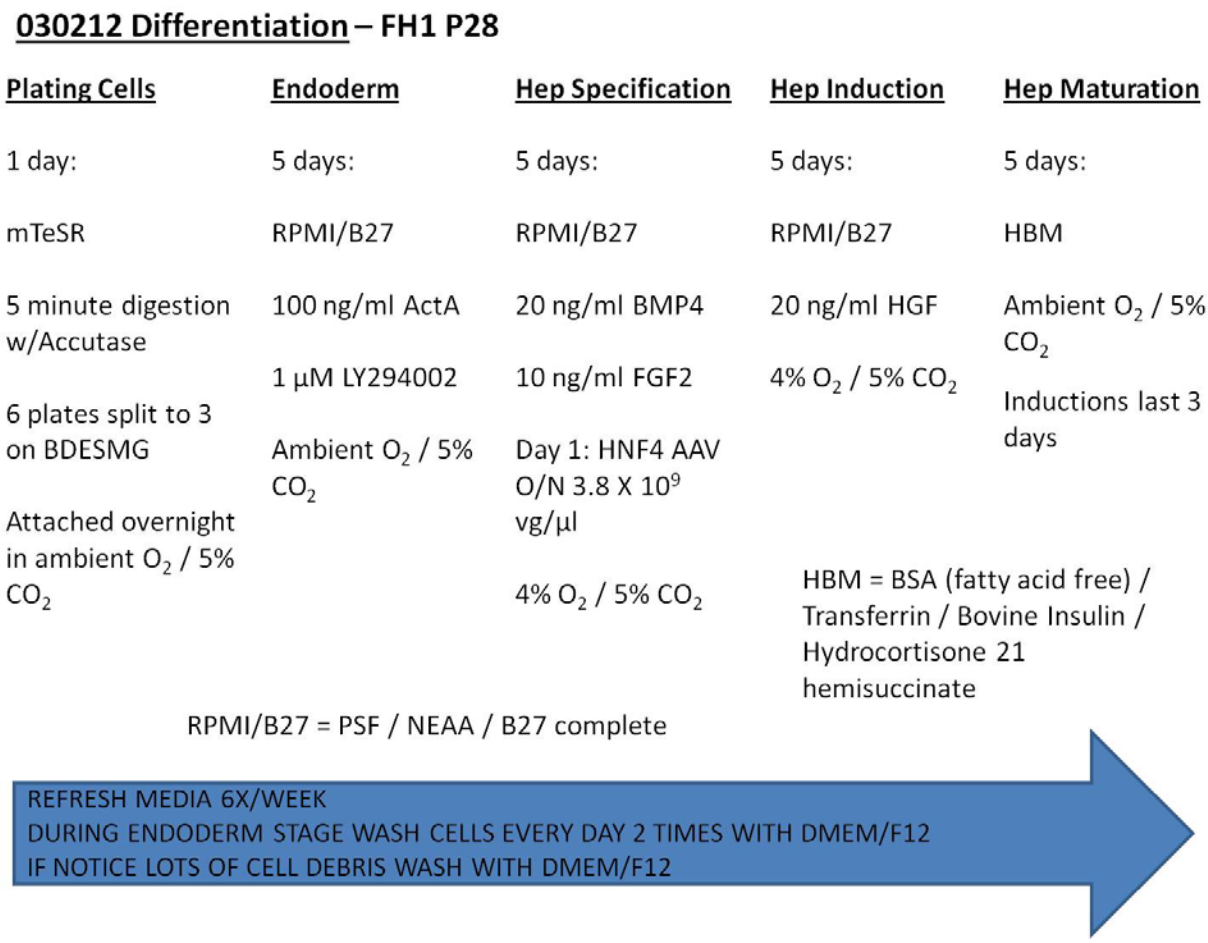


**Figure 45.** 030212 Differentiation of FH1 to Hepatocyte-like Cells
Outline of the 030212 differentiation to stem cell-derived hepatocytes. Figure includes cell line used and its passage number and media components used to differentiate the cells. HNF4 AAV was given overnight (O/N) on Day 1 of the hepatic specification stage.

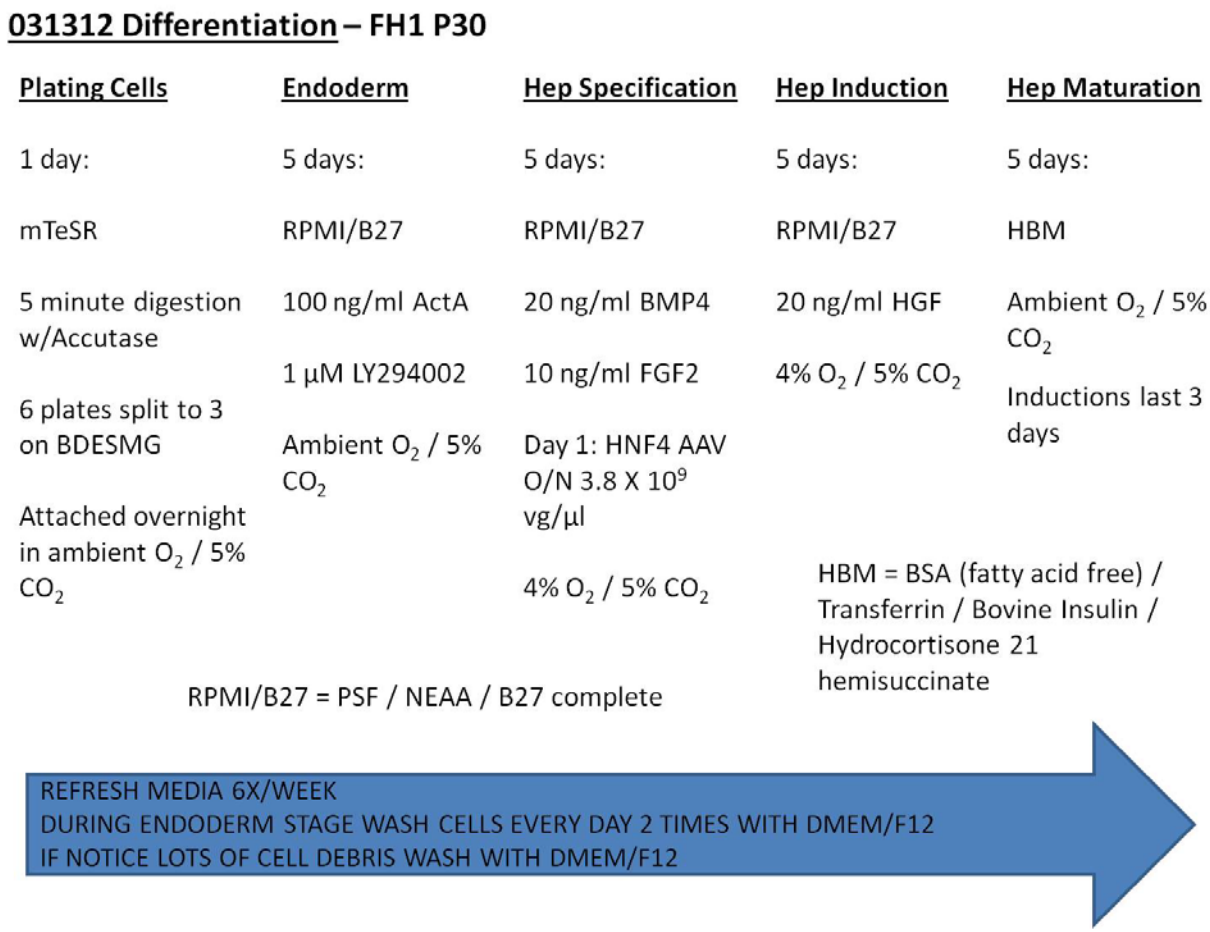


**Figure 46.** 031312 Differentiation of FH1 to Hepatocyte-like Cells
Outline of the 031312 differentiation to stem cell-derived hepatocytes. Figure includes cell line used and its passage number and media components used to differentiate the cells. HNF4 AAV was given overnight (O/N) on Day 1 of the hepatic specification stage.

### 3.3.8 Gene Expression

RNA was isolated using TRIZOL® reagent (Life Technologies, Carlsbad CA) according to the manufacturer's protocol. RNA integrity and purity were confirmed. DNase-I treated RNA of each sample were reverse-transcribed by PCR with RT-PCR reagents (Promega, Fitchburg, WI) according to manufacturer's protocol. The resulting cDNA was analyzed by quantitative

real-time PCR using specific TaqMan® assays and TaqMan® Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol. Ct values were entered into the following equation to determine the arbitrary unit value: $1 \times 10^9 \times e(-0.6931 \times Ct)$ [168] then normalized to cyclophilin A. All samples were run and analyzed on a ABI Prism 7000 Sequence Detection System. Table 17 summarizes the TaqMan® assays that were used:

**Table 17.** TaqMan® Assays Used for Chapter 3 Experiments of this Dissertation
Table summarizes the TaqMan® assays used for chapter 3 experiments and the associated gene symbols, gene name and assay ID numbers.

| Gene Symbol | Gene Name | Assay ID # |
|---|---|---|
| OCT4 | Octamer 4 | Hs03005111_g1 |
| SOX2 | SRY (sex determining region Y)-box 2 | Hs01053049_s1 |
| NANOG | Nanog homeobox | Hs02287400_g1 |
| SOX17 | SRY (sex determining region Y)-box 17 | Hs00751752_s1 |
| CXCR4 | Chemokine (C-X-C motif) receptor 4 | Hs00607978_s1 |
| HNF4A | Hepatocyte nuclear factor 4, alpha | Hs00230853_m1 |
| CYP1A1 | Cytochrome P450, family 1, subfamily A, polypeptide 1 | Hs00153120_m1 |
| CYP1A2 | Cytochrome P450, family 1, subfamily A, polypeptide 2 | Hs01070374_m1 |
| CYP3A4 | Cytochrome P450, family 3, subfamily A, polypeptide 4 | Hs00430021_m1 |
| CYP3A7 | Cytochrome P450, family 3, subfamily A, polypeptide 7 | Hs00426361_m1 |
| ALB | Albumin | Hs00609411_m1 |
| AFP | Alpha-fetoprotein | Hs00173490_m1 |
| CYP2B6 | Cytochrome P450, family 2, subfamily B, polypeptide 6 | Hs03044634_m1 |
| HNF3B (FOXA2) | Hepatocyte nuclear factor 3-beta (Forkhead box A2) | Hs00232764_m1 |

### 3.3.9 Drug Metabolism Studies

Each metabolite was measured in culture after three days of exposure to specific prototypical inducers (if applicable). Cells were incubated for 72 hours in the presence of specific inducers Rifampicin (RIF; 10 µM; CYP3A4; Sigma-Aldrich), Phenobarbital (PB; 1 µM; CYP3A4; Sigma-Aldrich) or β-naphthoflavone (BNF; 25 µM, CYP1A1/1A2; Sigma-Aldrich) in HMM media used to culture primary human hepatocytes. DMSO was used as vehicle control for all 3 specific inducers (0.1%; Sigma-Aldrich). Media was refreshed every 24 hours. Results are expressed as product mole/min and normalized per mg total cellular protein. Protein content was determined with the Bio-Rad Protein Assay kit (Bio-Rad) according to manufacturer's instructions.

### 3.3.10 Luminescent Cytochrome P450 Assays

Cells were lifted off of the tissue culture plates by a 5 minute accutase digestion and counted on a hemacytometer. Thirty thousand viable cells were added to each well of a 96-well plate and subsequent cytochrome P450 assays were performed in triplicate (P450-Glo™ Assays, Promega). The following assays were performed on hepatocytes in HBSS solution: CYP1A1 and CYP1A2. The following assays were performed on cells in HMM only solution: CYP2B6, CYP2C9, CYP3A4 and CYP3A7. 10 µL of each Cyp-Glo reagent was added to the each well of a 96-well plate that contained cells. The plate was incubated on a rocker in the incubator at 37°C for 30 minutes. After 30 minutes 50 µL of luciferase detection reagent without esterase (premixed with cysteine) was added to the wells that received CYP1A1, CYP1A2, CYP2B6, CYP2C9 and CYP3A7 specific compounds. 50 µL of luciferase detection reagent with esterase

was added to the wells that received CYP3A4 specific compound. The plate was incubated at room temperature for 30 minutes. After 30 minutes, the luminescent signal was read on a luminescent spectrometer (Synergy HT, Biotek Instruments, Inc., U.S.A.) equipped with Gen5 software. Results were normalized to total double strand DNA using Quanti-iT™ assay according to manufacturer's instructions. Luciferase detection reagents only were used as the negative control for assays.

### 3.3.11 EROD Assay – CYP1A1/1A2 Metabolism

Cytochrome P450-1A1/1A2 phase I activity was assessed by the conversion of 7-ethoxyresorufin to resorufin as described previously in HMM only [169]. EROD activity was measured in cells in culture after treatment for three days with specific 1A1/1A2 inducer BNF or vehicle control (DMSO). Briefly, cells are incubated for 1 hour at 37°C with 7-ethoxyresorufin solution (20 µM; Sigma Aldrich) and salicylamide (1.5 mM; Sigma-Aldrich). Salicylamide was added to prevent conjugation of the fluorescent product. After 1 hour incubation, the media was collected, clarified by centrifugation and frozen. 100 µL of each sample, analyzed in triplicate, was added to a 96-well white plate and analyzed on a fluorescent spectrometer (Synergy HT, Biotek Instruments, Inc., U.S.A.) equipped with Gen5 software at an excitation wavelength of 535 nm and an emission wavelength of 581 nm. Resorufin concentration was quantified by interpolating the $A_{581}$ values for the unknowns from a standard curve of resorufin prepared in HMM (resorufin [µg/ml] = 617844 * $A_{581}$ + 171.23; $R^2$ = 0.9996). Following sampling period, cells were harvested in phosphate buffer (0.1 M, pH 7.4) and stored at -20°C until protein content was determined. Results are expressed as picomoles of product formed/min normalized to total protein content.

### 3.3.12 Testosterone Metabolism – CYP3A4 Metabolism

Testosterone 6β-hydroxylase activity catalyzed by CYP3A4 was determined in cells in culture that were induced by RIF, PB or vehicle control (DMSO) as described above and previously [170]. Briefly, cells were exposed to testosterone (250 µM; Sigma-Aldrich) for 50 minutes at 37°C in HMM only. The media was collected, centrifuged to remove cells and fragments and stored at -20°C until analyzed by HPLC. Metabolite was identified by comparing retention times with 6β-hydroxytestosterone standard prepared in HMM. Results are expressed as picomoles of product/min normalized to protein content.

### 3.3.13 Ammonia Metabolism

Ammonia metabolism was determined *in vitro* through a colorimetric assay as described previously [171]. Differentiated cells were treated with 1 mM ammonium chloride and 1 mM ornithine for 2 hours in HMM only and one well was immediately centrifuged and the supernatant retained at “time 0” and then stored frozen. After 2 hours, each sample was collected, centrifuged and stored frozen until read. Each sample was read and prepared in triplicate on a spectrometer (Synergy HT, Biotek Instruments, Inc., U.S.A.) equipped with Gen5 software at a wavelength of 560 nm and results were compared to a linear calibration curve. The metabolism of ammonia was expressed as a percentage of ammonia metabolized after 2 hours and as picomoles/min normalized to total protein content.

### 3.3.14 Phase II Metabolism – Resorufin Metabolism

Phase II metabolism was determined by the metabolism of the fluorescent compound resorufin after incubation for 30 minutes at 37°C and measurement of the decrease in fluorescent signal in HMM only. A 50 ng/mL solution of resorufin was added to differentiated cells, and one well was immediately centrifuged and the supernatant retained at "time 0" and then stored frozen. Subsequent time point samples were centrifuged to remove cells and debris and then stored frozen until analyzed with the same conditions described for the EROD assay. Resorufin metabolism was quantified by comparing the fluorescent signal after 30 minutes to the "time 0" value. All the results are expressed as percentage of resorufin metabolized or as picomoles of product formed/min and normalized to total protein content.

## 3.4 RESULTS

### 3.4.1 Transplantation of Undifferentiated Fetal Hepatocyte-derived hiPSCs into FRG Mice

Undifferentiated fetal hepatocyte-derived hiPSCs were transplanted intrasplenically into (43-78 days old) FRG mice. Each animal was transplanted with 2 million cells and 4 mice were transplanted for each cell line. Survival was investigated and potential engraftment was analyzed by the human RNase P assay looking for human DNA in the mouse livers.

3 out of 4 mice transplanted with FH1 developed tumors (Figures 47 and 48). Tumor tissue (Figure 47; red arrow) and tumor adjacent tissue (Figure 47; green arrow) were carefully

dissected away from each other and snap frozen for DNA isolation. The animals that developed tumors lived for an average of 75 days post transplant (a range of 50-93 days) (Figure 48). Animals were sacraficed due to tumor burden. The one animal that did not develop a tumor lived for the duration of the study (150 days). Human DNA was detected in one transplanted animal's tumor adjacent tissue (≈ 0.9%) (Figure 48), which fell within the assays standard curve. Human DNA was found in all tumors, which consisted of 79.7-138.2% human DNA (data not shown).

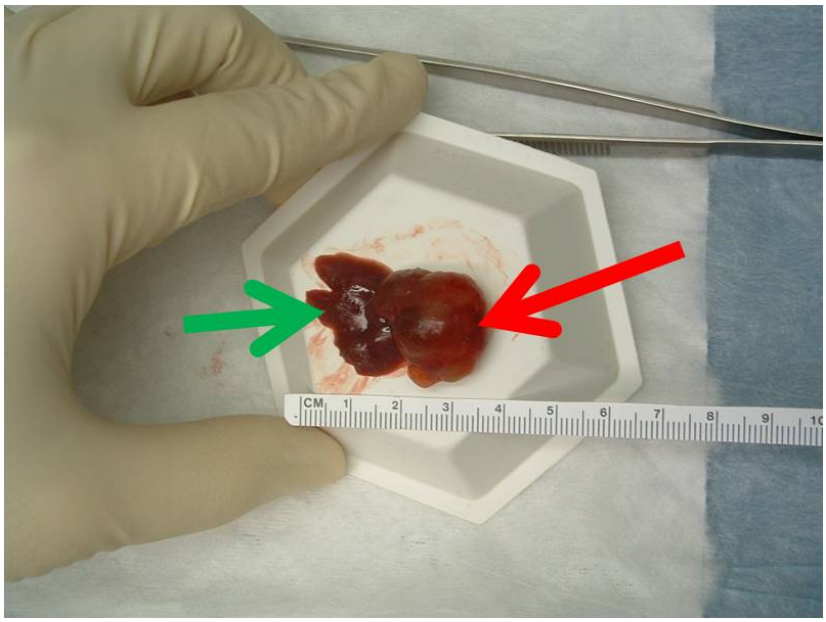


**Figure 47.** Tumor and Tumor Adjacent Tissue
This figure denotes the difference between tumor and tumor adjacent tissue. This figure is applicable to all *in vivo* transplants where tumors occurred. Tumor adjacent tissue is indicated by the green arrow and tumor tissue is indicated by the red arrow.

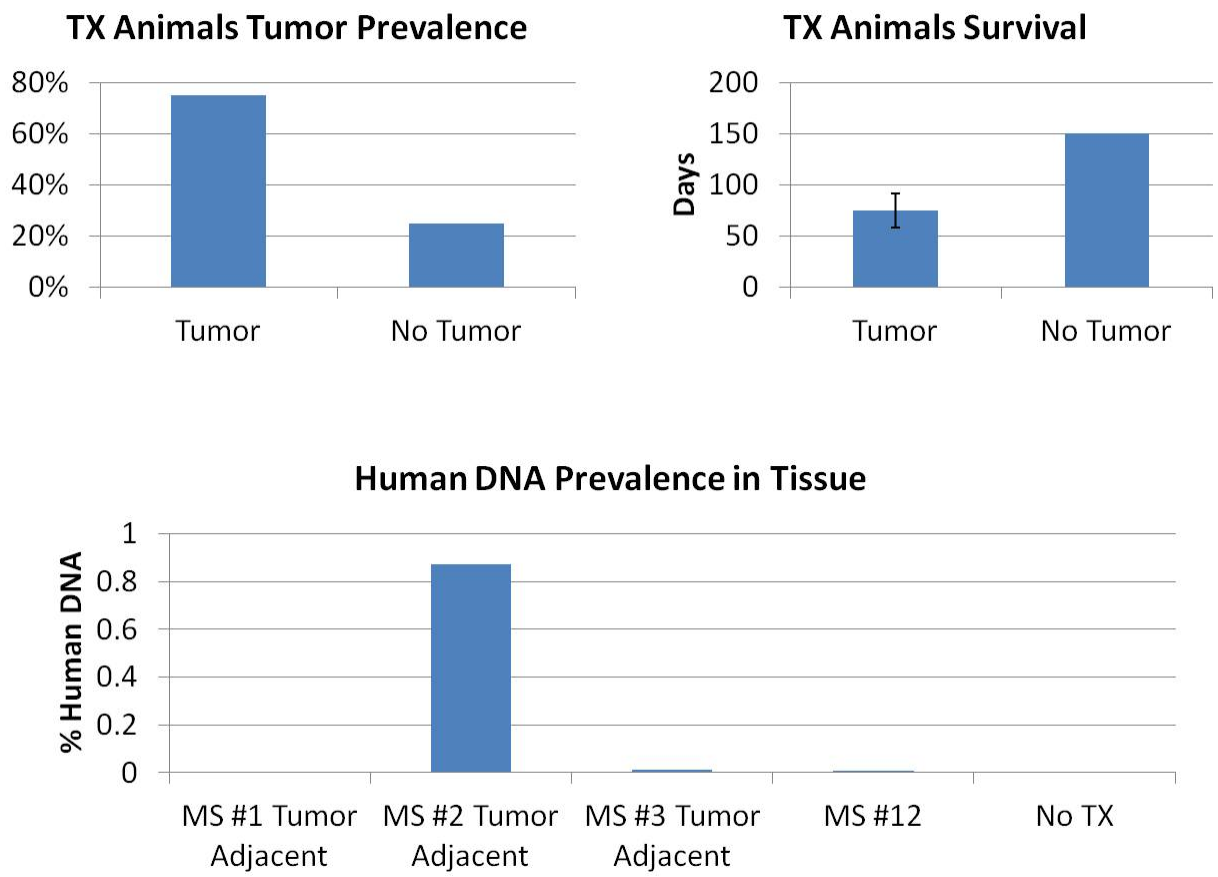


**Figure 48.** Tumor Prevalence, Animal Survival and Human DNA in FRG Mice Transplanted with Undifferentiated FH1
This figure summarizes the transplant data collected for undifferentiated FH1 intrasplenic transplantation to FRG mice.

0 out of 4 mice transplanted with FH2 developed tumors and all mice lived for the duration of the study (150 days). The animals' liver tissue was snap frozen and no human DNA was found (Figure 49).

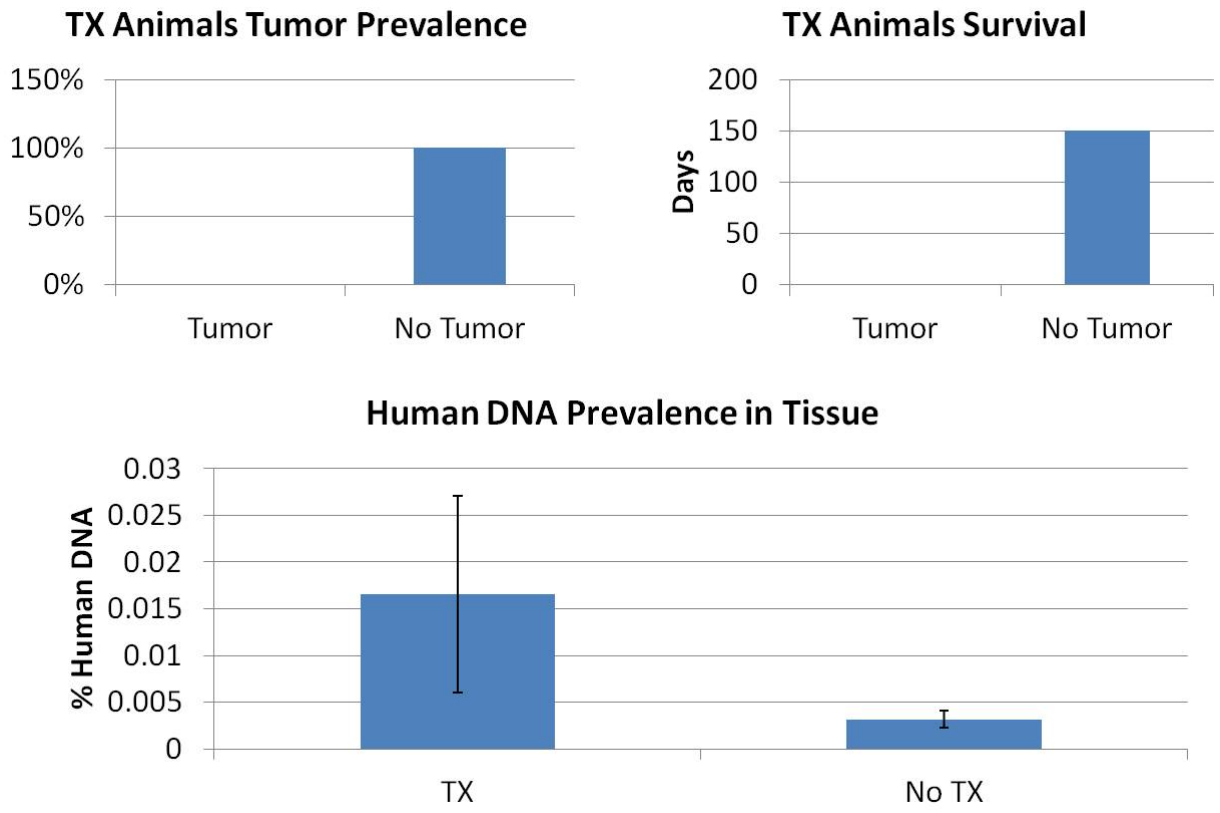


**Figure 49.** Tumor Prevalence, Animal Survival and Human DNA in FRG Mice Transplanted with Undifferentiated FH2
This figure summarizes the transplant data collected for undifferentiated FH2 intrasplenic transplantation to FRG mice.

### 3.4.2 Transplantation of Undifferentiated Fetal Hepatocyte-derived hiPSCs into NOD/SCID Mice

Undifferentiated fetal hepatocyte-derived hiPSCs were transplanted both IP and directly into the liver of 19 day old NOD/SCID mice. Each animal was transplanted with 2 million cells IP and 1 million cells to the left and right sides of the liver (2 million total). 3 mice were

transplanted for each cell line. Survival was investigated and potential engraftment was analyzed by the human RNase P assay looking for human DNA in the mouse livers.

0 out of 3 mice transplanted with FH1 developed tumors and all mice lived for the duration of the study (150 days). The animals' liver tissue was snap frozen and no human DNA was found (data not shown).

1 out of 3 mice transplanted with FH2 developed tumors. Tumor tissue (Figure 47; red arrow) and tumor adjacent tissue (Figure 47; green arrow) were carefully dissected away from each other and snap frozen for DNA isolation. All animals, even the one that developed a tumor, lived until the end of the study (150 days). The one animal that developed a tumor was sacrificed due to tumor burden. Human DNA was detected in one transplanted animal's tumor adjacent tissue (≈ 0.4%; data not shown), which fell within the assays standard curve. This animal's tumor consisted of > 100% human DNA (data not shown).

### 3.4.3 Transplantation of Definitive Endoderm derived from hiPSCs into FRG Mice

Undifferentiated FH2 cells were differentiated to definitive endoderm, the embryonic derivative of hepatocytes, using a step-wise protocol *in vitro* that consisted of RPMI medium supplemented with B27, non-essential amino acids, antibiotic-antimycotic, Activin A and the PI3K inhibitor LY294002. Over the course of the 5 day differentiation, the FH2 cells morphology changed dramatically. Undifferentiated FH2 cells grew in tight, compact colonies with high nuclei to cytoplasm ratio (Figure 50A). After accutase digestion, plating and growing to ≈ 85% confluence the cells resulted in colonies with a more distinct cytoplasm (Figure 50B). After 5 day differentiation to definitive endoderm the cells are much larger and their nuclei to cytoplasm ratio are drastically reduced (Figure 50C). Furthermore, the cells are no longer

growing in colonies. Cells that did not receive Activin A or LY294002 (RPMI basal medium only) did not display a characteristic definitive endoderm morphology as their nuclei to cytoplasm ratio remained constant and the well became over-grown with cells (Figure 50D).

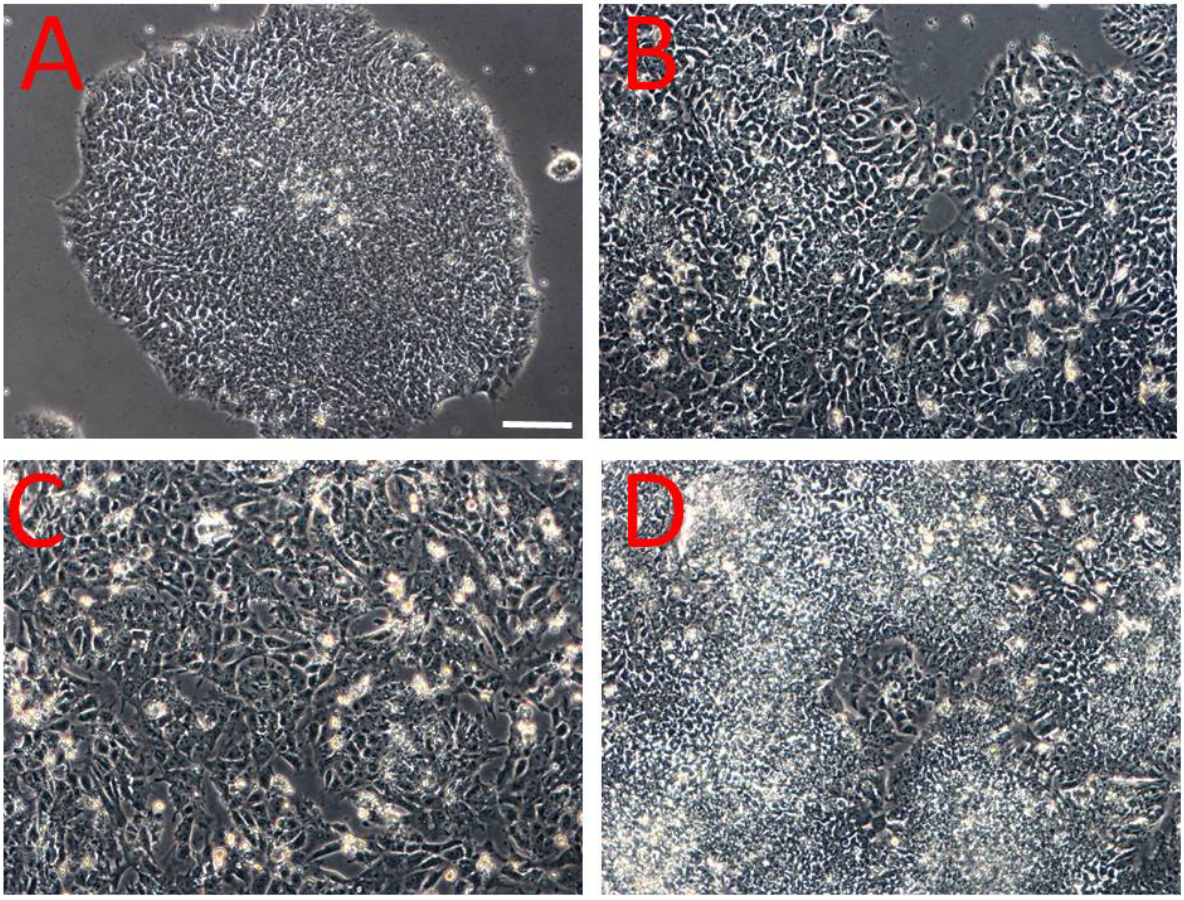


**Figure 50.** Morphology of FH2 Differentiated to Definitive Endoderm
This figure denotes the cellular morphology of FH2 throughout the course of differentiation to definitive endoderm. (A) Undifferentiated FH2. (B) FH2 after accutase digestion, plating and growing to ≈ 85% confluence. (C) Definitive endoderm. (D) Cells receiving only RPMI basal media. Scale bar is 100 µM.

When investigating gene expression, definitive endoderm highly expresses SOX17 and CXCR4 [74]. FH2-derived definitive endoderm saw 870 and 48 fold increases in SOX17 and

CXCR4 expression respectively compared to undifferentiated cells (Figure 51). Adult and fetal hepatocytes and IMR90 fibroblasts were negative controls of mRNA expression levels.

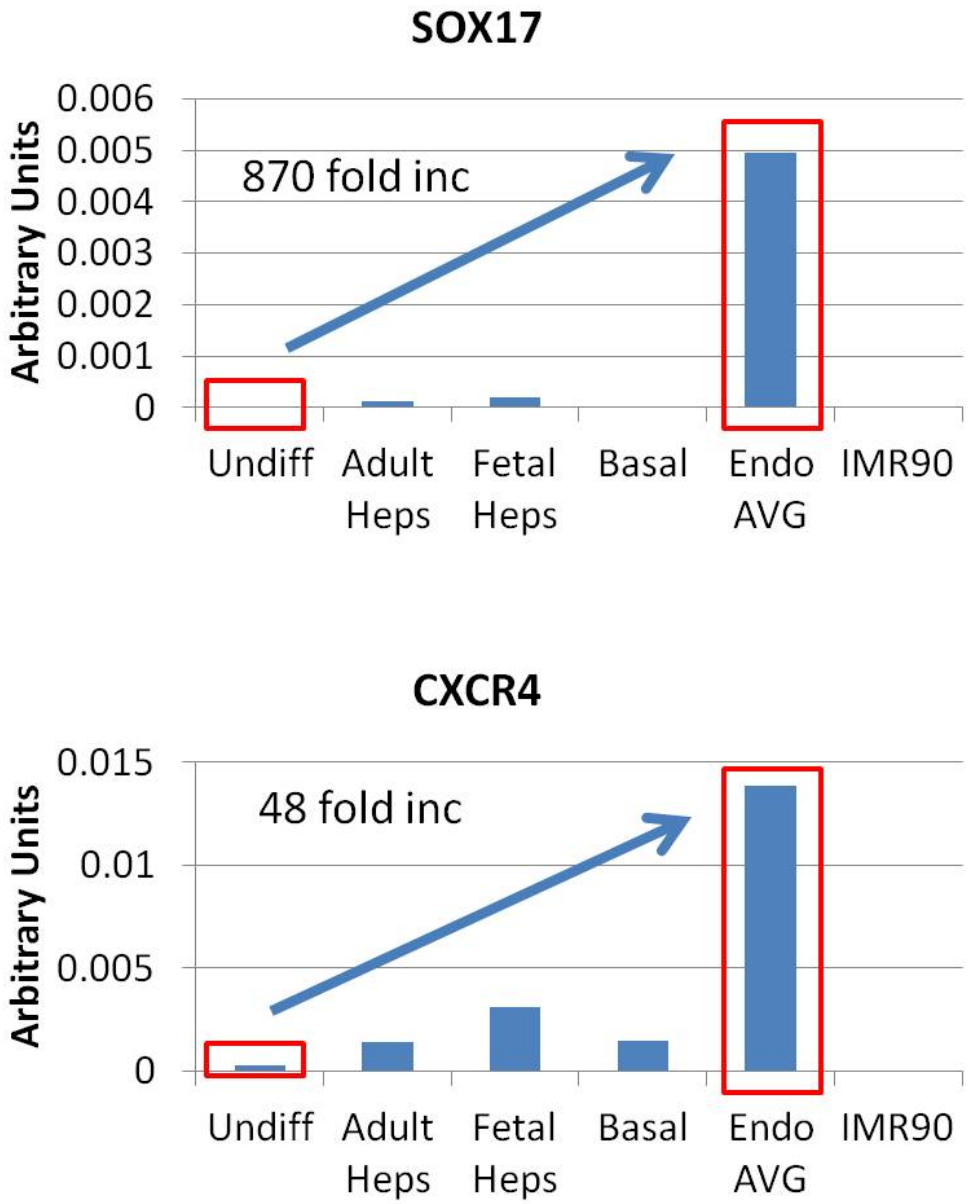


**Figure 51.** Definitive Endoderm-specific Gene Expression of FH2 Differentiated to Definitive Endoderm
This figure denotes definitive endoderm-specific gene expression of the definitive endoderm (Endo) derived from FH2. The differentiated cells showed 870 and 48 fold increases in SOX17 and CXCR4 expression respectively compared to undifferentiated cells. Adult and fetal hepatocytes and IMR90 fibroblasts were negative controls of expression levels. Levels are normalized to cyclophilin A.

When investigating pluripotency marker gene expression, over the course of differentiation to definitive endoderm the cells should decrease markers for pluripotency. FH2-derived definitive endoderm saw decrease in both OCT4 and SOX2 gene expression compared to undifferentiated cells (Figure 52). Adult and fetal hepatocytes and IMR90 fibroblasts were negative controls of expression levels.

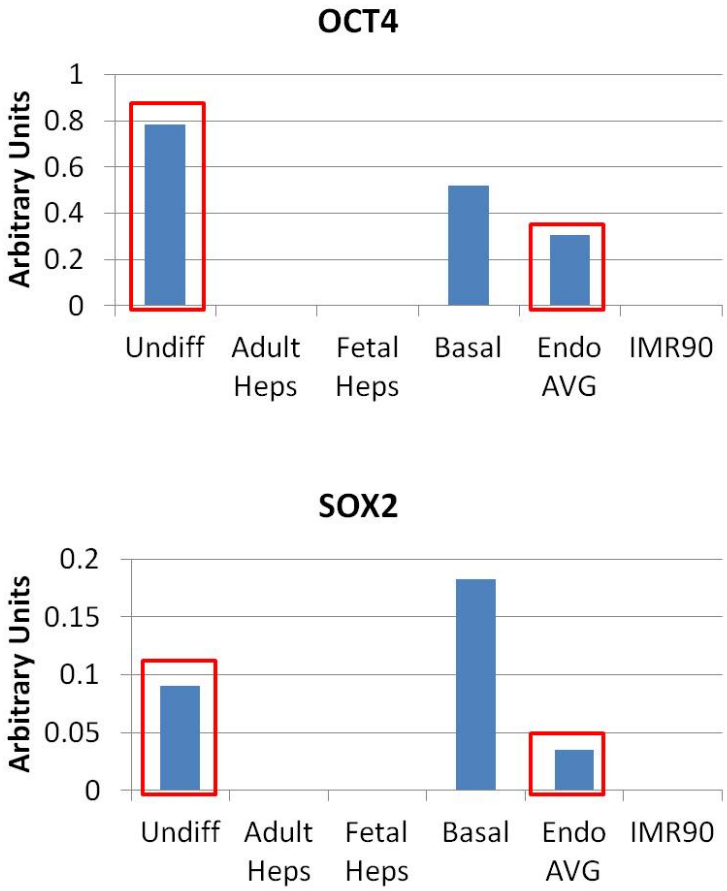


**Figure 52.** Pluripotency Marker Gene Expression of FH2 Differentiated to Definitive Endoderm
This figure denotes the pluripotency marker gene expression of the definitive endoderm (Endo) derived from FH2. The differentiated cells showed decreases in OCT4 and SOX2 expression respectively compared to undifferentiated cells. Adult and fetal hepatocytes and IMR90 fibroblasts were negative controls of expression levels. Levels are normalized to cyclophilin A.

FH2 cells were differentiated to definitive endoderm and 1 million cells were transplanted to each side of the livers of 19 day old NOD/SCID mice (n=7; 2 million cells total). Survival was investigated and potential engraftment was analyzed by the human RNase P assay looking for human DNA in the mouse livers as well as human albumin in the mouse serum.

3 out of 7 mice transplanted with FH2-derived definitive endoderm developed tumors. Tumor tissue (Figure 47; red arrow) and tumor adjacent tissue (Figure 47; green arrow) were carefully dissected away from each other and snap frozen for DNA isolation. Animals with tumors lived an average of 69 days (the range was 57-80). Animals that did develop tumors were sacrificed due to tumor burden. Animals that did not develop tumors lived until the end of the study (100 days). Human DNA was detected in one transplanted animal's tumor adjacent tissue (≈ 0.2%; Figure 53), which fell within the assays standard curve. This animal's tumor consisted of > 100% human DNA (data not shown). The animal that had human DNA in the tumor adjacent tissue also had 1 µg/mL of human albumin in its serum (Figure 53). 1 mg/mL albumin correlates with ≈ 20% repopulation of human cells within the mouse liver, therefore 1 µg/mL correlates with 0.2% repopulation with human hepatocytes derived from FH2-derived definitive endoderm.

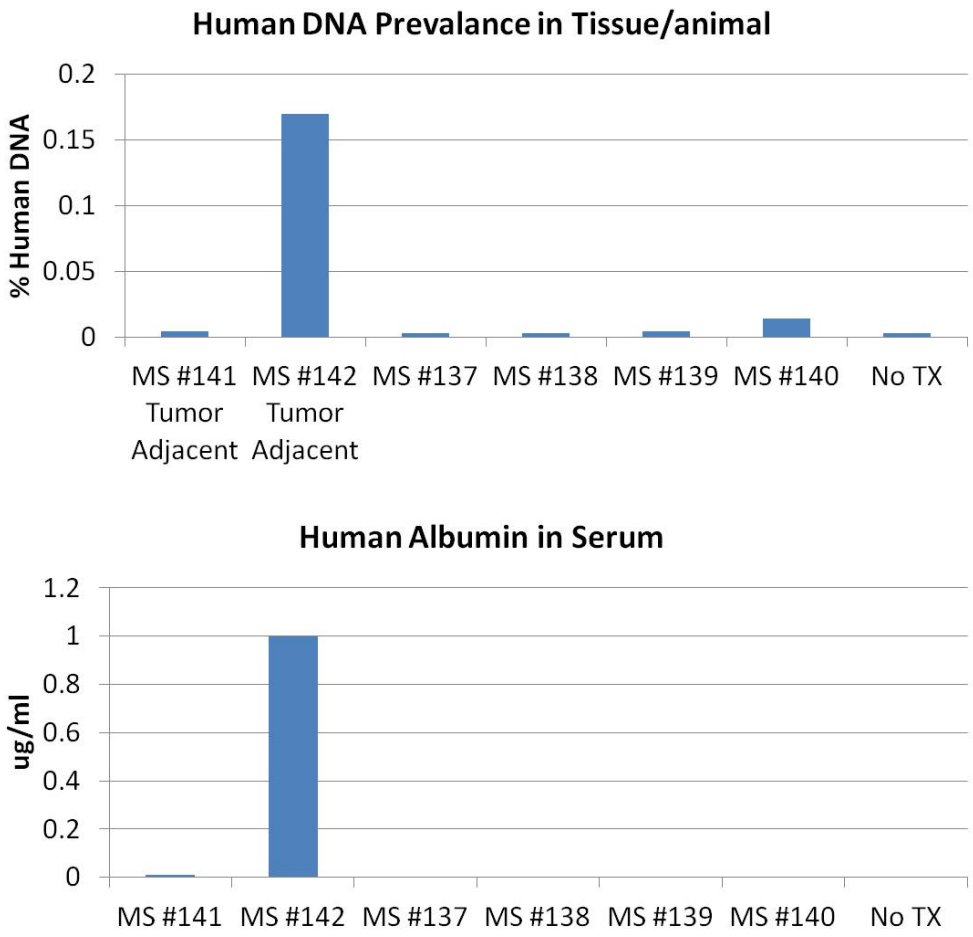


**Figure 53.** Human DNA Prevalence and Human Albumin in FRG Mice Transplanted with Definitive Endoderm Derived from FH2
This figure summarizes the human DNA prevalence and human albumin in FRG mice transplanted with definitive endoderm derived from FH2. Although preliminary, data suggests repopulation of MS #142.

### 3.4.4 Differentiation of FH2, FH12, FH14 and FH15 to Definitive Endoderm

FH2, FH12, FH14 and FH15 were differentiated to definitive endoderm *in vitro*. They show characteristic decrease in nuclei to cytoplasm ratio and increase in cell size that is consistent with definitive endoderm morphology (data not shown). They have also been shown to have definitive endoderm gene expression with large inductions of SOX17 and CXCR4

expression when compared to their undifferentiated counterparts. Table 18 summarizes each cell line's fold change from their undifferentiated cell counterparts in terms of SOX17 and CXCR4 expression. Adult and fetal hepatocytes and IMR90 fibroblasts were used as controls for gene expression (data not shown). All data in the table was normalized to cyclophilin A expression.

**Table 18.** Cell Lines Differentiated to Definitive Endoderm and their Associated Fold Changes in SOX17 and CXCR4 Gene Expresssion Compared to their Undifferentiated Cell Counterparts

| Cell Lines | Fold Δ SOX17 | Fold Δ CXCR4 |
|---|---|---|
| FH2 | 870 | 48 |
| FH12 | 32 | 6 |
| FH14 | 71 | 10 |
| FH15 | 538 | 13 |

### 3.4.5 Differentiation of Fetal Hepatocyte-derived hiPSCs to Hepatocyte-like Cells

6 unique differentiation protocols were used to differentiate fetal hepatocyte-derived hiPSCs to hepatocyte-like cells. This was done because after each protocol was completed only limited liver specific gene expression and function of hepatocyte-like cells resulted. Here we describe the data for one of the six experiments, 012512 differentiation, which is representative of each of the other five not shown. Briefly, the differentiation protocol entailed the following: FH2 cells were lifted off the tissue culture plates by a 5 minute digestion with accutase and plated in mTeSR™1 and allowed to come to ≈ 85% confluence after six days. This followed a 5 day endoderm induction with Activin A and LY294002, a 5 day hepatic specification with BMP4 and FGF2, a 5 day hepatic induction with HGF (half the cells received an AAV

expressing human HNF4α on day 1 of this stage) and finally a 5 day hepatic maturation in HMM+ (adult hepatocyte media). Throughout the induction cell morphology changed as expected. The differentiating cells became larger and their nuclei to cytoplasm ratio decreased. By the end of the protocol the cells had a hepatocyte-like morphology with a cobblestone like morphology, however the cells were still relatively small compared to a primary hepatocyte. Some areas of the plate even had cells that had bile canalicular-like structures with neighboring cells (data not shown).

Real time gene expression analysis was performed to look at the ability of the differentiating cells to form definitive endoderm in culture. Therefore gene expression of SOX17 and CXCR4 was analyzed. For this experiment, there were 30 and 4 fold increases in SOX17 and CXCR4 gene expression compared to undifferentiated cells (Figure 54). This is a drastic reduction from a previous experiment with this cell line that showed 870 and 48 fold inductions respectively at this stage in differentiation (Figure 51).

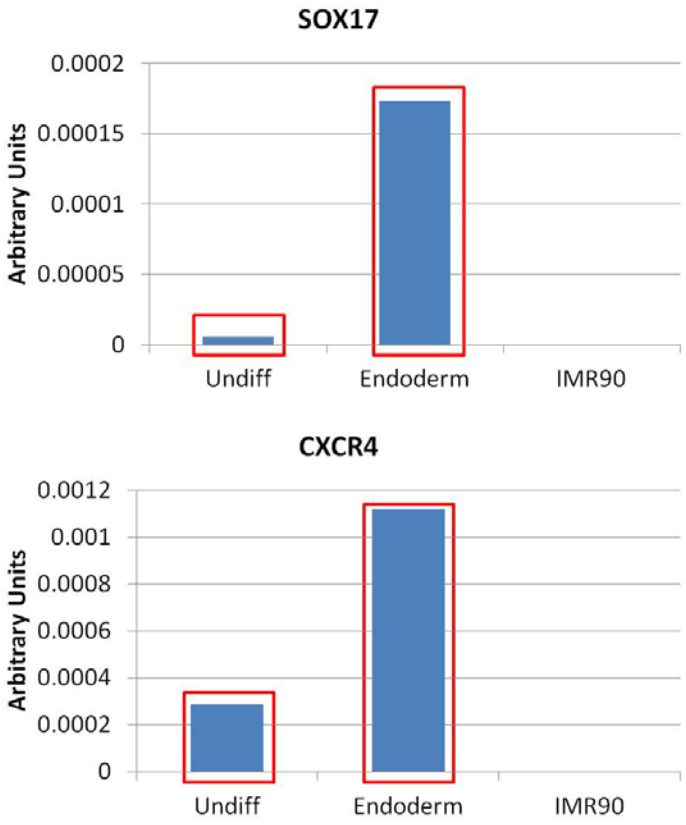


**Figure 54.** 012512 Differentiation Experiment Endoderm Marker Gene Expression
This figure denotes the endoderm marker gene expression for the 012512 differentiation experiment of FH2. The differentiated cells show an increase in endoderm marker expression at the endoderm stage; however this increase is not what is seen in pervious differentiation experiments. IMR90 fibroblasts were used as a negative control of expression levels. Levels are normalized to cyclophilin A.

Liver-specific gene expression at the end of the differentiation protocol was investigated on both the cells that received HNF4α AAV and the cells that did not. When looking at HNF4α gene expression, cells that received HNF4α AAV on Day 1 of the hepatic induction stage had a 251% increase in HNF4α gene expression compared to primary adult hepatocytes (Figure 55). In contrast, cells that did not receive the HNF4α AAV only had a 0.4% HNF4α gene expression compared to primary adult hepatocytes, thus demonstrating that the HNF4α AAV was "jump starting" HNF4α gene expression in the cells that were exposed to the virus (Figure 55).

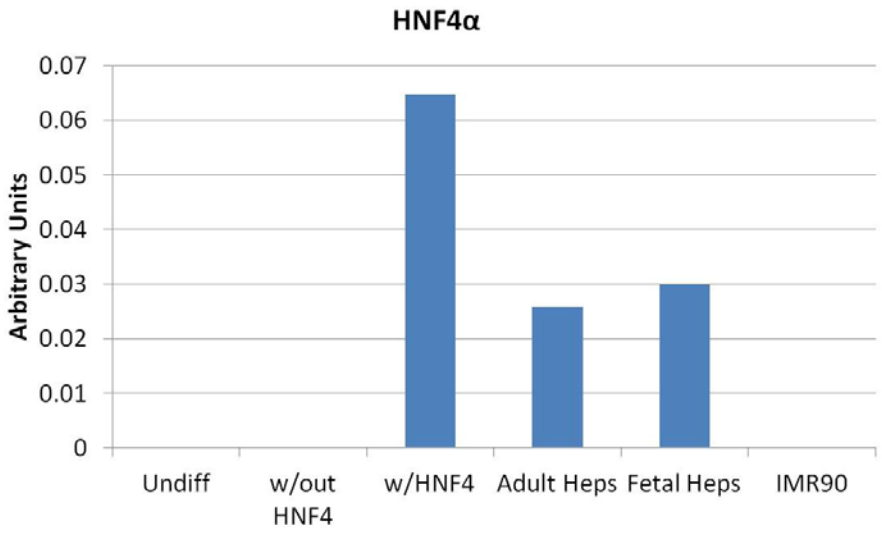


**Figure 55.** 012512 Differentiation Experiment HNF4α Gene Expression
This figure denotes the HNF4α gene expression at the end of the differentiation protocol for the 012512 differentiation experiment of FH2. Cells that received HNF4α AAV had a significant increase in HNF4α gene expression compared to cells that did not receive the virus. Adult and fetal hepatocytes were used as positive controls of expression and IMR90 fibroblasts were used as a negative control. Levels are normalized to cyclophilin A.

Other liver-specific gene expression was investigated on the differentiated cells at the end of the protocol. Table 19 summarizes the liver-specific gene expression of the differentiated cells that did or did not receive HNF4α AAV at the end of the differentiation protocol. Each marker is broken down by percentage compared to primary adult hepatocyte expression levels. There was limited expression of liver-specific markers in the differentiated cells in all markers analyzed.

**Table 19.** Liver-Specific Gene Expression of the Differentiated Cells at End of 012512 Differentiation Protocol
Each marker is broken down by percentage compared to primary adult hepatocyte expression levels and whether or not the cells received HNF4α AAV.

| Liver Marker | Percent Adult w/HNF4α AAV | Percent Adult w/out HNF4α AAV |
|---|---|---|
| CYP1A1 | 16% | 20% |
| CYP1A2 | 0.1% | 0.1% |
| CYP3A4 | 0.02% | 0.002% |
| CYP3A7 | 0.1% | Undetected |

| | | |
|---|---|---|
| Albumin | 0.0007% | 0.001% |
| AFP | 11,588% | 17,225% |
| CYP2B6 | 0.3% | 0.3% |
| HNF3β | 4.6% | 3.2% |

Gene expression of the pluripotency markers was analyzed throughout the course of the differentiation and, as expected, OCT4, SOX2 and NANOG expression decreased throughout the course of the protocol (data not shown).

The differentiated cells were also investigated to see if they would have characteristic hepatocyte drug-specific inductions. Some inductions were seen with the hepatocyte-like cells; however none of the inductions seen were on the same order of magnitude as primary adult hepatocytes (data not shown).

The differentiated cells were also investigated for metabolic activity capabilities in various metabolic activity assays: ammonia metabolism, EROD metabolism, testosterone metabolism, resorufin metabolism and luminescent cytochrome P450 assays. As was seen in the gene expression analysis, differentiated cells showed limited or no metabolic activity in all assays performed. If there was activity, it was not on the same order of magnitude as a primary adult hepatocyte (data not shown).

Each protocol was ranked based on liver specific-gene expression to determine the most efficient differentiation protocol out the six performed. For example, each protocol was ranked 1 through 8 for ability to express CYP1A1. The experiment yielding highest expression of CYP1A1 was given a score of 1 and the lowest received a score of 8. This was done for each liver-specific marker analyzed. Each protocol's score was added together for each marker to get an overall score. Table 20 summarizes the total scores for each specific protocol. The protocol with the lowest score was determined to be the most efficient protocol for differentiation of fetal

hepatocyte –derived hiPSCs to stem cell derived hepatocytes in terms of ability of the differentiated cells to express liver-specific markers. 013112 Differentiation with HNF4α AAV was determined to be the most efficient protocol to date using this scoring method.

**Table 20.** Comparing Every Performed Differentiation Experiment for its Ability to Differentiate Fetal Hepatocyte-derived hiPSCs to Stem Cell-derived hepatocyte-like cells
Each protocol was ranked based on liver specific-gene expression to determine the most efficient differentiation protocol out the six performed.

| Experiment | Hepatocyte Markers Rank (total score) |
|---|---|
| 013112 – w/ HNF4α | 1 (38) |
| 012512 – w/ HNF4α | 2 (41) |
| 012012 | 3 (53) |
| 022112 | 3 (53) |
| 012512 – w/out HNF4α | 4 (54) |
| 013112 – w/out HNF4α | 5 (58) |
| 030212 | 6 (67) |
| 031312 | 7 (68) |

## 3.5 DISCUSSION

Two different mouse models were used to investigate whether undifferentiated fetal hepatocyte-derived hiPSCs could engraft within mouse livers (FRG and NOD/SCID models). Although extremely preliminary, undifferentiated hiPSCs could be transplanted into mice and human DNA could be found in liver tissue in both models. However engraftment was always associated with tumorigenesis. Furthermore, an alternative explanation for human DNA being found in tumor adjacent tissue is that when dissecting away the tumor tissue from the tumor adjacent tissue, some of the tumor tissue contaminated the tumor adjacent samples thus resulting in the tiny

amount of human DNA in the tumor adjacent samples. Although beyond the scope of this thesis, future experiments should be performed to see if transplantation of undifferentiated cells results in human albumin in the mouse serum, which would further support that undifferentiated cells can engraft in the mouse liver and differentiate to hepatocytes expressing albumin. Furthermore, upon animal sacrifice, one can analyze human albumin in tissue sections to provide direct evidence of cell differentiation in terms of the ability of the transplanted cells to produce human albumin.

Fetal hepatocyte-derived hiPSCs were differentiated to endoderm and subsequently transplanted to FRG mice to investigate if the endoderm can engraft within the mouse liver. Once again human DNA was found in the tumor adjacent tissue. However in this same animal human albumin was found in the blood serum that correlated to 0.2% repopulation. Although, once again, engraftment was associated with tumorigenesis, these data suggest that differentiated fetal hepatocyte-derived hiPSCs have the ability to engraft in mouse livers.

Taken together, these three transplant experiments show that engraftment of fetal hepatocyte-derived hiPSCs in the liver is possible, albeit at the cost of tumorigenesis in the animal. These results agree with other published work reporting poor engraftment of stem cell-derived hepatocytes and undifferentiated iPSCs. These data also suggest that if these hiPSCs were differentiated to stem cell-derived hepatocytes, these fully differentiated cells would have the potential to engraft within the mouse liver and therefore be useful for regenerative medicine purposes if issues of tumorigenicity can be overcome.

Fetal hepatocyte-derived hiPSC lines FH2, FH12, FH14 and FH15 were differentiated to definitive endoderm *in vitro*. SOX17 and CXCR4 gene expression levels were induced among these lines, however induction levels were different when comparing all lines. Importantly, and

as expected, higher SOX17 inductions correlated with higher CXCR4 inductions. These results agree with the current literature that says different hiPSC lines have better abilities to differentiate than others. Since these fetal hepatocyte-derived hiPSC lines can be differentiated to definitive endoderm in culture, it is expected that these cells can be further differentiated to hepatocyte-like cells.

When full differentiation of fetal hepatocyte-derived hiPSCs was performed limited results were obtained. A total of 6 unique experiments were performed that generated limited hepatocyte-like characteristics of the cells in terms of their ability to express liver-specific markers or perform liver-specific metabolism. It has been determined that HNF4α can be induced in these hiPSCs by giving the cells an AAV expressing HNF4α, however this HNF4α expression alone is not enough to jumpstart "full differentiation". All differentiation protocols outlined in the methods section resulted in data trends similar to the ones outlined in Table 19. Furthermore all performed metabolic activity assays in all differentiations showed the same trends as the gene expression data found in Table 19.

In order to get full hepatic differentiation of hiPSCs, proper definitive endoderm formation is critical. An explanation for why none of these differentiation protocols efficiently formed hepatocyte-like cells is that each full differentiation experiment resulted in limited endoderm formation, in terms of SOX17 and CXCR4 mRNA expression. All endoderm formation in all hepatocyte differentiation experiments was similar to the one found in Figure 54. Future experiments should be performed to show the large definitive endoderm inductions as seen in Figure 51. Cells that achieve this large induction should be able to be differentiated more efficiently to hepatocyte-like cells.

# 4.0 CONCLUSIONS AND FUTURE RESEARCH

## 4.1 DISCUSSION AND SUMMARY

Cell therapy has a chance to take a huge burden off the liver transplant waiting list. If we can identify which diseases can be treated with cell replacement therapies then we can concentrate on performing OLTs on those not corrected by cellular therapy. Therefore all other diseases can be treated with cell therapy, where theoretically one organ donor can treat multiple patients if enough cells can be isolated from the tissue, thus further taking the burden off the transplant waiting list. Moreover, if hESC and hiPSC technology can become clinically relevant doctors would not have to rely on organ donors to treat patients with cell therapy. Furthermore, hiPSC technology allows for the creation of patient-specific cells for autologous transplants that offers the potential of not needing immunosuppression.

If the cell source, techniques and protocols become available, hiPSCs will have countless numbers of real life applications if they can be differentiated to hepatocytes that are indistinguishable from primary cells. These stem cell-derived hepatocytes can be an unlimited source of cells for basic hepatic biology research and drug discovery. They could also be used for transplant to treat liver-based metabolic disorders or liver failure. This cell replacement therapy could be curative or used as a “bridge to transplant” to keep the patient alive until a donor liver becomes available for OLT. More importantly, disease-specific hiPSCs could be made to give researchers a limitless source of disease-specific hepatocytes to study new disease pathologies or to come up with drug interventions to treat underlying disease symptoms. Patient specific cells could be created and corrected for their genetic defect and transplanted back to the patient without the need for immunosuppression since the transplant is autologous.

Recent reports in mice have determined that fetal hepatocyte-derived iPSCs contain superior re-differentiation capacity and fetal hepatocytes have superior reprogramming capacity

when enriched for progenitor cell populations. Therefore if human fetal hepatocytes can be reprogrammed to iPSCs, then this cell source may represent the best cell source to generate human stem cell-derived hepatocytes.

Here, we report for the first time the derivation of hiPSCs from human fetal hepatocytes in entirely feeder-free conditions. Furthermore derivation of fetal hepatocyte-derived hiPSCs can be done using only OCT4, SOX2 and NANOG. Fetal hepatocytes reprogram significantly more efficiently and reproducibly than adult hepatocytes and are a potential new source of disease specific cells, since many terminated pregnancies are done so because of genetic defects. Finally we have reported for the first time the generation of a metabolic disease specific hiPSC from an adult hepatocyte. The established hiPSC cell lines from both fetal (n=37) and adult (n=3) hepatocytes will be an invaluable source for all of the reasons stated above.

We also have preliminary data showing that undifferentiated fetal hepatocyte-derived hiPSCs and hiPSCs differentiated to endoderm can engraft within mouse livers; although engraftment is always associated with tumorigenesis. This suggests that if successful hepatic differentiation is performed on these hiPSCs then they should have the ability to engraft within mouse livers and therefore be useful for regenerative medicine applications if issues with tumorigenesis can be solved.

Finally, full hepatic differentiation was performed on these fetal hepatocyte-derived hiPSCs however limited differentiation was observed in culture. Differentiated cells showed limited expression of liver-specific markers and performed poorly in different liver-specific metabolism assays. It has been concluded that the limited differentiation was most likely due to lack of good endoderm formation in the experiments where differentiation was carried out the entire way. Since there are a total of 40 distinct cells lines (n= 37 and 3 derived from fetal and

adult hepatocytes respectively) the best lines in terms of hepatic differentiation potential could be selected.

## 4.2 FUTURE RESEARCH RECOMMENDATIONS

Here we have reported that human fetal hepatocytes reprogram more efficiently and reproducibly than adult hepatocytes, however we have not determined the mechanism for this phenomenon. We know that it is not transduction efficiency or virus toxicity related. Furthermore we know it is not due to reprogramming factor stochiometry differences or differences in expression of BAF factor complex members. However it could be due to other known important pathways in reprogramming. These pathways include: p53 and its various downstream targets [129, 133, 136], innate immune response pathways and regulators [130], pathways downstream of hypoxia inducible factors [135], MEK-ERK and TGFβ pathways [132], retinoic acid signaling [134], AID-dependent DNA demethylation [137], downstream targets of the orphan nuclear receptor Esrrb [138], and up-regulation of epithelial cell adhesion molecule complex proteins [139]. Any one of these pathways could be responsible for why human fetal hepatocytes reprogram more efficiently than adult and should be subsequently investigated.

We only investigated engraftment of undifferentiated hiPSCs and hiPSCs differentiated to definitive endoderm. Although preliminary, potential engraftment was always associated with tumorigenesis. In repopulated animals we can investigate whether there is human hepatocyte-specific gene expression in the tumor adjacent tissue to determine if the cells contributing to the human serum albumin and human DNA are also expressing other liver genes, thus suggesting the transplanted cells have differentiated to a hepatic phenotype. The tumors from these animals

have been fixed and stored in paraffin blocks. H & E's should be made to determine the type of tumor it is; whether it is a teratoma or not. Furthermore, experiments should be performed to see if hiPSCs, when fully differentiated, can engraft in our various mouse models without tumorigenesis. If they still do then differentiated cells can be separated based on SSEA-5 expression. Recently, a group has discovered a monoclonal antibody against SSEA-5, and separation based on SSEA-5 expression through FACS greatly reduced teratoma-formation potential of poorly differentiated hESC cultures [160]. Therefore, if transplantation of differentiated hiPSCs still leads to tumor formation, differentiated cells can be separated using this antibody to see if engraftment can occur without tumor formation. In repopulated animals engraftment can be assessed by human DNA found in the liver tissue as well as human albumin in the serum. In highly repopulated animals, serial liver sections can be stained with a human specific cytokeratin-18 antibody to identify human derived cells that have engrafted within the recipient mouse liver. This would be the direct evidence needed to say that hiPSC-derived hepatocytes can engraft in recipient livers.

Finally, transplantation of undifferentiated or differentiated hiPSCs can be accompanied with transplantation of primary non-parenchymal cells. The idea here is that these primary non-parenchymal cells will provide support to the undifferentiated or differentiated cells. This may help increase differentiation or engraftment of the recipient animal.

One issue with the current hepatic differentiation of fetal hepatocyte-derived hiPSCs is that every full differentiation experiment resulted in poor induction of definitive endoderm. Full differentiation experiments should be repeated to ensure high quality definitive endoderm induction. If this occurs, more efficient and reproducible hepatic differentiation of fetal hepatocyte-derived hiPSCs should be obtained. This will result in differentiated cells having

higher expression of liver-specific genes and metabolism that is equivalent to primary adult cells in culture in the various metabolic assays we investigated.

Finally, there are multiple reports of hepatic differentiation protocols for both hiPSCs and hESCs. We based our differentiation on a modified protocol from Si-Tayeb and colleagues [153]. However future differentiation experiments can utilize other published protocols or other modifications to current protocols. Ideas include the use of: TRA1-60 microbeads to purify the starting cell population, various extracellular matrices to provide extracellular support during the differentiation process, viruses delivering important liver specific transcription factors (like the HNF4α AAV) or human liver non-parenchymal cell co-culture. Undifferentiated hiPSCs could also be treated with valproic acid or other histone deacetylase inhibitors to reactivate important silenced genes that are necessary for true developmental potential of the hiPSC lines. For example reactivating the 12qF1 locus increases mouse iPSC developmental potentials [162].

Finally, we have generated a disease specific hiPSC line from hepatocytes of a patient diagnosed with CN-1 syndrome. Experiments should be undertaken to see if this hiPSC line can form stem cell-derived hepatocytes *in vitro* that contain both normal hepatocyte activity as well as disease specific activity. These cells once differentiated should be able to metabolize ammonia, however should be defective in generating bilirubin glucuronides. An important control in demonstrating this disease specific behavior is differentiating a hiPSC without CN-1 and showing that when differentiated it can generate bilirubin glucuronides. Once this is established, the hiPSCs can be corrected for their genetic defect in the UGT1A1 enzyme using zinc finger nucleases. Once corrected for their defect one should be able to differentiate them back to hepatocytes that have the ability to form bilirubin glucuronides in culture. This data, once generated, would pave the way for patient specific therapies. These therapies would be

based on generating patient-specific hiPSCs, correcting them for their defect and differentiating them to hepatocytes that can be used for autologous transplant back to the patient without the need for immunosuppression or an invasive OLT to correct the metabolic defect.

Furthermore this CN-1 hiPSC line can be differentiated to the hepatic lineage and transplanted to our FRG mouse model. Animals that are highly repopulated with disease specific cells should display CN-1 symptoms, like inability to conjugate bilirubin. This mouse model of CN-1 disease would be a superior animal model to those currently available because underlying disease pathologies are due to the presence of human cells. These repopulated animals would provide the microenvironment milieu to support the tissue's physiological function within the context of the whole organism, enabling greater understanding of disease pathogenesis and providing a platform for preclinical testing of CN-1 drug candidates.

It is obvious that a multidisciplinary approach is going to be needed to differentiate these fetal hepatocyte-derived hiPSCs to hepatocyte-like cells that resemble primary adult hepatocytes in gene expression and metabolic activity. The fact that there is a bank of 40 hiPSC lines shows there is promise that one of the lines will be useful in forming a stem cell-derived hepatocyte that can be used for drug discovery or regenerative medicine purposes.

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