ISOLATION AND CHARACTERISATION OF

HUMAN FETAL LIVER PROGENITOR STEM CELLS

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NATIONAL UNIVERSITY OF SINGAPORE

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Summary

Fetal liver progenitor cells are the most natural precursors of hepatocytes and bile duct cells. They hold tremendous promise in their ability to provide a continual source of cells for cellular transplantation, bioartificial assisted liver devices, gene therapy for metabolic disorders, drug testing in pharmaceutical industries and even for studies in understanding mechanisms regulating normal liver development, specification and maturation.

We thus aimed to isolate and characterize the population of progenitor cells from fetal liver and determine their role as a stem cell for liver repair and regeneration.

Mid-trimester human fetal livers were cultured up to 3 months to allow enrichment of progenitor cells. A novel technique combining chemical selection with GS418 and mechanical harvesting was designed and successfully isolated these cells. Progenitor cells are small cells (10um) with high nuclear-cytoplasmic ratio of 46 hour doubling time. In optimized cultures on NIH 3T3 (mouse embryonal fibroblasts cells) feeder layers, clonal colonies validated by Humara X assay could be maintained up to 100 population doublings or 20 passages.

Progenitor cells were then characterized by immunofluorescence, western blot and RT-PCR. They had markers consistent with a stem cell signature including CD34, thy-1, c-kit and SSEA-4. They appeared to have a mixed epithelial-mesenchymal phenotype being positive for EPCAM, CK18, CK19 as well as vimentin and CD44. Interestingly they had yet to specify into hepatic fate as they were negative for albumin and alphafetoprotein,

and do not express HNF transcription factors specific for hepatic lineage.(HNF1α,3β and 4α).

Progenitor cells were able to differentiate to become hepatocytes and cholangioytes via mesenchymal-epithelial transition. Hepatocytes were positive for albumin, Hep-par and alpha-1-antitrypsin and showed consistent features on electron microscopy. Functional studies including albumin secretion, glycogen storage, P450 enzymes inducibility and glucose 6 phosphastase activity were also positive. Cholangiocyte differentiation was correspondingly determined with positivity for CK7, CK19, GGT and characteristic microvilli on electron microscopy. Differentiation into mesodermal lineages was also successful with lineage confirmed with at least 2 modalitites. Fat differentiation was positive for oil globules and PPARgamma RTPCR; cartilage differentiation was confirmed with identification of collagen II and mucopolysaccharides; bone differentiation was confirmed with calcium deposition and osteopontin RTPCR and endothelium differentiation confirmed with identification of VWF and CD31.

Progenitor cells were transplanted into Rag2γc double knockout mice treated with retrorsine and carbon tetracholoride. Successful engraftment, integration and function of transplanted human cells was determined with human specific albumin, Hep-Par and ZO-1 immunofluorescence, RT PCR of liver samples, ELISA of mice serum and in situ hybridization for human chromosome. Transplantation up to 9 months showed prolonged though variable survival with no evidence of fusion or aneuploidy.

These progenitor cells exist in fetal liver de novo and using lineage tracing, do not appear to have been derived from hepatocytes, MSC or HSC cells. Given their mixed phenotype,

they may have arisen from mesendoderm This is the first reported long term culture of clonal population of human fetal liver progenitor cells fulfilling stem cell properties of self-regneration, plasticity and functional offsprings both in vitro and in vivo.

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CHAPTER 1

Background:

Liver cirrhosis and end-stage liver failure constitute a major cause of mortality and morbidity worldwide. There are currently 400 million Hepatitis B carriers in the world, of which 1 million die every year from its complications.[1-2] Hepatitis B is endemic in Singapore with up to 4% of the population being carriers of the virus[3]. In the west, chronic Hepatitis C will reach epidemic proportions in the next 2 decades. When end stage liver cirrhosis develops, the only cure lies in liver transplantation. However, the scarcity of suitable organ grafts has resulted in an exponential increase in the waiting list for liver transplant worldwide. Patients are waiting longer for a transplant and more patients are dying while on the waiting list[4].

The promise of regenerative medicine has led to significant advances over the last two decades in the understanding of stem/progenitor cell and spurred interest in trying to obtain liver cells *in vitro* for therapeutic application. In fact, the liver is an organ with tremendous regenerative potential. Liver hepatocytes can repair and regenerate itself when the liver is injured or after liver resection surgery. When the injury is severe and normal hepatocytes can no longer regenerate the liver, a distinct progenitor cell compartment is activated to attempt to repair and regenerate the liver[5].

Yet hepatocytes, despite their innate regenerative potential have very limited use in this respect as they are notoriously difficult to grow in culture and they dedifferentiate rapidly, losing their hepatocytic functions. As a result, progenitor/ stem cells hold tremendous promise as they can potentially provide a continual source of cells for a

variety of therapeutic uses. These uses include cellular therapy where cells are transplanted directly into patients with liver failure[6], gene therapy for rescue of genetic or metabolic disorders[7] or employed in bioartificial liver device for liver dialysis[8], especially where liver transplant is not available or possible. In addition, large numbers of human hepatocytes are needed for toxicology studies for drug development in the pharmaceutical industry and even for studies in understanding mechanisms regulating normal liver development, specification and maturation. (Figure 1)

As a result of stem cells being the potential answer to these unmet needs, there has been significant efforts to identify and utilize stem cells that can be expanded easily in culture and subsequently manipulated to give rise to hepatocytes, either by directed differentiation (hepatocyte linage stem/ progenitor cells) or transdifferentiation (stem cells from other lineages).[9]

1.1 Definition Of A Stem Cell

Potten and Loeffler[10] describe stem cells as undifferentiated cells capable of: (i) proliferation; (ii) self-maintenance; (iii) the production of a large number of differentiated, functional progeny; (iv) regenerating the tissue after injury; and (v) flexibility in choice of these options (i.e. plasticity) (Table 1). Stem cells can be further classified into pluripotent or multipotent stem cells by virtue of how primitive they are.

Progenitor cells, a term often used interchangeably with stem cells is generally accepted to refer to descendants of stem cells.[11] Progenitor cells are still highly proliferative and can differentiate into specialized cells but they cannot self-regenerate and are less plastic compared to stem cells. Adult stem cells, particularly those

derived from specialized tissues, should strictly be called progenitor cells until they fulfill the strict definition of a true stem cell. A paradox then exist such that stem cells that are totipotent with the highest proliferative potential to scale up, have the highest risks of oncogenicity limiting their transition to clinical use. Stem cells which are committed tissue specific cells have lower tumorigenic risk but are less plastic and may have limited regenerative potential limiting their ability to scale up. (Figure 2)

1.2 Potential Liver Stem Cell candidates

The fledging field of liver stem cells has, however, been dogged by two major problems: (i) inconsistencies in the immunophenotype of liver progenitor cells; and (ii) lack of understanding of the true physiological role of variously described stem/progenitor cell populations, especially stem cells derived from extra-hepatic transdifferentiation or from protocols that require unconventional prolonged culture.

1.2.1 Hepatocytes

Interestingly, hepatocytes are the main cell that undergoes mitosis and repair of the liver in most liver injury including surgical resection of the liver[12-13]. While they are dormant in normal state with as few as one out of 2000-3000 differentiated adult hepatocytes dividing to maintain the physiological liver mass, they can undergo division up to 100x when activated[5]. In fact, they can give rise to biliary type epithelium[14] in injury. Transplantation of mouse adult hepatocytes showed that they are able to engraft into urokinase-type plasminogen activator-transgenic (uPA) mice, undergoing more than 70 replications, repopulate the recipient liver, and even be serially transplanted fulfilling one of the key characteristics of a stem cell[15]. They can also repopulate injury animal model where up to 90% of uPA-Severe Combined Immunodeficiency (SCID) mice liver can be repopulated with human liver cells

under severe selection pressure and immunosupression[16]. Their limited ability to expand in vitro however, limits their clinical usefulness as healthy liver grafts from which they can be derived are limited and any use of liver graft for cellular transplantation will further compound the problem of graft shortage.

1.2.2 Intrinsic Liver Progenitor Cells

Work in rodents by Fausto[17], Sell[18] and others showed that, in animal models of liver injury where the proliferation of the resident hepatocytes was inhibited, a progenitor population of oval cells was activated and can be identified as "oval cells" population. Models of injury that can activate and expand this population of cells utilises the principle of inhibiting replication of the resident hepatocyte and providing a regenerative stimulus through liver injury. These strategies include combinations of chemical injury, such as retrorsine[19], 2-acetylaminoflurene (AAF)[20], carbon tetrachloride[21], galactosamine[22]; dietary models such as choline-deficient diet[23], 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)[24] and/or its modification with ethionine supplementation[25] and partial hepatectomy[20].

These oval cells, with slight variations between species, are positive for albumin, alpha-fetoprotein (AFP), cytokeratins (CK) 7, 8, 18 and 19 as well as progenitor markers such as OV6 (rat), A6 (mouse) and G7[5]. These cells have been shown to be bipotential in both in vitro and in vivo setting to be able to give rise to both hepatocytes and bile duct epithelium.[26-28]. Other markers that have been reported include hematopoietic markers such as CD34, C-kit, Sca-1 and CD90 (thy-1), as well as AC-133.[29-31] Even mesenchymal markers such as CD44 and even vimentin have been described[32]. The variability of markers have called into question of issues such as interspecies variation, non-clonal cultures, culture artefacts[32-33]; or in the latter

case, true mesenchymal–epithelial transdifferentiation[34]. Newer studies on these progenitor cells have identified new surface markers that allow flow sorting of these cells to form colony forming units, a concept similar to hematopoietic stem cells.[35] Over the last few years, evidence has emerged that these oval cells can be isolated from normal adult liver without injury stimulus[36-37] lending evidence to the de novo entity of progenitor stem cells in adult liver.[38]

The equivalent progenitor cells in adult human liver have been localised to the terminal bile ductules, known as canals of Hering, near the portal triads in zone 1 of the hepatic acinus[39]. They are believed to be a dormant cell compartment and are activated when the adult hepatocytes are not able to repair and regenerate the injured liver. They are believed to play a role in liver repair in liver diseases such as alcoholic liver disease, fatty liver, viral hepatitis or fulminant hepatic failure[40-41]. They proliferate and give rise to two distinct lineages: ductular daughter cells appear as ductular hyperplasia while hepatocytic lineages show progressive differentiation along a linear pathway[42]. More recent success utilizing EPCAM and NCAM have reported the isolation of a population of cells from normal or cirrhotic liver that have bipotential differentiation capacity[43-44]. They were able to repopulate animal models of liver injury and are negative for albumin and AFP but positive for CD44, NCAM, claudin and AC133.

1.2.3 Fetal Liver

Knowledge on fetal liver progenitor cells has come mainly from work on rodent fetal livers. Hepatoblast cell lines have been derived from embryonic age ED12 rat[45] and ED9.5 mouse embryonic livers[46]. These cells are albumin, AFP, and CK19 positive,

and as expected, have bipotential differentiation capability. Various approaches have been attempted to isolate these progenitor cells by positive selection, negative panning or by culture selection. Suzuki et al[47] used fluorescent activated sort and reported that the CD45–TER119–CD49f+/lowCD29+CD117–c-met+ fraction gave rise to the highest number of colony forming units. Strick-Marchand et al[28] isolated a bipotential mouse epithelial line from fetal livers. These cells, like those from Suzuki et al[47], were able to give rise to both hepatocyte and biliary epithelium *in vivo*. Using the fetal pig model, Talbot et al were able to derive hepatocytes from 8 day epiblast[48].

Using human fetal livers, Malhi et al[49] isolated fetal liver cells that could be cryopreserved, were bipotential and could repopulate the liver of animal models of liver disease. Lazaro et al[50] was able to maintain long term cultures of primary fetal livers. In their culture, they saw a subpopulation of progenitor cells which they suggested to be fetal liver stem cells. Nowak et al[51] selected CD117+/CD34+/Lin- cells from fetal livers and showed they had similar *in vitro* and *in vivo* bipotential capability. Schmelzer et al[44] used EPCAM to sort and isolate progenitor cells and estimated that these cells constituted 2-5% of the mid-trimester fetal livers.

The potential of these rodent fetal progenitor cells can be glimpsed from repopulation models where cryopreserved fetal hepatoblasts transplanted into normal adult livers were able to achieve repopulation of up to 14%, even with no selection pressure.[52] This has tremendous implications for gene therapy for metabolic defects and end stage liver diseases.

1.2.4 The Embryonic Stem Cell

Embryonic stem cells from the inner cell mass of embryos can be coaxed into the endodermal lineages using a variety of culture technique. These cells can be in turn, differentiated into functional hepatocytes fairly efficiently with both *in vivo* and *in vitro* techniques[53-54] including variations of serial differentiation protocol of sodium butyrate (human ES cell)[55], activin, FGF2, BMP-2, HGF and oncostatin M (human ES cell)[56-57] and/or coculture with fetal thy-1 mesenchymal cells[58], human liver non-parenchymal cell lines[59] or mature hepatoctes[60].

Differentiated cells can produce AFP, transferrin and albumin[61-63]. Transplantation of these cells in murine models of liver injury showed successful engraftment[64-68] with efficiency ranging from 0.06% to 2%. Clinical improvement in the levels of albumin and bilirubin were measurable with ES cell–derived hepatocyte transfusion[61, 63-64]. In addition, survival benefits were demonstrable with reversal of acute liver failure when embryonic derived hepatocytes were incorporated in bioartificial liver devices transplanted subcutaneously into mice subjected to 90% hepatectomy[69] or administered as extracorporeal liver dialysis[60] in rats treated with galactosamine.

Despite these encouraging successes, ES cells derived hepatocytes have been reported to have limited functionality and maturation[67]. Other challenges include the ability to generate sufficient numbers of differentiated cells for transplant, the ethical concerns of using blastocyst in their derivation. More importantly, the concerns with teratoma formation remains a formidable challenge. While removal of undifferentiated ES cells (Oct3/4 positive) before transplantation significantly decreased the teratoma

formation[66], it is possible that a single residual ES cells transplanted into the body will eventually give rise to a teratoma[62, 64].

1.2.5 Induced Pluripotent Stem Cell

Induced pluripotent stem (iPS) cells are generated from reverse reprogramming of somatic cells of individuals by using defined transcription factors[70-72]. These cells are reported to be similar to ESCs and can be differentiated into hepatocyte lineage cells using similar protocols. These cells demonstrated liver cell functions including albumin secretion, glycogen synthesis, urea production and inducible cytochrome P450 activity and were comparable to that of the human ES cell-derived hepatic cells. They could also be transplanted into animal models and shown to engraft in the liver. [73-75]. The obvious advantage of these cells are that they can be derived autologously from the fibroblast of the same patient thus obviating the need for immunosuppression as well as being free from the ethical concerns of ES cells. However concerns with teratoma formation and lentivirus use still need to be overcome.

1.2.6 The Bone Marrow Hematopoietic Stem Cell (Table 3)

The promise of bone marrow stem cell as a candidate for liver regeneration was first fueled by initial reports of hepatocytes having a bone marrow origin as liver cells of female patients who had received male bone marrow transplants were found to contain XY sex chromosomes[76-77]. The first case of proof that these bone marrow stem cells can repopulate an injured liver was shown by Lagasse et al[78], using the hereditary type I tyrosinemia (FAH) model which exerts an extreme selection pressure on bone marrow cells. However, this repair process was subsequently shown

to be primarily mediated by fusion with the monocyte subpopulation and not transdifferentiation of bone marrow stem cells[79].

On the other hand, Harris et al[80] used a sophisticated cre/lox system to show that it is possible to find epithelial cells derived from bone marrow with no evidence of fusion. Since then, many studies and critical reviews[81,82-85] have pointed out that, although it is possible to transdifferentiate bone marrow stem cells to produce hepatocyte-like cells, the weight of evidence suggest that most transdifferentiation phenomena are due to fusion events. Further, this process contributes only minimally to liver regeneration and, in the absence of continued overwhelming selection pressure, is rapidly lost. Although there is still controversy over whether transdifferentiation of bone marrow cells can occur *in vivo* without fusion, depending on the model used[80, 86], it is fair to conclude that, to date, there is no conclusive evidence that hematopoietic stem cells play an important physiological or pathological role in liver injury and repair via transdifferentiation[5, 87].

1.2.7 The Umbilical Cord Stem Cell (Table 3)

Arising from the excitement that hematopoietic stem cells can become hepatocytes, umbilical cord blood became the next potential candidate. Indeed, many groups have reported successful generation of hepatocytes from the umbilical cord blood. *In vivo* liver repopulation has been shown in in CCl_4 , 2AAF and Fas ligand (Fas-L) liver injury models[88-90]. However, the true stem cell fraction, whether it is the hematopoietic, mesenchymal or a distinct somatic progenitor population in cord blood, has not been defined. Both CD34+ and CD34- populations were reported as able to give rise to "hepatocytes"[91]. While mesenchymal stem cell fraction was

specifically studied [92-94] as being the candidate liver precursor cells in some studies, others reported that fractions defined by beta2microglob+/c-met+[89], CD34+/lin-[95] and C1qRp[90] harbored the pluripotent stem cell.

Survival benefit has been claimed in rodent studies[96], but more interestingly, similar transplantation experiments in mammals such as fetal sheep[97] and goats[95], have also shown promise. Almeida-Porada et al[97] described multipotent cells derived from human cord blood and were able to create a chimeric model in preimmune fetal sheep where human hepatocytes constituted as much as 20% of the liver 11 months after transplantation. Like the bone marrow story, cell fusion has been implicated as the mechanism by which human cells are seen in the recipient's liver. Yet in studies specifically looking at cell fusion, reports have been conflicting; some observed cell fusion in most cells[98], some claim no evidence of cell fusion,[99] and yet others observed both scenarios[100].

1.2.8 The Mesenchymal Stem Cell (Table 3)

Mesenchymal stem cells derived from bone marrow or fetal liver have been shown to differentiate into liver cells in both in vitro and invivo experiments.[101-105]. Aurich et al[102] differentiated mesenchymal stem cells from human bone marrow into hepatocytes using pro-hepatocytic growth medium, and transplanted these cells into Rag2 mice. The engrafted cells continued to express human liver specific functions and showed no evidence of fusion. It is interesting that liver stem cells have been increasingly reported to carry both endodermal and mesenchymal markers[32]. A multipotent progenitor cells from the bone marrow, termed MAPCs have been

reported to by able to differentiate into hepatocytes as well as a wide variety of other cell types.[106-107]

Multiple reports have claimed that bone marrow mesenchymal cells when transplanted into animal models of liver cirrhosis, ameliorate and reverse the disease. [6, 108]. It is not known, however, whether other mechanisms may be at play that might prove useful in bone marrow mesenchymal therapy for injured liver Sakaida et al[6] reported improvement of liver cirrhosis claiming both reduction in fibrosis due to MMP-9 expression and transdifferentiation into hepatocytes[81]. Similar studies also lend support to the contention that bone marrow-derived cells, especially the mesenchymal stem cell component can enter the fibrous bands in cirrhotic liver models and aid in regression of fibrosis[109-110]. Yet other studies have found no benefit from these bone marrow mesenchymal stem cells[111] and some have even cautioned that bone marrow contributes significantly to hepatic stellate cell and myofibroblast population in the liver. If such is the case, it may also participate in and accelerate the fibrotic process.[112-113]

Arising from the promising data in animals, bone marrow or peripheral stem cells were transplanted into human patients with cirrhosis or acute liver injury. [114-117] and significant improvement in liver child pugh score or functions were reported. While there was data suggesting that there was improved liver proliferation and repair, it could not be ascertained if the effect was due to mesenchymal cells differentiating into hepatocytes or a immunomodulatory reparative effect of the trranpslanted cells on native hepatocytes. To support the latter hypothesis, there have been several publications[118-119] supporting the role of mesenchymal cells as repair assist cells and

perhaps conditioned medium from the mesenchymal stem cells may be sufficient to aid liver repair.

1.2.9 The Adipose Tissue Mesenchymal Stem Cell (Table 3)

In addition, mesenchymal lineage stem cells such as adipose tissue derived stem cells have also been coaxed to form hepatocyte-like cells[120-123].[124]. These cells appear to be similar to mesenchymal stem cell yet are easier to convert to functional hepatocytes and have better liver function. This obviously opens up the possibility of autologous transplantation as adipose cells can be obtained from the same patient with liver failure.

1.3 The Developing Human Liver

The liver bud is formed from the ventral wall of the endoderm around D20 of gestation[125]. Receiving FGF and BMP signals from the heart and septum transversum, it specifies for hepatic lineage and invaginates the septum transversum. It is believed that the endodermal cells differentiate to form hepatoblasts which are bipotential and further differentiate to form hepatocytes and bile ducts along the ductal plates[126]. Mesodermal cells from the septum transversum form the non-parenchymal fraction including endothelial cells, fibroblasts and stromal cells. In addition, hematopoietic stem cells colonize the liver and reside in the liver through most part of the gestation period, leaving for the bone marrow when the liver matures and is no longer conducive for hematopoiesis[127]. A tight interactive paracrine regulation thus exist between hepatoblast, mesenchymal cells, hematopoietic cells and extracellular matrix driving survival, proliferation and differentiation of the various lineages that constitute the liver.

1.4 Current Knowledge on Human Fetal Liver Progenitor Cells.

At the point of initiating this project and perhaps even to date, true human liver stem cells have not been isolated and their characteristics and physiological roles are hitherto, unknown.

Human fetal liver progenitor cells have several advantages over other stem cell sources. The progenitor cells in the adult liver are hypothesized to be similar to fetal progenitor cells which are remnants of the developing embryonic liver trapped in their "progenitor" niche. Although adult progenitor cells are plentiful in advanced liver disease, the accumulation of DNA mutations[128] and shortening of telomeres in these adult cells means their equivalent counterpart in the fetal liver is more attractive as the ideal stem cell candidate for cell therapy applications. In addition, fetal liver progenitor cells have committed to the liver lineage and are further downstream in the differentiation process compared to the more pluripotent ES cell. As such, they would be technically easier to differentiate into fully functional cells with lower risks of teratoma or malignant transformation[129]. Last but not least, fetal liver stem/progenitor cells are the natural putative cells that give rise to the adult liver during physiological development of the human liver. Systematic understanding of the physiological mechanisms and changes that control this physiological differentiation of fetal liver progenitor cells to differentiated cells is critical in allowing us to manipulate *in vitro* stem cell differentiation, regardless of the stem cell source.

1.5 Hypothesis:

The distinct population of putative liver stem cells in the adult liver arose during fetal development and represents a unique subpopulation in the fetal liver. Derived directly from the endodermal layer, these stem cells self-regenerate and transit into hepatoblasts before differentiating into mature hepatocytes and biliary cells. While dormant in the adult liver unless activated in injury, these cells would be actively replicating and readily identified in the normal fetal liver when the liver bud is expanding to form the organ. These cells would be more committed and would be easier to differentiate into functional hepatocytes and bile duct cells but yet retain self-regenerative potential to allow scale up *in vitro*. They would have lost their totipotency but be less likely to be oncogenic and thus be more useful in transition to clinical applications.

By identifying these cells in fetal liver and studying their characteristics, it would be possible to isolate them using sorting techniques based on their surface molecular signature. The ability to maintain these cells *in vitro* would allow expansion of these cells yielding full characterisation of their self regenerative capacity, plasticity and potential to differentiate into functional cells both *in vitro* and *in vivo*. Studying these unadulterated physiological stem cells from the fetal liver will yield important understanding of the environment cues that control stemness as well as differentiation. This knowledge can then be applied *in vitro* to other stem cell candidates or during scale–up production of *ex vivo* hepatocytes.

1.6 Overall Aims of Study

The specific aims of the study are thus:

1) To identify, isolate and purify the liver progenitor population in the human fetal liver for characterisation.
2) To expand fetal liver progenitor cell, demonstrating self proliferative potential and determine the ideal *in vitro* culture conditions.
3) To demonstrate precursor- progeny relationship – potential for differentiation into at least 2 tissue types - hepatocytes, bile duct cells as well as exploration of their plasticity.
4) To determine functional capabilities of progenies of hepatocytes and cholangiocytes from these progenitor cells.
5) To demonstrate *in vivo* functions of these progenitor cells - transplantability, durability of transplant and potential for serial transplantation.

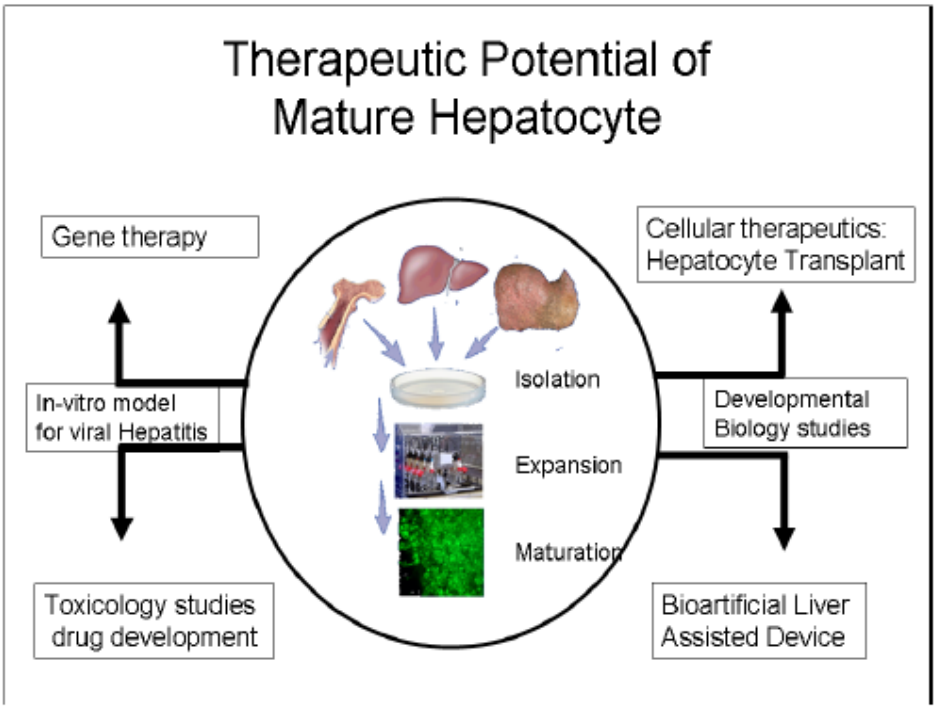


Figure 1.1 Therapeutic Potential of in vitro Hepatocytes from Stem Cells

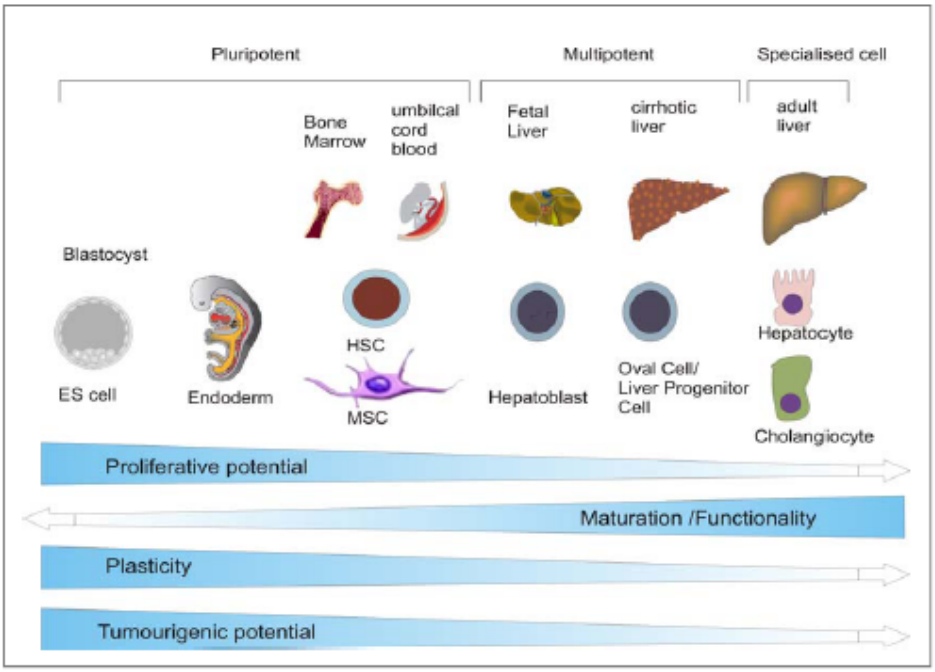


Figure 1.2. Developmental Hierarchy of Liver Cells.

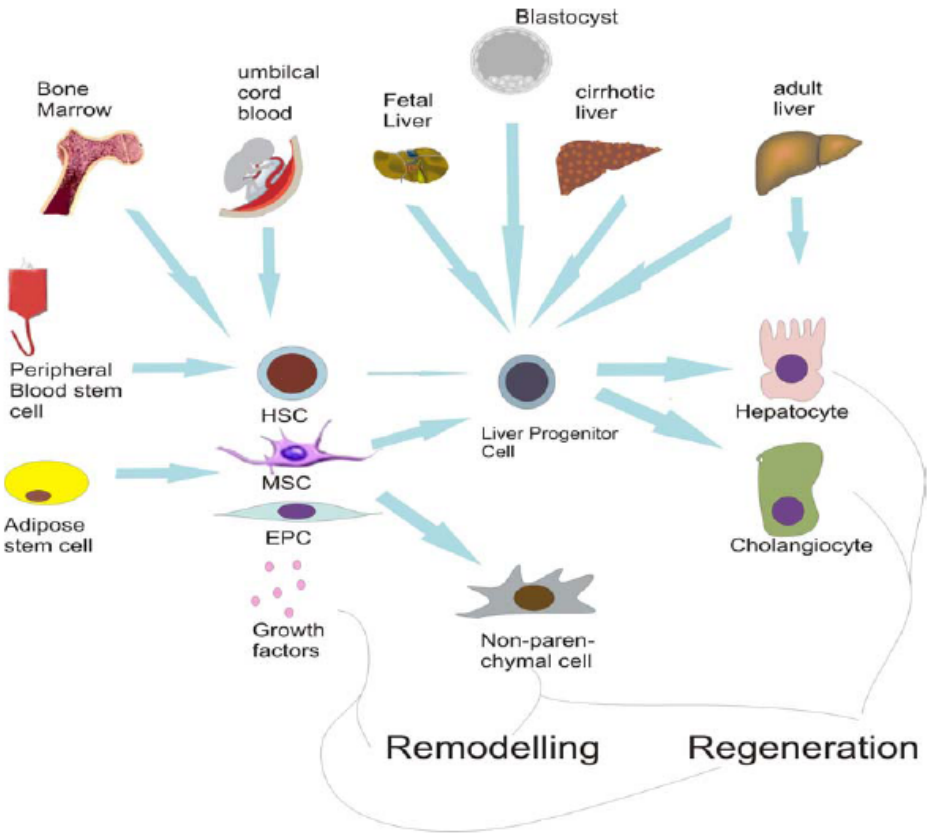


Figure 1.3: Potential Sources of Stem Cells for Clinical Therapy

Readily available without major ethical concerns
Reproducible, reliable and simple isolation technique
Definitive immunophenotypic signature, such as unique surface marker
Allows clonal derivation
Ability to expand in vitro
Can be cryopreserved with high viability after thawing
Bipotency with ability to differentiate into functional hepatocytes and cholangiocytes
Demonstrate functional integration in in vivo studies with absence of cell fusion or horizontal gene transfer
High repopulation potential in transplant studies
Demonstrable clinically relevant therapeutic effect in injury models
No risks of malignancy over time

Table 1 Characteristic of an Ideal Liver Stem Cell

Chapter 2

Isolating the Human Fetal Liver Stem Cell

Long term fetal liver cultures reported by Lazaro et al[50] have raised the possibility of a distinct cell population that became prominent after 1- 3 month of culture. These cells had high nuclear-cytoplasmic ratio and a higher replication rate than hepatoblasts and were suggestive of blast cell population.

However fetal liver stem cells have never been isolated and little is known about their immunophenotype, culture conditions and growth potential. As such, to determine that these were indeed stem cells, strategy was undertaken to allow these cells to enrich in primary fetal culture and expand to sizeable numbers. Attempts will then be made to purify these cells for characterisation and systematic exploration of ideal culture conditions.

2.1 Expansion of progenitor cell in primary hepatic cultures

We observed the growth of "blast" cells in their native state in primary hepatic cultures and maintained them up to 6 months. (Figure 2.1)

In primary cultures at density of 10^4 cells per cm2, the plate became confluent within a week with a predominant mixed population of small and large hepatocyte colonies. Interspersed in between these colonies were non-parenchymal cells such as ductal biliary cells, fibroblasts and endothelial cells. Hematopoietic cells were constantly formed from the hematopoietic stem cells in culture and despite washing and changes in medium, persisted till second week before they disappeared. Among the hepatocytic cells, clusters of small dense cells started to appear by D3 to 5 in

cultures. In the initial first 2 weeks, they became more prominent as they were proliferating faster than the hepatocytes. These cells had high nuclear cytoplasmic ration and had been shown previously to have higher BrdU staining. (Figure 2.2)

By about 3 weeks, the mesenchymal fraction started to increase and overgrow the epithelial cells forming a meshwork of multilayered cells. While some of the epithelial cells die off, a new population of small distinct high-nuclear-cytoplasmic ratio cells forms on top of the multilayered cultures and became confluent by about 6 weeks. Interestingly, a distinct increase in the viscosity of the culture supernatant is always noted to precede the expansion of these progenitor cells. Although not tested, it was believed that the viscosity was due to extracellular matrix secreted by non-parenchymal cells.

2.2 Isolation

To isolate the progenitor cells, 3 month cultures with confluent blast cells were dissociated and passaged. As these epithelial cells were extremely sticky and formed tight junctions, dissociation by breaking them up into small individual cells was extremely difficult. Of the variety of dissociation techniques employed, the ideal dissociation was the protocol of collagenase with dispase dissociation for 30 min interspersed with mechanical disruption. Adding cold trypsin increases the efficiency yield but dropped the viability of cells. (Table 2)

2.3 Purification technique

Once cells were dissociated into single or small 2-3 cell clusters, they were purified with a variety of techniques.

2.3.1 Flow cytometry

Sorting was performed separately using c-met and CD 34 antibodies and secondarily labeled with Alexa 488 and controlled with similar idiotype and propidium iodide. . Flow cytometry sorting resulted in 10^4 cells from 12 million cells and were cultured at intermediate density at 10^3 cells/cm2. Sparse intermittent cells were seen attaching at Day 3 but expanded over 2 weeks after plating. They showed a bizarre morphology with spindle shaped and flattened cells initially that reorganized to epitheloid configuration. Eventually however, fibroblast overgrew the plate. As cells had to be broken down to single cells before flow sorting, most of the epithelial 2-3 cell clumps were filtered off resulting in significant loss of cells. Majority of the single cells that were sorted did not attach and were not viable in culture. In addition, uncertainty over the changing morphology of the cells and risks of contamination from a Flow Activated Cytometry Sorting resulted in abandonment of this technique.(Figure 2.3)

2.3.2 Chemical isolation

In direct passage or subculture of enriched primary fetal hepatocyte cultures, epithelial blast cell clusters expanded seemingly from clonal origin amidst the fibroblast non-parenchymal cells. With time however, fibroblast again overgrew the cultures. Chemical selection was thus attempted by testing cultures with proline[130] and geneticin[131] to inhibit fibroblast growth. Of the methods used, geneticin had the best effect at 50ng/ml when at D5-7, fibroblasts showed apoptotic cell death leaving behind viable blast cell colonies. Persistence of geneticin or stronger dose of geneticin resulted in delayed but eventual death of blast cell colonies as well. Withdrawal of

geneticin at D7 resulted in survival of blast cell colonies but they undergo spontaneous differentiation with increase in cytoplasm and assumption of hepatocyte like morphology and immunophenotype. These differentiated cells drop in their replication rate compared to their parent "blast cells" and eventually senesce. (Figure 2.4)

It thus appeared that fibroblasts may be acting as a feeder layer allowing for clonal expansion of the progenitor cells. Geneticin was more toxic to the fibroblast than progenitor cells and thus at D7, resulted in death of the fibroblast feeders while progenitor cells were still alive and can be salvaged by removing geneticin. However, the loss of the feeder layers resulted in spontaneous differentiation of these cells.

2.3.3 Mechanical Isolation

We thus postulated that within the window period of D5-D7 when feeder cells were inhibited, progenitor cells can be mechanically isolated using cut and paste technique, a technique we adapted over from ES cell cultures. Using micro-surgical manipulation under an inverted microscope, colonies with "high quality stem cell morphologies can be cut into 4-5 cell clusters and lifted off and transferred to healthy feeder layers where they can survive and expand. Clusters of progenitor cells up to 100 cells colonies can be obtained in 1-2 weeks and can be further propagated up to more than 20 passages. (Figure 2.5)

2.4 Ideal culture condition (Table 3)

Human fetal liver progenitor cells behaved like Embryonic Stem cells. Cells at the periphery of the colony in direct contact with plastic expressed albumin and AFP and

showed spontaneous differentiation. Differentiated cells became hepatocytic like but decreased in replication rate and senesced after 2-3 weeks. To maintain them in progenitor state to maintain continued regeneration, the ideal culture conditions were systematically explored. Determination of regeneration and immunophenotype were performed at D7 using % of attachment, colony size increase, cell numbers with XTT assay, morphology assessment and immunophenotype with EPCAM and CD44.

2.4.1 Feeder layer/ Density:

Fetal liver progenitor cells spontaneously differentiated when in low density cultures with cells taking on a bizarre large morphology. Progenitor morphology and immunophenotype is maintained only in center of clusters, As a result, congruous with ES cell cultures, feeder layers were used to provide the cell-cell contact as well as support of the progenitor cell cultures. Human fetal liver fibroblast, amniotic cells and mesenchymal stem cells were tested at densities from 10^3/cm2 to 10^6/cm2. Cultured progenitor cells were observed for morphology and immunophenotyped. Irradiated or mitomycin treated NIH3T3 cells (American Type Culture Collection, Manassas, VA) at 180,000 cells/cm2 were determined to be most supportive of progenitor cultures. (Figure 2.6A, Figure 2.6C)

2.4.2 Culture medium/Supplement

Fetal liver culture medium as reported in Lazaro et al[50] was adapted for progenitor cultures by removing dexamethasone, a component believed to enhance liver differentiation. As essential growth factors were not known, 20% serum FBS which contained most growth factors was added to cultures with complex fatty acids. bFGF was added at 20ng/ml fresh daily and was found to be critical in maintaining

progenitor cell morphology. In addition, conditioned medium from 6 month fetal cultures were essential to augment replication. (Table 3)

2.4.3 Extracellular matrix

The optimal matrix that supported progenitor cell cultures was tested using laminin (5ug/ml), collagen (1:5), fibronectin (5ug/ml), gelatin (5ug/ml) and hyaluronic acid (Suplasyn, Bioniche Life Sciences Inc., London, Ontario, Canada). Without feeder layers, laminin and hyaluronic acid maintained morphology and immunophenotype of EPCAM and CD44 up to 7 days. Collagen by itself resulted in differentiation of cells with acquirement of albumin expression by D3. Fibronectin and gelatin resulted in loss of morphology and immunophenotype. Hyaluronic acid maintained immunophenotype but resulted in biliary type morphology. With feeder layers, combination using sequential layering of collagen and laminin was determined to be the optimal matrix for attachment and maintenance of fetal liver progenitor cells. (Figure 2.6B)

2.5 Interpretation

As human fetal liver progenitor cells had never been isolated and reported, an extensive systematic study was carried out to determine the ideal conditions that supported optimal cultures of these cells. To obtain enough cells for study, we maintained primary cultures of these cells allowing progenitor cells to enrich in culture naturally. The primary culture method involved a complex culture system where non-mesenchymal cells formed a natural feeder layer and loss of hepatocytes in culture simulated an injury system similar to cirrhosis condition where these cells are

activated, presumably from growth factors and extracellular matrix from non-parenchymal cells.

The challenge however with primary culture technique was that the progenitor cells, being epithelial, formed very tight junctions and was extremely difficult to dissociate for isolation. Flow cytometry and magnetic sorting were designed for hematogenous single cells and were not optimal for epithelial cells as aggressive enzymatic dissociation resulted in death of these cells, stripping of antigen and compromised further culture of these cells. Progenitor cells attachment and growth kinetics were also better in clumps rather than isolated cells, a feature consistent with human ES cells.

Subculture of these cells (passaging) allowed resolute expansion of these progenitor cells on the fibroblast feeder layers up to D9 but cultures are in turn overrun by the fibroblast and analysis was difficult. Use of chemical selection to inhibit fibroblast result in loss of feeder layer and spontaneous differentiation occur.

To overcome these limitations, we designed a novel technique of chemical-mechanical isolation. Relative inhibition of fibroblast with geneticin opened up a window period at D4-6 when progenitor cells are expanding and can be picked and transported to feeder layers and expanded. Using this technique, we were able to reliably harvest progenitor cells from >90% of our cultures, expand them for more than 20 passages and were the first to report successful isolation and culture of human fetal liver progenitor cells.

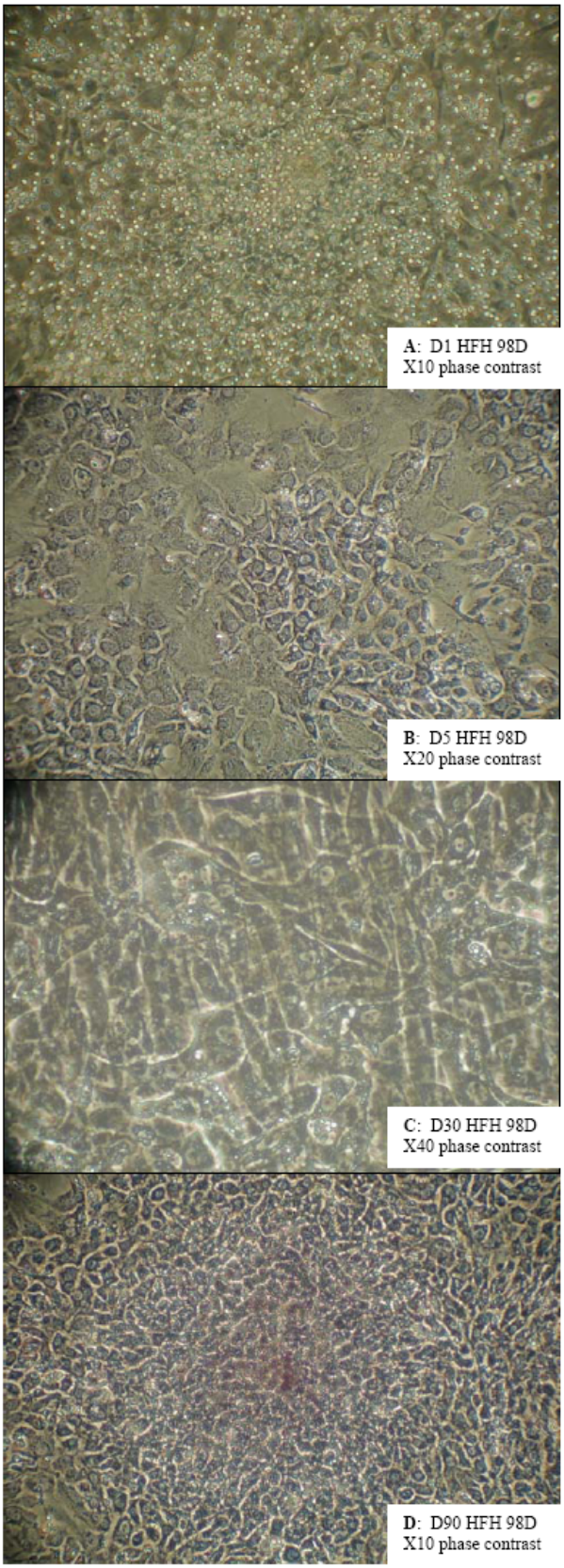


Figure 2.1. Primary Fetal Hepatocyte Culture

A: D1: Culture plate is obscured by hematopoietic cells. As these cells are non-adherent, they are washed off progressively.

B: D5. Cultures become confluent with mixture of large and small hepatocytes interspersed with non-parenchymal cells.

C: D30 Fibroblast grow over hepatocytes and form multi-layered thick culture system and acts as the feeder layer for progenitor cells to enrich.

D: D60: Progenitor cells become confluent over the cultures and are seen as uniform small blast like cells.

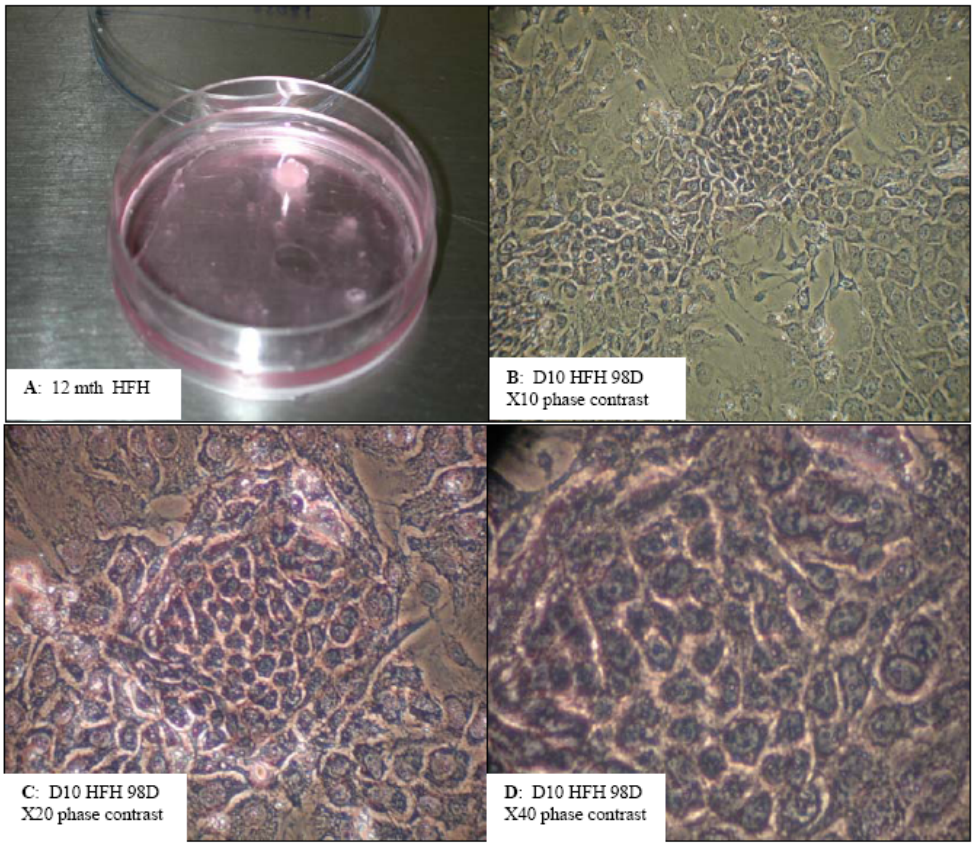


Figure 2.2 Human Fetal Progenitor Cells in Primary Hepatocyte Culture.

A: Primary hepatocyte culture up to 12 months form a multi-layered tissue complex. B. Clusters of progenitor cells can be observed from Day 3. They appear almost like a clonal colony, expanding in numbers. They have high-nuclear cytoplasmic ratio with scanty cytoplasm, have high granularity on inverse microscope and are very different from small and large heptocytes.(C,D)

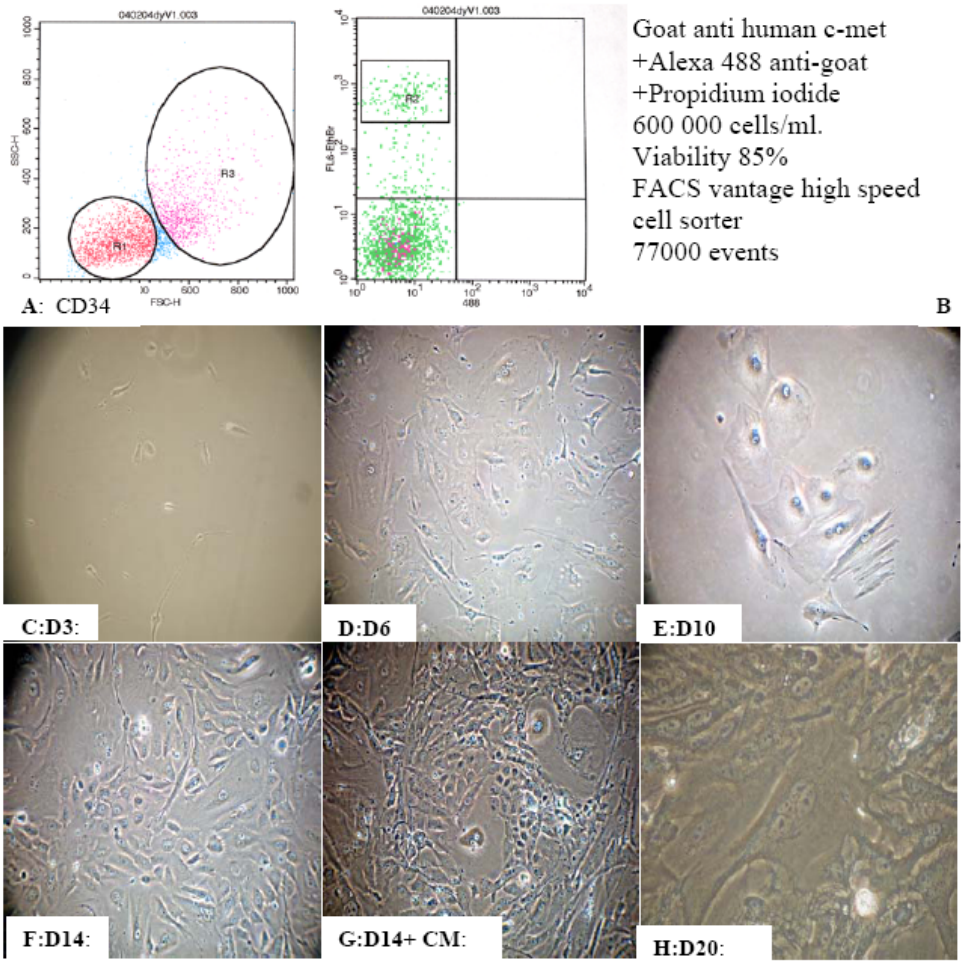


Figure 2.3 Flow Cytometry Sorting of Human Fetal Progenitor Cells .

A: 77000 cells were obtained from sorting 12 million cells. Plated at low density, very few cells were viable post sorting. Expansion of cells occurred over 2 weeks but cells were of bizarre morphology. (C-F). Addition of conditioned medium made them more epithelial in morphology (G) but by 3 weeks, cells had become irregular and were overrun by fibroblasts (H).

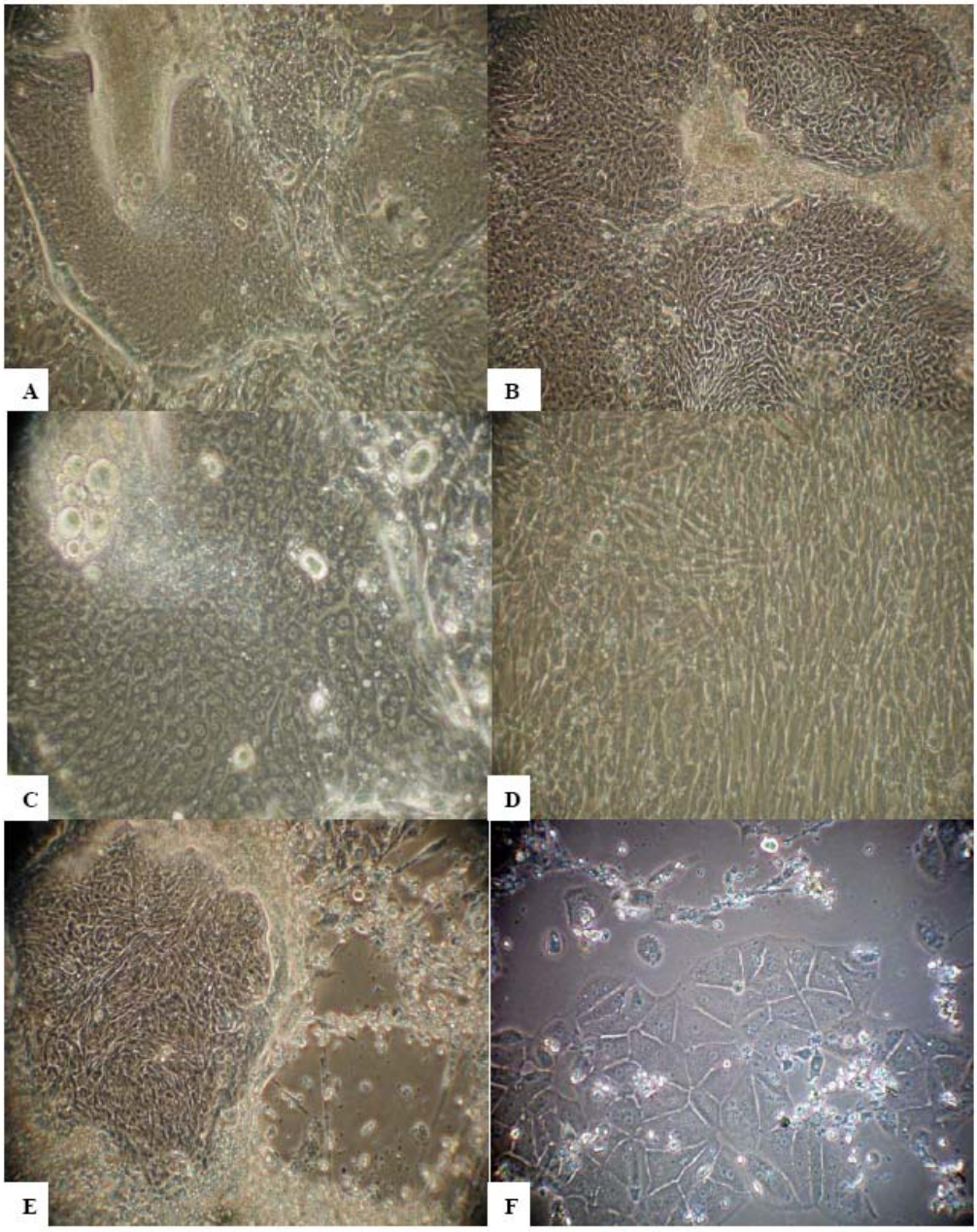


Figure 2.4 Passage of Fetal Liver Progenitor Cells and Chemical Isolation.

A-C: P2 Passage cultures showed attachment and proliferation of small blast cell colonies on underlying fibroblasts. D: Continuation of P2 cultures result in fibroblast overgrowth and loss of progenitor cells. E: Chemical selection with geneticin 50ng/ml selectively inhibits the fibroblast with death of fibroblast at D5– D7. F: Persistence of culture without feeders result in spontaneous differentiation and eventual death.

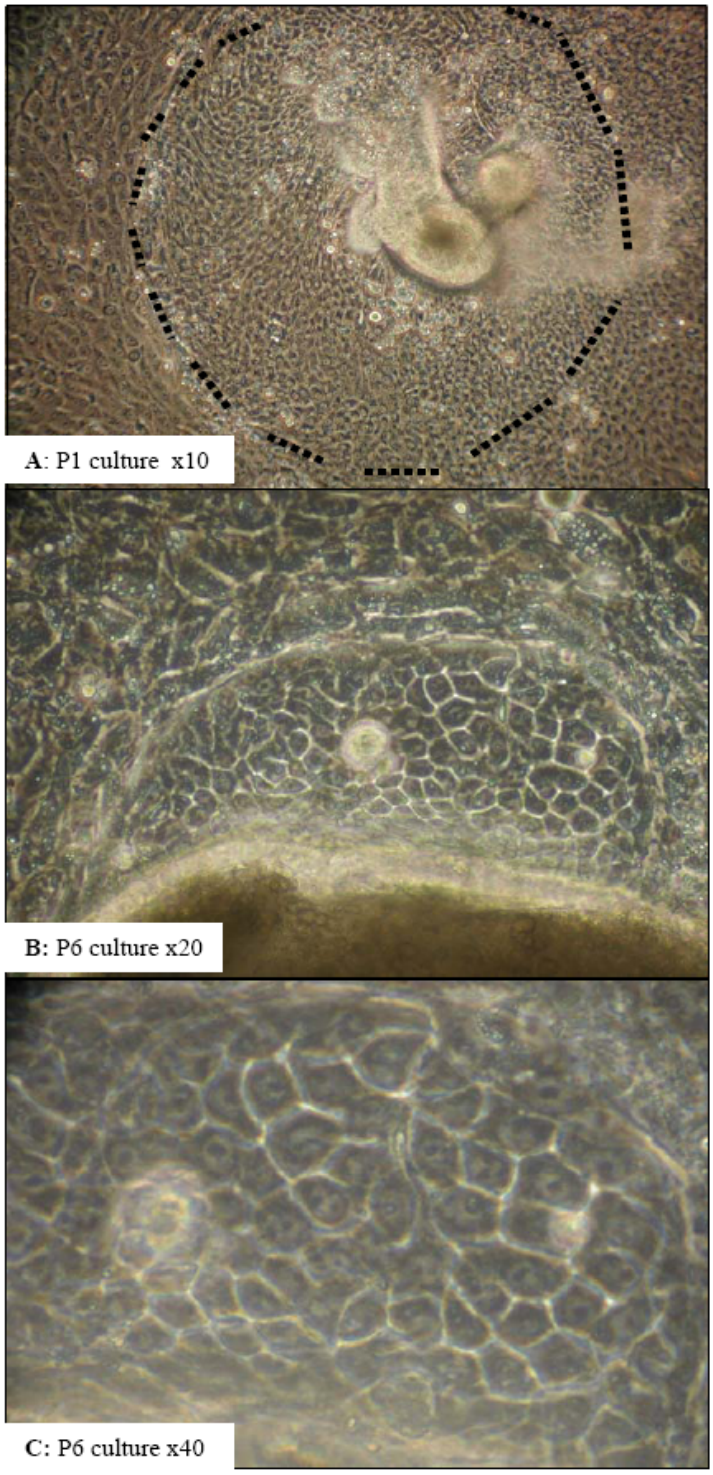


Figure 2.5. Mechanical Isolation of Progenitor cells

Progenitor cell cluster are picked off and transferred onto replication arrested feeders. Before feeder layers degenerated, progenitor cells are mechanically dissected using micromanipulation under microscope and passaged onto new feeder plates.

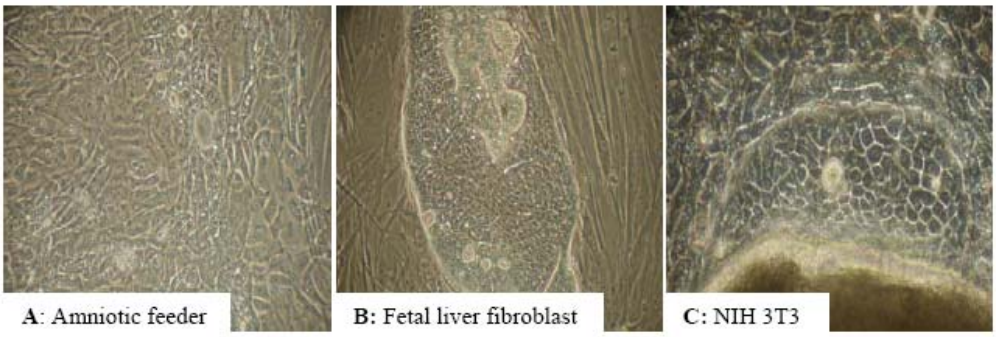


Figure 2.6A. Ideal Culture Condition: Feeder layer
NIH 3T3 feeder are most ideal for maintaining morphology and regeneration

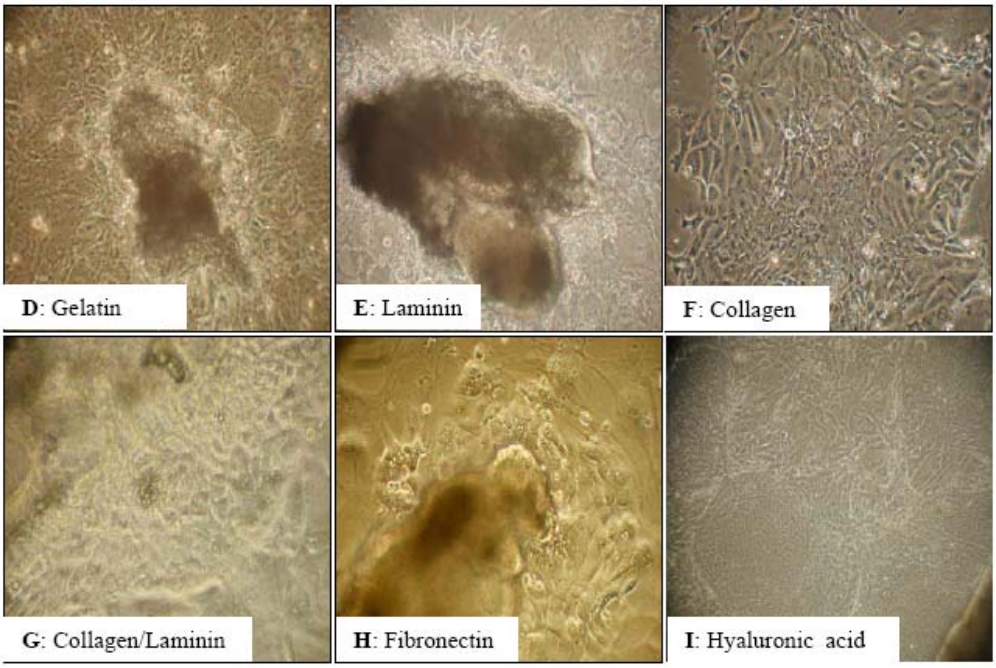


Figure 2.6B. Ideal Culture Condition: Extracellular Matrix
Collagen and laminin are most ideal for maintaining morphology and regeneration

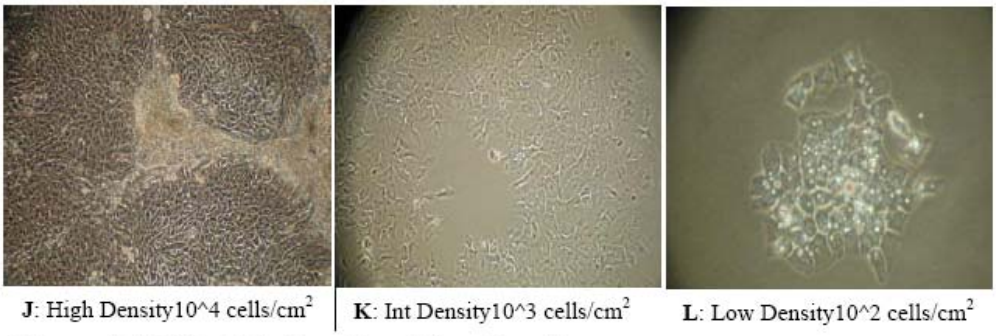


Figure 2.6C. Ideal Culture Condition: Density
High density at >10^3 cells/cm2 is needed to prevent spontaneous differentiation

Method	Duration	Efficiency/ Viability
Trypsin	10 min- 20 min	+/ +
Collagenase/ Dispase	10 min – 40 min	++ / ++
Trypsin/EDTA / collagenase	2/20 – 10/ 30	++ / +
Cold trypsin/ EDTA	4h-12h/ 30 min	+/+
Cold trypsin/ EDTA/ collagenase/dispase/	30 min/ 10mins	+++/ ++
collagenase/dispase + mechanical disruption	30 min	+++/ +++

Table 2: Comparing Different Dissociation Techniques And Their Efficiency In Breaking Up Cultures Into Single Cell And The Impact On The Viability Of Cells.

	Attachment	Regeneration	Morphology	EPCAM/ CD44	Albumin/ CK7
Collagen	+++	++	Large cells	+/-	+++/-
Laminin	++	+++	Maintained	++/++	-/-
Fibronectin	++	+	Lost	-/++	+/-
Gelatin	++	-	Lost	-/+	+/-
HA	+	++	Biliary	++/++	-/+
Collagen/Lam	+++	+++	Maintained	++/++	-/-
Matrigel	++	+++	Large cells	++/++	+/-
Amniotic Cells	++	+	Maintained	++/++	-/-
NIH3T3	++	++	Maintained	++/++	-/-
Fetal Liver fibroblast	++	++	Large cells	+/-	+++/-
EGF		++	Maintained	++/++	-/-
FGF		++	Maintained	++/++	-/-
Dexa		+	Large cells	+/-	+++/-
Oncostatin		-	Large cells	+/-	+++/-

Table 3 Characterisation Of Ideal Culture Conditions For Human Fetal Liver Progenitor Cells.

Extracellular Matrix	Collagen (1:4 dilution coating)/ Laminin 5ug/cm2
Feeder Layer	NIH 3T3 (Irradiated or mitomycin treated) 10^3/cm2
Medium	50% Fetal progenitor Culture medium
	William's E medium
	High Glucose
	Glutamine
	Sodium pyruvate
	Sodium bicarbonate
	Ascorbic acid
	Insulin-transferrrin and selenium + linoleic acid
	Complex fatty acid
	EGF 20ng/ml
	bFGF 20ng/ml
	30% Conditioned medium from 6 month primary fetal hepatocyte culture
	20% Fetal bovine serum

Table 4 Final Culture Conditions For Human Fetal Liver Progenitor Cells

CHAPTER 3

Characterisation of the Human Fetal Liver Stem Cell

3.1 Morphology of Fetal Liver Progenitor Cells

Human fetal liver progenitor cells are small cells at 10um compared to hepatocytes which were 60-100um in diameter. They grow in tight clusters forming a heaped up 3-dimensinal clump of cells rather than a monolayer, a feature of human stem cells. They have a large nucleus with scanty cytoplasm exhibiting high nuclear-cytoplasmic ratio. Electron microscopy confirmed the scanty cytoplasm and the lack of organelles such as mitochondria and endoplasmic reticulum typical of hepatocytes. In addition, no bile canaliculi are noted in these cell clusters. (Figure 3.1)

3.2 Self-regeneration

To determine cell regeneration, cells were maintained in culture and passaged using the technique described. They could be maintained for up to 20 passages for more than 6 months. BrdU labeling and Ki67 showed an increase in proliferation rate of these cells compared to the hepatoblast or non-parenchymal cells in fetal liver. To measure the growth curve of these cells, we used 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) assay which used calorimetric quantitation of cell metabolism to determine the cell numbers in culture. As the progenitor cells were grown on irradiated feeder layers, subtraction of activity from the feeder layers was performed by using a control plate with same number of feeder layers but no progenitor cells and followed through in parallel. From the assay, the doubling time of these cells was estimated to be 46 hours, which is typical of human stem cells.

Self regenerative capacity was determined by comparing cells from Passage 0 (source cells), Passage 5 and subsequently Passage 10 cells. To determine that regeneration resulted in the same cells with subsequent passages, cells were immunophenotyped for EPCAM, CD44 and albumin and confirmed that continued culture of these cells did not lose their immunophenotype. Similarly, cells from different passages maintained the same plasticity which is described later in detail. (Figure 3.2)

3.3 Are progenitor cells clonally derived?

Using the technique described, each cluster of progenitor cells appeared to arise from a single cell. Further isolation was performed with serial dilution to isolate single cells on feeder layer cultures. Following the cultures serially, colonies from a single cell were obtained and subjected to clonality validation using the Humara assay[132] on female specimens. This assay capitalizes on the random lyonisation of one of the X chromosomes in females. In females with heterozygous Humara X allele, lysing the DNA at specific site with HhaI on the unmethylated X chromosome will reveal 2 different length fragments of the allele with nested RT PCR. A colony of cells, by random chance, would express both fragment length. A single cell and its clonal progeny would however have only 1 fragment length as the same X chromosome is lyonised. We raised 3 progenitor cell colonies from a single heterozygous female specimen (no. 18849). Using an inactivation ratio (density ratio of least-intense band to more-intense band, before compared with after digestion) of <0.4[41], all three colonies derived showed nonrandom skewed inactivation of the unmethylated Humara X allele, confirming that colonies derived using this technique were likely to be clonal in origin. (Figure 3.3)

3.4 Determination Of Telomere Length

To test the self-regenerative extent of these cells, we tested the telomere length of colonies from P0,P5 and P10 colonies. In differentiated human cells, as telomerase is inactive, telomere length became progressively shorter with repeated divisions. On the other hand, progenitor stem cells express telomerase and are able to maintain the telomere length. Southern blot analysis for the telomere length was performed using a commercial kit. Colonies of progenitor cells that had been passaged up to 20x and maintained over 6 months showed that the telomere length was maintained across 100 population doublings and the telomere length was comparable to that of a positive control stem cell population. (Figure 3.3)

3.4 Stem Cell characteristics

To explore the stem cell characteristics in these cells, clusters of cells were cryopreserved either directly or with commercial scaffold. Sections were then cut and immunofluorescence or immunohistochemistry performed, with confirmation by either RT-PCR (when confirming negativity) or immunoblotting (when confirming positivity). Fetal liver progenitor cells were positive for stem cell markers CD34, Thy-1, SSEA-4 and c-kit. Oct-4 staining was equivocal and was classified as negative for stringency. These markers are consistent with a stem-cell signature[133] and have been reported in mouse liver progenitor cells. These cells appear to be committed to endodermal lineage and express a variety of epithelial markers including CK18, CK19, EPCAM and E-cadherin. Yet surprisingly, they were negative for albumin and alphafetoprotein which was contradictory to reported liver stem cell markers known at that time. (Figure 3.4)

To determine that these were not non-parenchymal cells, cells were stained for CD44h (a mesenchymal marker then), vimentin and anti smooth muscle actin. Progenitor cells are strongly positive for CD44 and vimentin but negative for anti-smooth muscle actin, CD105, CD73, CD31 and Sca-1.

To determine that they were indeed committed liver progenitor cells, we performed RT-PCR on HNF 1alpha, 1 beta, 3beta and 4alpha. HNF 1alpha, 3beta and 4alpha are the earliest transcripts in liver cell specification. To our surprise, these transcripts were absent in our cells. (Figure 3.5)

3.5 Interpretation

The isolated fetal liver cells we have isolated are typical of human progenitor cells. They are small with high nuclear-cytoplasmic ratio. Like human stem cells, their half life is moderate about 46 hours although they regenerate much faster than mature hepatocytes. They self-regenerate and are maintained up to 20 passages over 6 months, in which they undergo more than 100 population doublings with maintenance of immunophenotype, morphology, differentiation potential and telomere length. They have a stem cell signature that is similar to what has been reported in murine liver oval cells. Yet unlike murine oval cells or even the surrounding hepatoblast or the more differentiated hepatocytes, they do not appear to have entered liver specification yet, testing negative for albumin, AFP and are negative even for the earliest transcription factors that specify the liver lineage. Instead, they carry a mixed epithelial-mesenchymal marker set that has previously not been described before. The human fetal liver progenitor cell isolated thus represents a distinct population of self-regenerating progenitor cells. (Table 5)

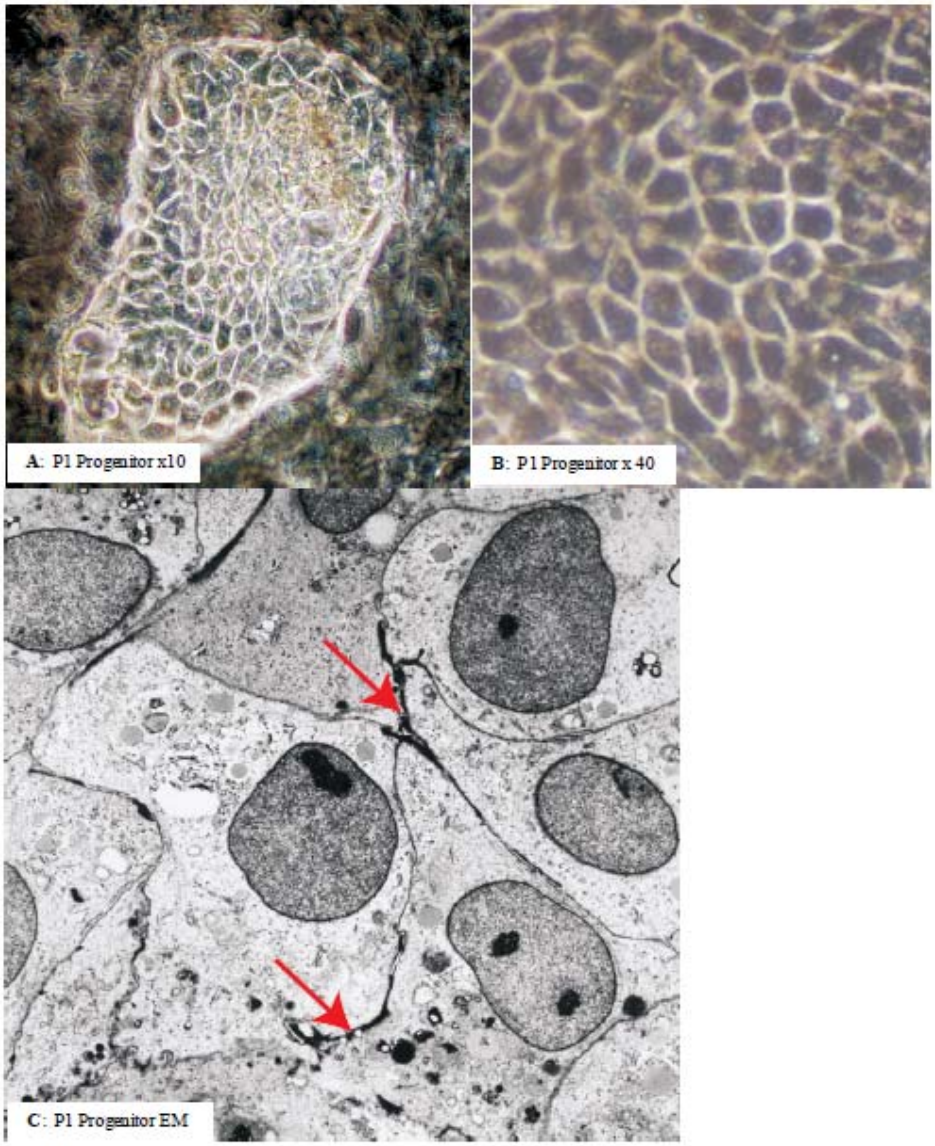


Figure 3.1 Morphology of Human Fetal Liver Progenitor cells

Human liver progenitor cells are small cells with a diameter of 10um. They grow in 3 -dimensional clusters rather than monolayers, forming tight junctions (red arrows) between each other. On electron microscopy, they show high nuclear-cytoplasmic ratio with scanty cytoplasm, lack of organelles and canaliculus between cells.

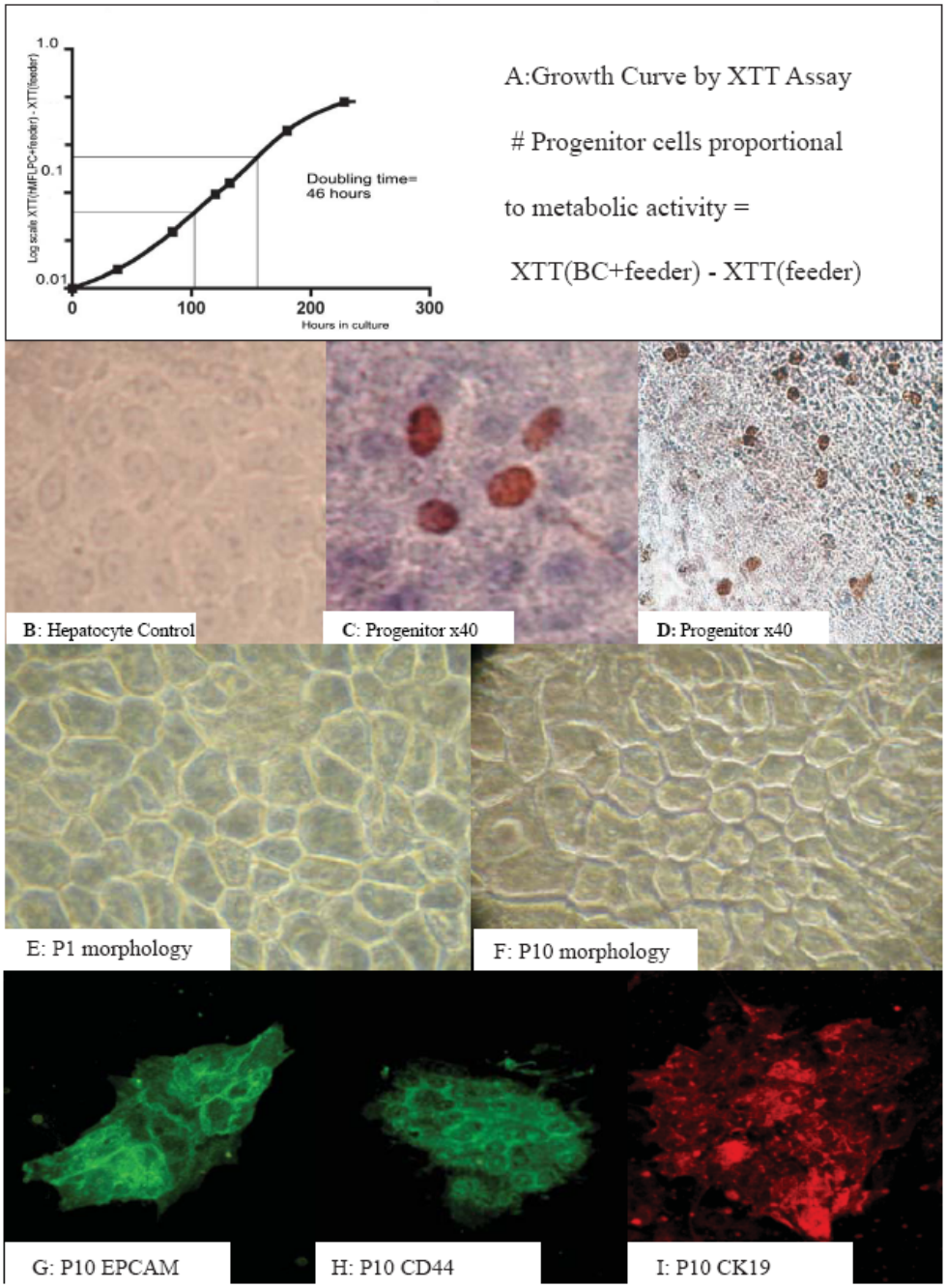
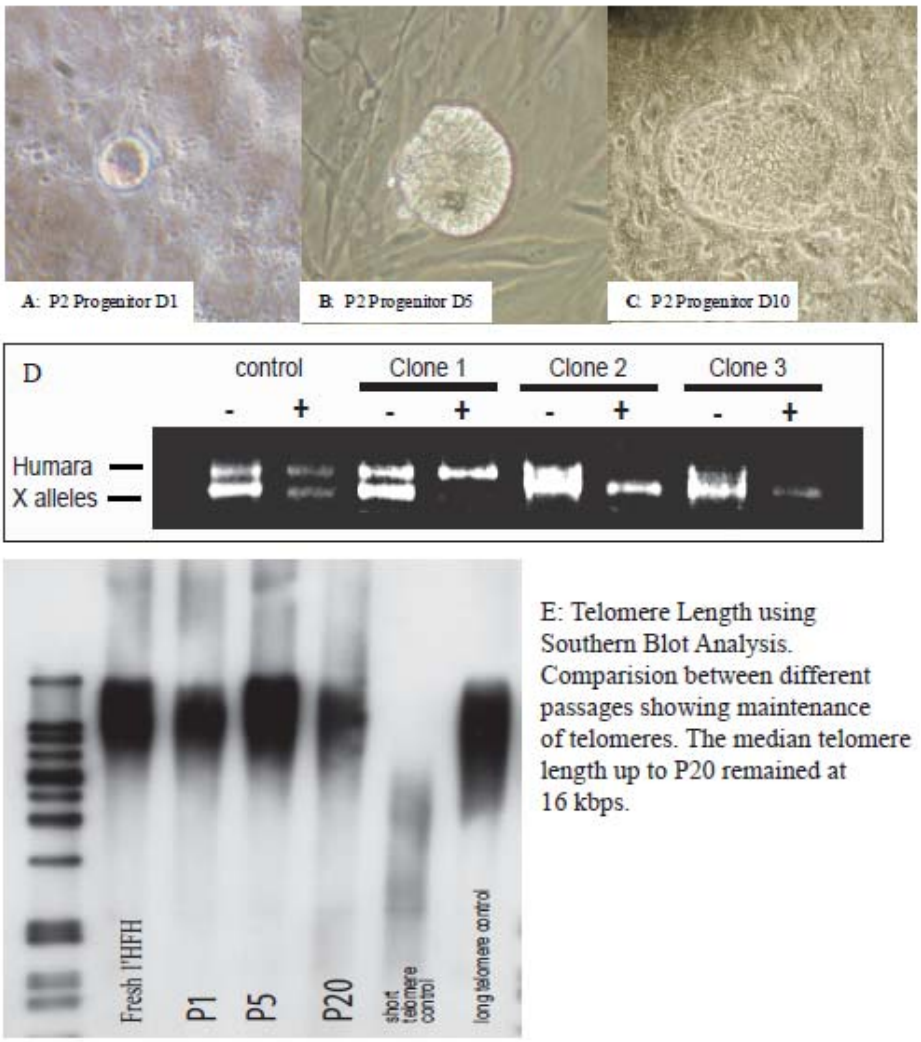


Figure 3.2 Self -Regenerative Potential of Fetal liver Progenitor cells.

A: Growth curve shows a doubling time of 46 hours. B-D. Progenitor cells have higher proliferative capacity compared to adult hepatocytes. They have similar morphology (E-F) between P1and P10 colonies and maintain their immunophenotype staining positive for EPCAM, CD44 and CK19 even after 10 passages.



E: Telomere Length using Southern Blot Analysis. Comparision between different passages showing maintenance of telomeres. The median telomere length up to P20 remained at 16 kbps.

Figure 3.3 Clonality and Telomere Length of Fetal liver Progenitor Cells

(A-C) Derivation of clonal colonies of fetal progenitor cells using serial dilution of progenitor cells culture. A colony is seen forming from a single cell. (D): Clonality of colonies was performed using Humara nested RTPCR assay on Humara X allele. Digestion with Hha1 in heterozygous female will yield 2 bands (control) unless cells were clonal and the same X chromosome is non-randomly lyonised. (E)Telomere length from colonies of different passages were assayed using southern blot and showed maintenance of telomere length across 20 passages.

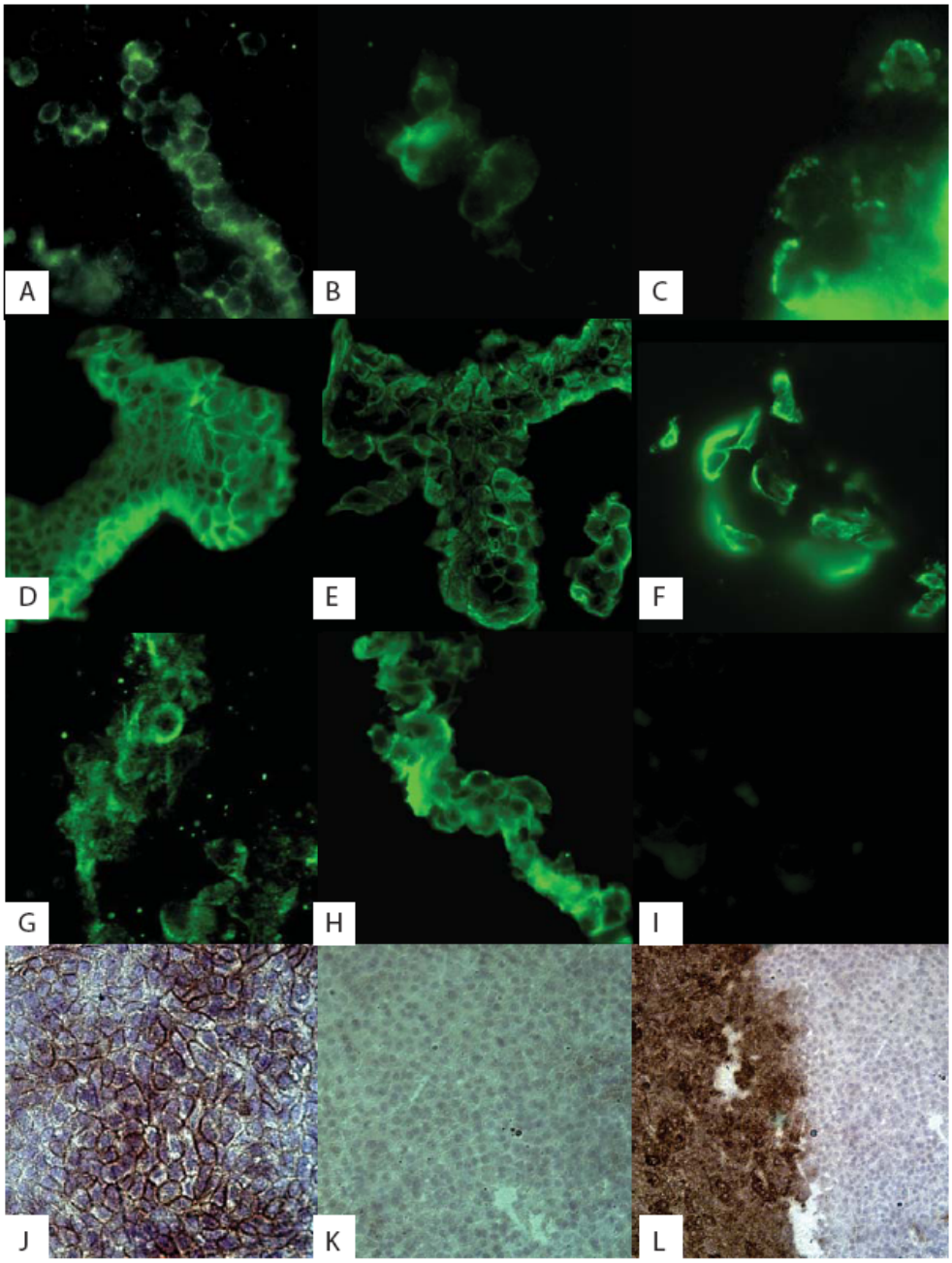


Figure 3.4 Immunophenotype of Human Fetal liver Progenitor Cells

Progenitor cells are positive for stem cell signatures such as CD34 (A), Thy-1 /CD90 (B), SSEA-4 (C) and epithelial markers EPCAM (D), CK18 (E) and CK 19 (F). They are also positive for CD44 (G), c-kit (H) and c-met (J) but negative for CD45 (I), AFP (K) and albumin (L).

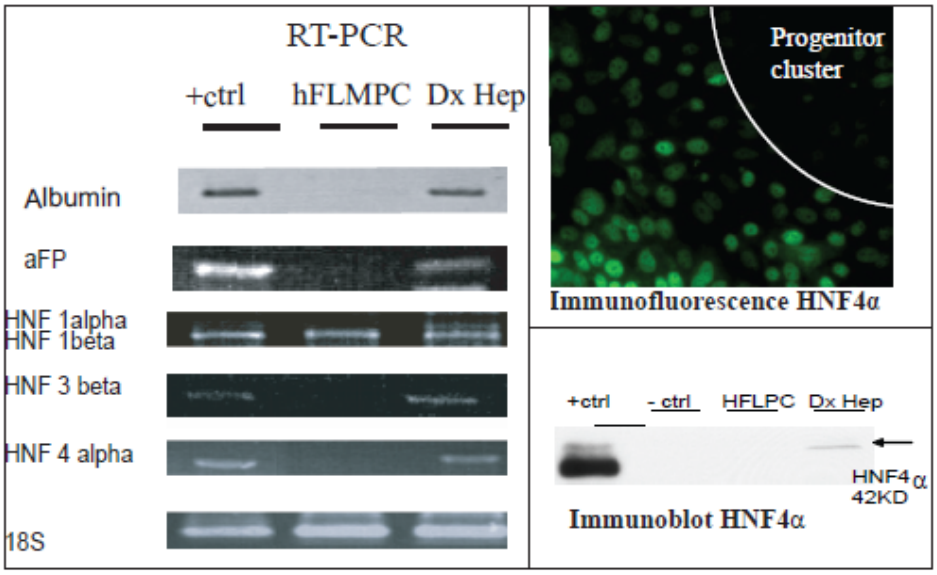


Figure 3.5. Progenitor Cells have not specified to Hepatocytic Lineage

Human fetal liver progenitor cells are albumin and alphafetoprotein negative on immunophenotyping and are confirmed by negativity of mRNA by RT-PCR. They are also absent for HNF1apha, HNF 3beta and HNF4alpha, the 3 key transcription factors that specify for hepatocytic lineage. Immunofluorescence of HNF4alpha (hepatocytic endodermal marker) is negative in the progenitor cluster but positive in the surrounding differentiated cell. This suggest that fetal liver progenitor cells have yet to specify into a liver lineage.

	Fetal liver progenitor cells	Fetal liver hepatoblast/ hepatocyte	Fetal liver Non-parenchymal	Adult Liver
CD34	+	-	Endothelial Hematopoietic	Endothelial
CD 90 (Thy-1)	+	-	++	-
SSEA-4	+	-	-	-
Oct-/4	-	-	-	-
CD 117 c-kit	+	-	Hematopoietic	-
CD 326 EPCAM	++ (membrane)	+ cytoplasmic	-	-
CD44h	++	-	++	mesenchymal
Ck19	++	-	Biliary cells	Biliary
HNF 4alpha	-	++	-	++
E cadherin	++	++	-	++
CK18	++	++	-	++
Albumin	-	+	-	++
CK7	-	-	Biliary	Biliary

Table 5. Markers of Progenitor Cells Compared to other Cells in Fetal Liver

CHAPTER 4

Proving progenitor-progeny relationship

One of the main criteria of a stem cell is that it must be able to give rise to at least 2 different functional cell types through differentiation[10]. In the liver, the 2 readily differentiated specialized cell types are the hepatocyte and bile duct cells. The challenge is that while the liver performs many functions, few of these functions are exclusive to the liver and to prove successful and meaningful differentiation, one would require exhaustive evidence that progeny cells can perform the myriad of functions of mature hepatocyte or bile duct cells.

4.1 Hepatocyte Differentiation

4.1.1 Can liver progenitor cells differentiate into hepatocytes?

To induce differentiation, progenitor cell clumps were transferred onto collagen plates at low density. Hepatocyte differentiating medium based on William's E medium was used with withdrawal of FGF, conditioned medium and complex fatty acids. Dexamethasone 10% as well as oncostatin M^{50} was then added to cultures. Cells migrated into monolayer configuration and became much bigger in size by D3. At D8, they took on a polygonal morphology and are indistinguishable from adult hepatocytes by D15. Similar to adult hepatocytes in culture, these differentiated cells stopped proliferating, became irregularly shaped, developed vacuoles and were lost after 3 weeks. This phenomenon is precisely similar to adult hepatocytes and underlines the current limitation of being unable to maintain differentiated hepatocytes in culture. (Figure 4.1)

As fetal liver progenitor cells differentiated to hepatocytes, they started to express albumin at D3 and by D21, most of the cells were strongly positive for albumin. At the same time, AFP which was negative in progenitor cells, became transiently positive at D3-D5 but disappeared as full maturation occurred, recapitulating what happens in normal liver development. These trends were corroborated by immunoblotting and RT-PCR (Figure 4.2)

On re-assessing the transcripts that specified for hepatocytic lineage, HNF1beta, 3beta and 4alpha, which were not detectable in the progenitor state are now positive and indicate cell fate commitment into hepatocytic lineage. (Figure 4.2)

4.1.2 Mesenchymal-Epithelial transition during hepatocytic differentiation

To determine whether epithelial and mesenchymal markers are co expressed in the same cells and what happened to the mesenchymal markers as these progenitor cells transit into hepatocytes, we co-labeled albumin and vimentin with different colour probes and tracked their expression during D0-D12 of hepatocytic differentiation. As reported, progenitor cells were negative for albumin but positive for vimentin. By D3, progenitor cells were positive for both albumin and vimentin with co-localisation of both antigens in the same cell. Upon further differentiation, vimentin and CD44 were down regulated and were lost completely when albumin became fully expressed. This phenotypic change is characteristic of mesenchymal–epithelial transition and suggests that fetal liver progenitor cells represent transitional cells between mesenchymal and endodermal lineages. (Figure 4.3)

4.1.3 Functional Analysis of differentiated Hepatocytes.

We tested progenitor cells, partially differentiated (D6) and fully differentiated cells D21) extensively for hepatocytic functions.

4.1.3.1 Protein Function

Hepatocytes are known to express albumin and although many cells may be coaxed into turning on the albumin gene expression with promoters, secretion of albumin protein is one of the key functions of mature hepatocytes. Albumin concentration in culture supernatant was assayed using quantitative ELISA and showed a accumulative secretion of albumin from 180 ng/ml at D5 and 1.2g/ml at D15. (Figure 4.4)

Differentiated cells also progressively expressed alpha-1 anti-trypsin with differentiation. This was demonstrated in both immunofluorescence and immunoblotting. (Figure 4.4)

4.1.3.2 Glycogen Storage

Hepatocytes are able to convert glucose into glycogen stores and this was detectable in differentiated hepatocytes using Periodic Acid Schiff (PAS) stain. To confirm the specificity of glycogen, the purple colouration is lost after digestion of glycogen by diastase. (Figure 4.5)

4.1.3.3 Liver specific cytochrome P450

Hepatocytes play an important role in drug metabolism and excretion. Most of the drug and toxins metabolism is via the cytochrome P450 pathways. While none of the P450 enzymes are specific for the liver, the inducibility of these P450 enzymes such

as the ubiquitous 3A4 and more specific 2B6 is a specific feature of hepatocytes. Instead of demonstrating upregulation of CYP mRNA, we tested the protein expression of 3A4 as well as the functional effects of 2B6 on pentoxyresorufin-O-dependentylase (PROD) before and after induction with phenobarbital to demonstrate functional inducibility. Induction of differentiated cells increased Cytochrome 3A4 proteins by 1.6 x using semi-quantitative density assay of immunoblot while calorimetric PROD assay showed up-regulation of 2B6 activity by 1.4x. (Figure 4.5)

4.1.3.4 Electron microscopy

While single genes and proteins may be turned on in cells by molecular manipulation, only hepatocytes form anatomical bile canaliculus with neighboring cells. These bile canaliculus are conduit systems to carry bile and bile salts from the hepatocytes as they organize into hepatic cords. With hepatocyte differentiation protocol, electron microscopy showed that progenitor cells show an increase in size and reduction of nuclear-cytoplasmic ratio due to relative increase in cytoplasm. Endoplasmic reticulum, mitochondria and vesicles increase in numbers. Electron microscopy also showed definite characteristic presence of bile canaliculus between neighbouring cells, a feature not present in progenitor cells. (Figure 4.6-4.7)

4.1.3.5 Infectibility with Hepatitis B Virus

Hepatitis B virus has almost exclusive specificity for human hepatocytes and infectibility of cells is tantamount to a test of hepatocyte phenotype. To test this feature, differentiated hepatocytes were cultured with Hepatitis B virus isolated from serum of patients with high titres of chronic hepatitis B. Differentiated hepatocytes were washed extensively and supernatant and cells collected over time. New batches

of hepatocytes were exposed sequentially to determine durability of infection and true viral replication in these cells. HBsAg using ELISA as well as HBV DNA using quantitative RT PCR) was positive up to 16 days in culture. Subsequent new batches of hepatocytes exposed to supernatant of these cells showed infection with positivity and successive increase in HBsAg and HBV DNA titres. HBc Ag was detectable by immunofluorescence in nucleus as well as cytoplasm of infected cells indicating entry into nucleus and viral replication within the cytoplasm. To further confirm true infection, integration and replication of Hepatitis B virus in the cell, cccDNA, a marker of viral integration, was detected in cell lysates of infected cells using RT PCR. Electron microscopy also showed corroborative presence of Hepatitis B viral particles in endosomes of hepatocytes differentiated from progenitor cells. (Figure 4.8-4.9)

The potential of having such an *in vitro* culture system has significant implications in studying Hepatitis B virus pathogenesis as well as in development of new drugs for Hepatitis B. As demonstration of principle of proof of this potential application, differentiated hepatocytes were infected together with antivirals such as lamivudine or adefovir. As expected, antivirals blunted HBV DNA production into the supernatant in keeping with suppression of Hepatitis B replication. (Figure 4.9)

4.2 Bile Duct Differentiation - Can Fetal liver Progenitor Cells form bile ducts?

Progenitor/ Stem cells are precursor cells that are able to give rise to at least two different specialized cell types in the tissue/organ structure. To prove that the cells derived from the fetal liver were indeed liver progenitor cells, *in vitro* differentiation of progenitor cells into biliary cholangiocytes were undertaken. Liver progenitor cells

were cultured in bilayered 3D collagen gels to provide polarity signals to these cells. Differentiation medium consisted of Williams E, insulin plus dexamethasone, conditioned medium from fetal hepatocyte culture 50% and pulse bFGF 40ng/ml. Unlike previously reported protocols that did not work in our hands, pulsed basic Fibroblast Growth Factor (FGF) was used, adopting the embryological developmental principle of providing a FGF gradient for inducing directional branching and tube formation.

4.2.1 Morphology

Progenitor cells in cholangiocyte differentiation culture took on a very different morphology. Cords of cells streaked out linearly and reorganized into cystic and tubular structures. By D7, the cells were arranged in 2 forms: 3-dimenstional cyst like structures were seen while in other areas, branching tubes were seen with canalization in the middle. The internal lining of these cyst-like structures became columnar in orientation and electron microscopy confirmed the presence of microvilli in the luminal pole, a key feature of bile duct cells. (Figure 4.10-4.11)

4.2.2 Immunophenotype

Immunostaining with CK19 and CK7, markers of bile duct epithelium, were performed on D7 differentiated cells. CK19 are positive in progenitor cells but are lost in differentiated hepatocytes. In these cells, they continue to be positive and at the same time, cells started to express CK7 indicating a mature biliary phenotype. (Figure 4.12)

4.2.3 Gamma glutamyl transferase. γGT/GGT

Gamma glumayl transferase is involved in the transfer of amino acids across the cellular membrane. It is present in all cells but is particularly concentrated in hepatocytes, bile duct cells and kidney. GGT was assayed using Rutenberg's histochemical staining[134] and showed that while human fetal liver progenitor cells were weakly positive for GGT, the intensity of GGT was greatly enhanced in the branching tubes obtained in bile duct differentiation cultures. (Figure 4.12)

4.3 Interpretation

By changing environmental cues, we were able to differentiate progenitor cells into two separate progenies of functional hepatocytes and biliary cholangiocytes. These differentiated progenies showed consistent morphology, tissue organization as well as molecular immunophenotype consistent with their differentiated cell type. In addition, we performed extensive functional analysis to confirm that these cells *in vitro* were functional differentiated cells. The human fetal liver progenitor cells have fulfilled the 2 main criteria for stem cells: Self regeneration and ability to differentiate into at least 2 functional progeny cells.

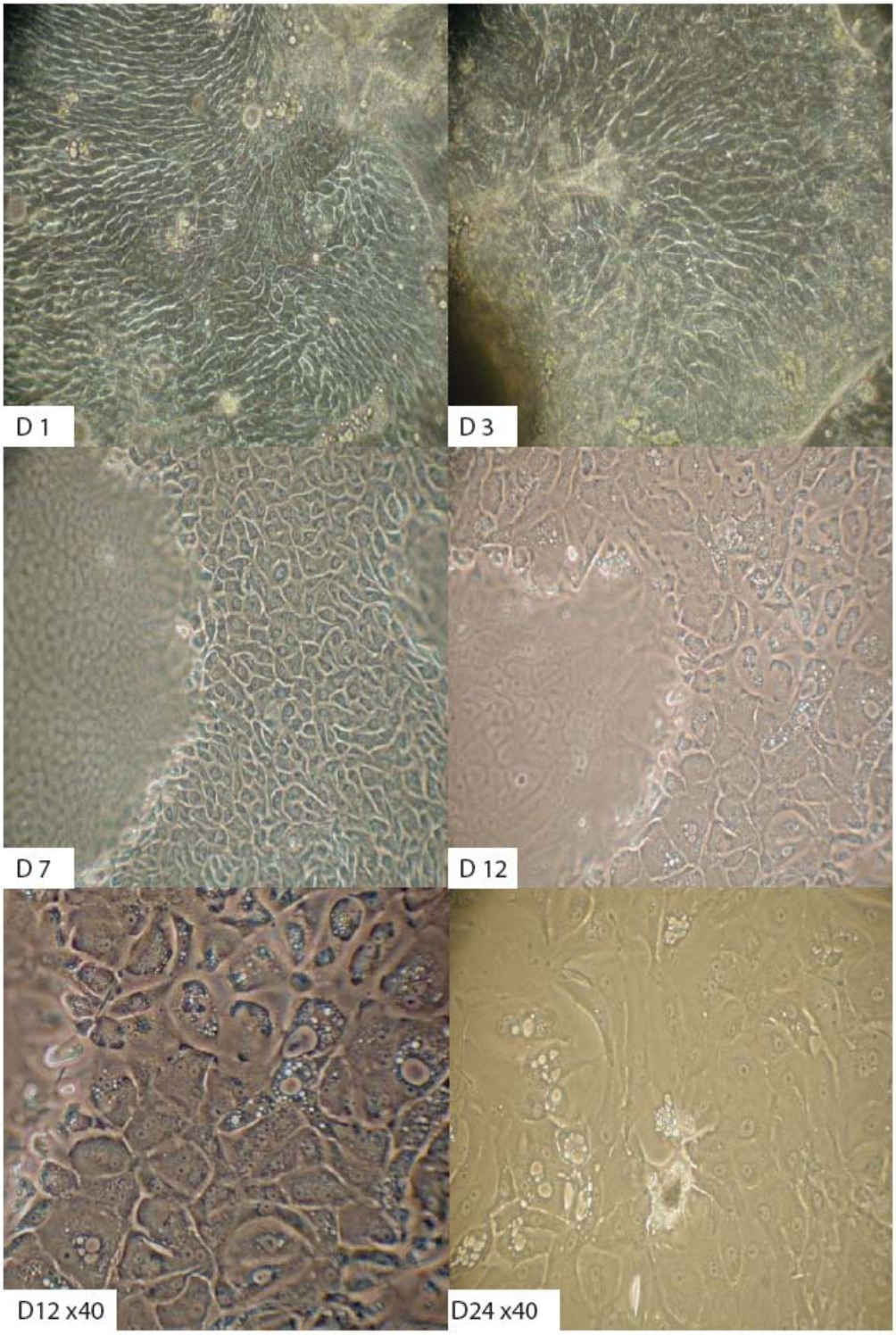


Figure 4.1 Differentiation into Hepatocytes

Progenitor cells increase in size and becomes polygonal in shape and grows into 2 dimension monolayer culture, senescing eventually like adult hepatocytes.

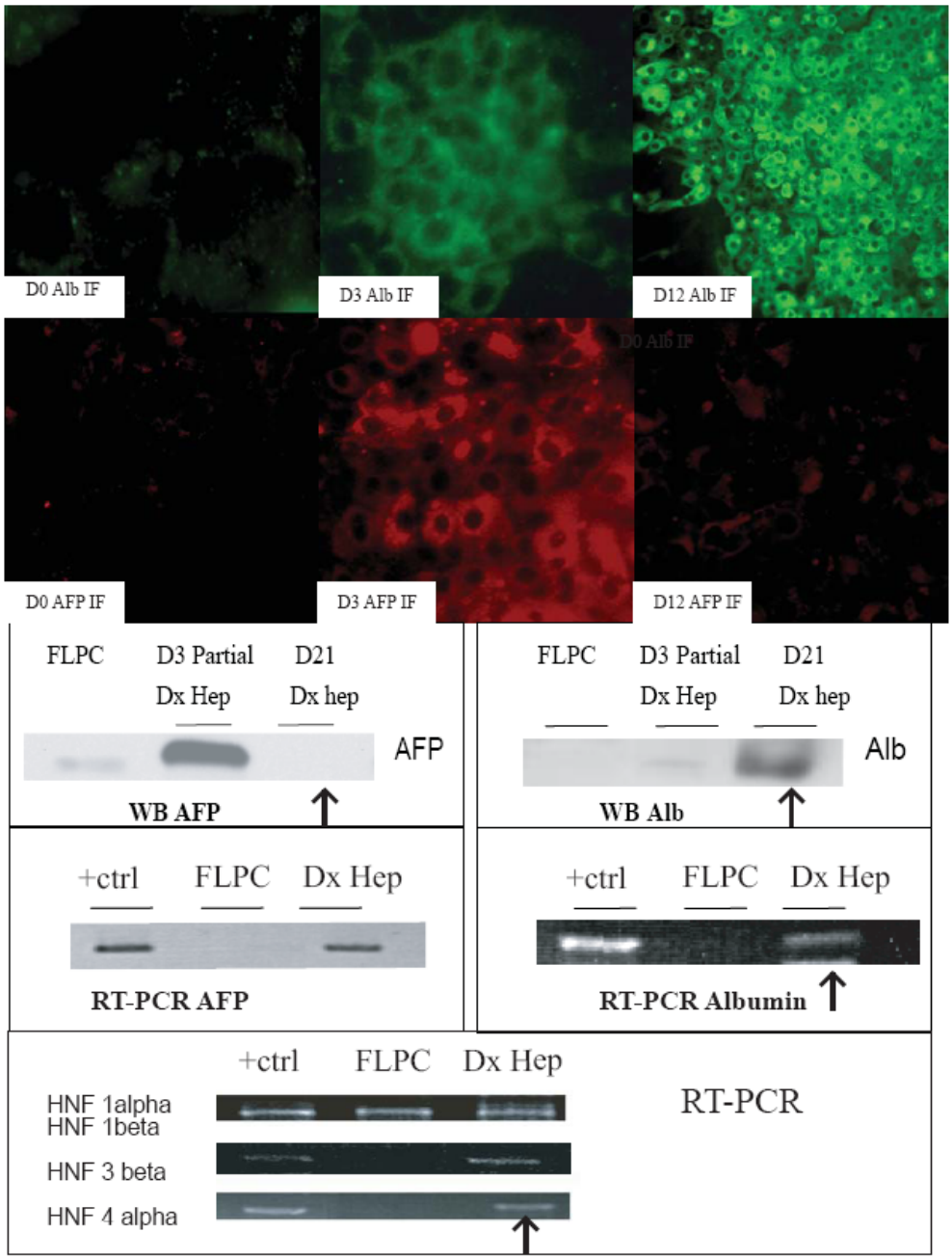


Figure 4.2. Differentiation into Hepatocytes II

Progenitor cells start to express albumin weakly by D3 and are strongly positive by D12. This is corroborated by western blot and RT-PCR for mRNA. AFP becomes transiently positive during maturation but disappears at D12 reflecting real-life physiological maturation. Hepatocytic specification transcription factors HNF 1alpha, 3beta and 4alpha, which were all negative in fetal progenitor cells, are upregulated with differentation.

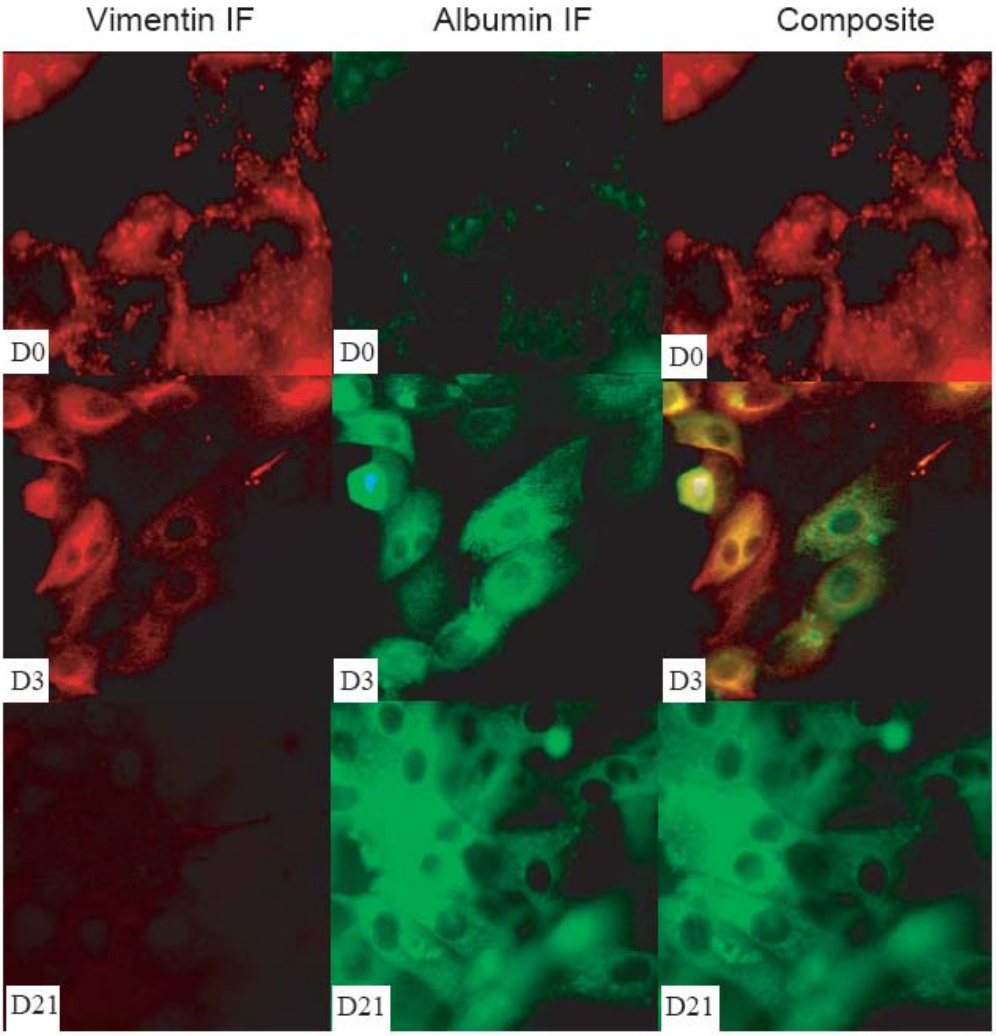


Figure 4.3 Mesenchymal-Epithelial Transition During Differentiation

Co-labeling performed by Vimentin IF with Alexa594 (red) and Albumin IF by Alexa-488 (green). At D0, progenitor cells are vimentin postive but albumin negative. With differentiation at D3, albumin is expressed and co-localise with vimentin postive cells (orange cells on composite image). With differentiation by D21, vimentin is lost and cells have switched over to full epithelial liver lineage expressing albumin.

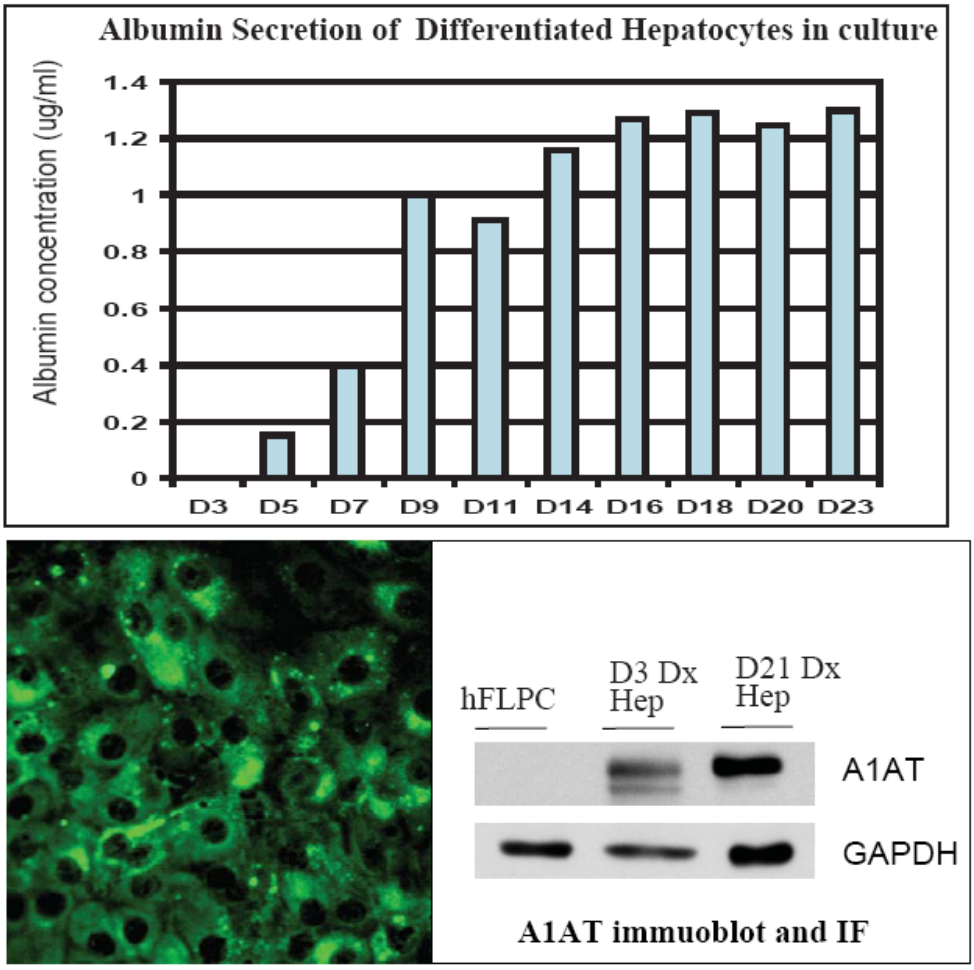


Figure 4.4 Functional Characterisation of Differentiated Hepatocytes - Protein function

Albumin is a key protein secreted by hepatocytes. In differentiation culture, cells start to secrete albumin into the sulture supernatant and is detectable by ELISA at D5. As sulture supernatant is colected and changed every 2 days, new albumin is continuously being secreted. Levels peak at about D16 and drops as cell senesce. Alpha-1- antitrypsin is expressed in liver and is positive only on differntiated hepatocytes and not on progenitor cells. hFLPC, human fetal liver progenitors.

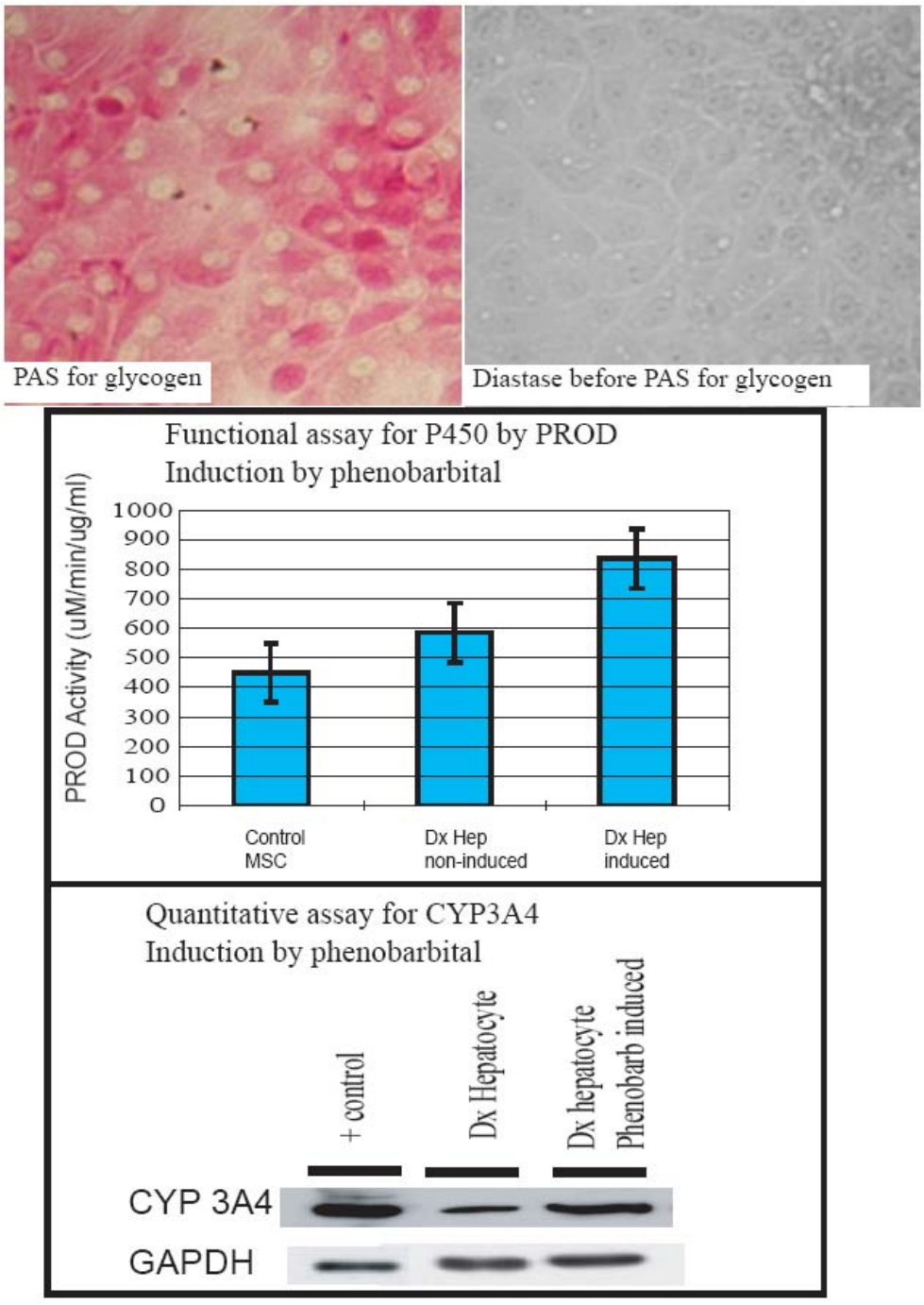


Figure 4.5 Functional Characterisation of Differentiated Hepatocytes- Glycogen and P450 Enzymes Inducibility

Differenitated hepatocytes stored glycogen which stain positive for PAS stain and specificity is confirmed with disatase treatment which digest away the glycogen. Quantitive and functional analysis of Cytochrome P450 enzymes indicate liver specific inducibility of both protein quantity and function with phenobarbital.

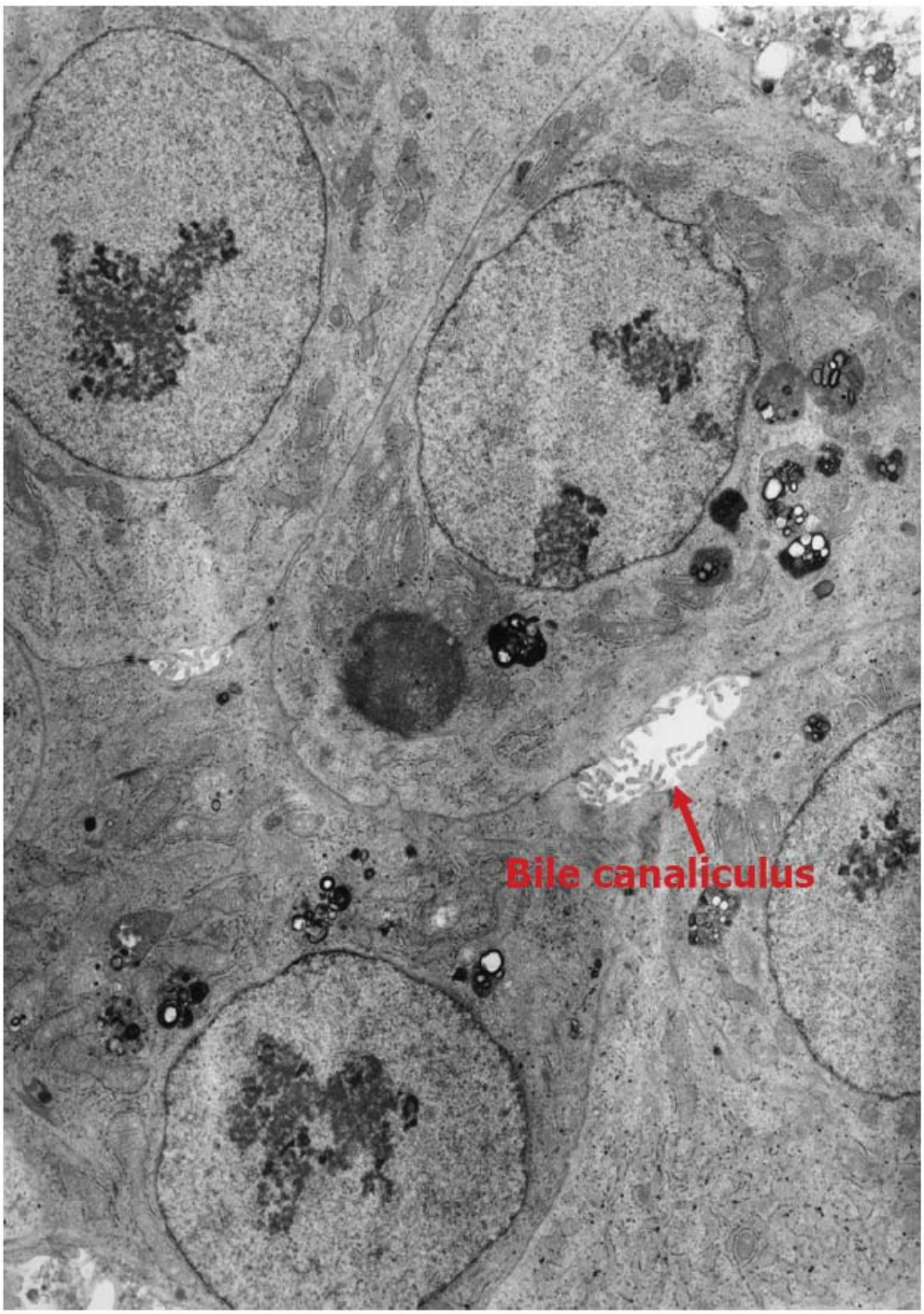


Figure 4.6 Electron Microscopy of Differentiated Hepatocytes

Differenitated hepatocytes show an increase in cellular organelles, namely mitochondria and endoplasmic reticulum and vesicles. The key characteristic of functional hepatocytes is formation of bile canaliculus with neighbouring cells.

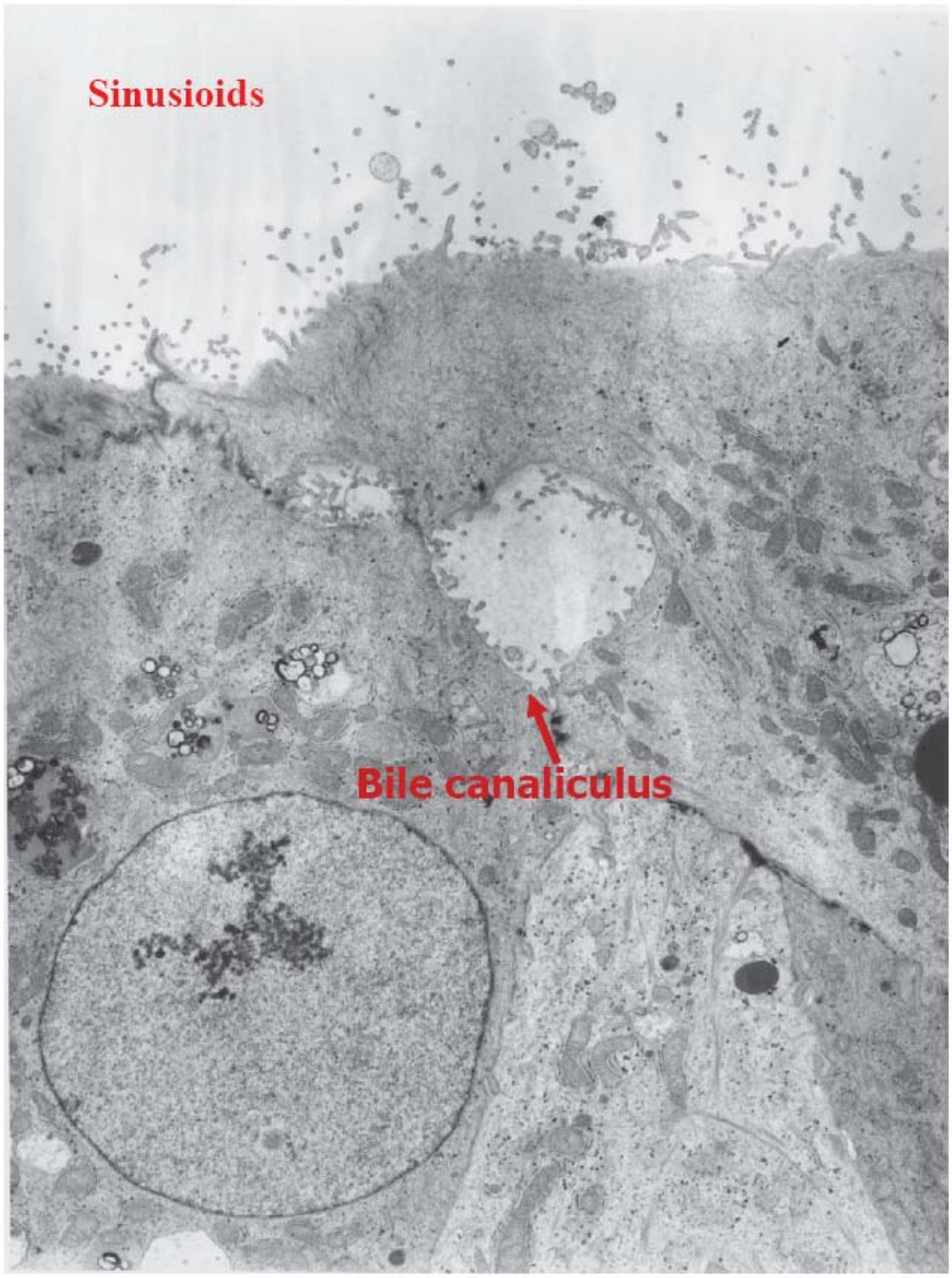


Figure 4.7 Electron Microscopy of Differentiated Hepatocytes II

Differenitated Hepatocytes arranging in polar axis. Finger projections are noted in the sinusoidal surface while canaliculus form on adjacent walls with neighbouring hepatocytes.

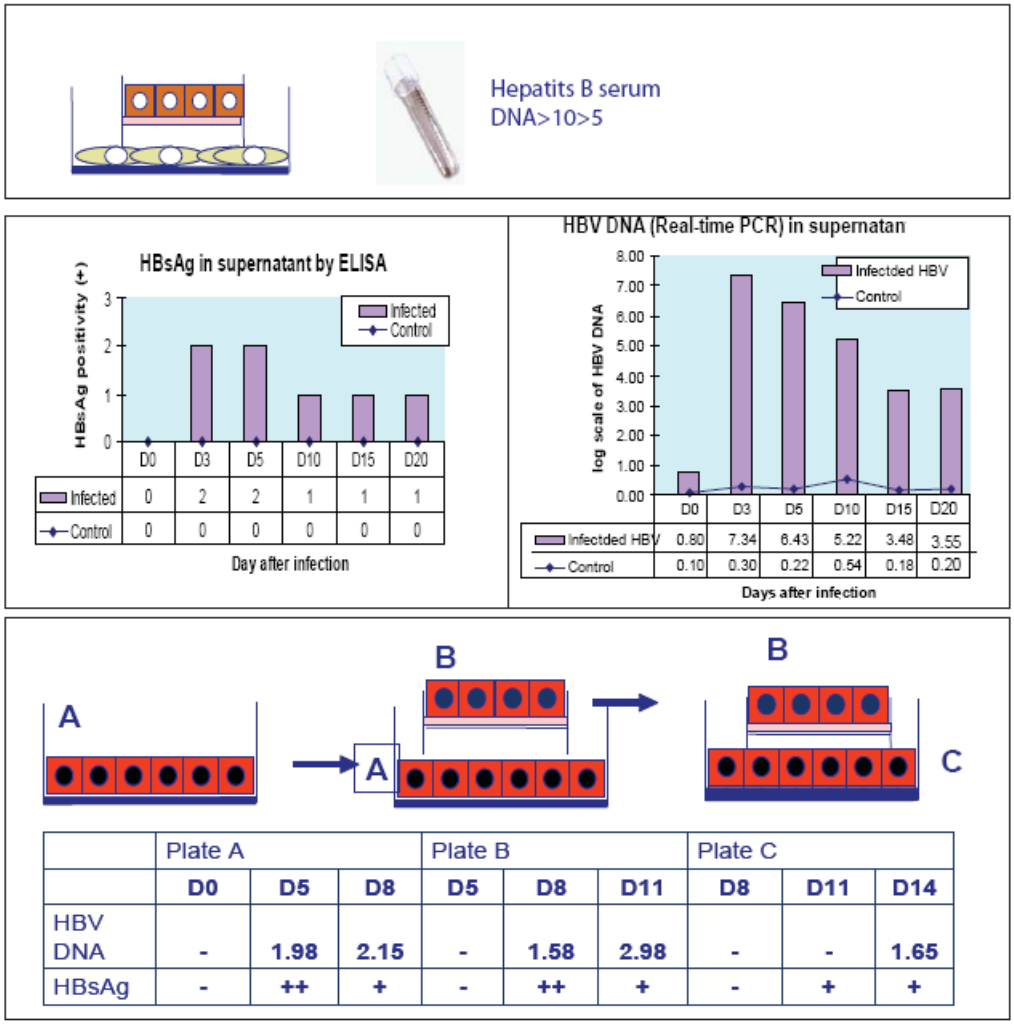


	Plate A			Plate B			Plate C		
	D0	D5	D8	D5	D8	D11	D8	D11	D14
HBV DNA	-	1.98	2.15	-	1.58	2.98	-	-	1.65
HBsAg	-	++	+	-	++	+	-	+	+

Figure 4.8 Infectibility with Hepatits B virus

Differenitated hepatocytes are cultured using fetal fibroblast as feeder layers to maintain cultures from senescing. Human serum with HBV DNA>10^5 copis/ml was added to culture and supernatant assayed for HBsAg and HBV DNA (RTPCR). Infection peaked at D3 with maximal HBsAg and HBV DNA by to 7 logs copies/ml. To ensure that readings were not due to HBV virus being carried over in medium, we serially infected fresh batches of hepatocytes at very low tires. Assay shows that serial infection can be achieved with continuous replication of virus in new batches of exposed differentiated hepatocytes.

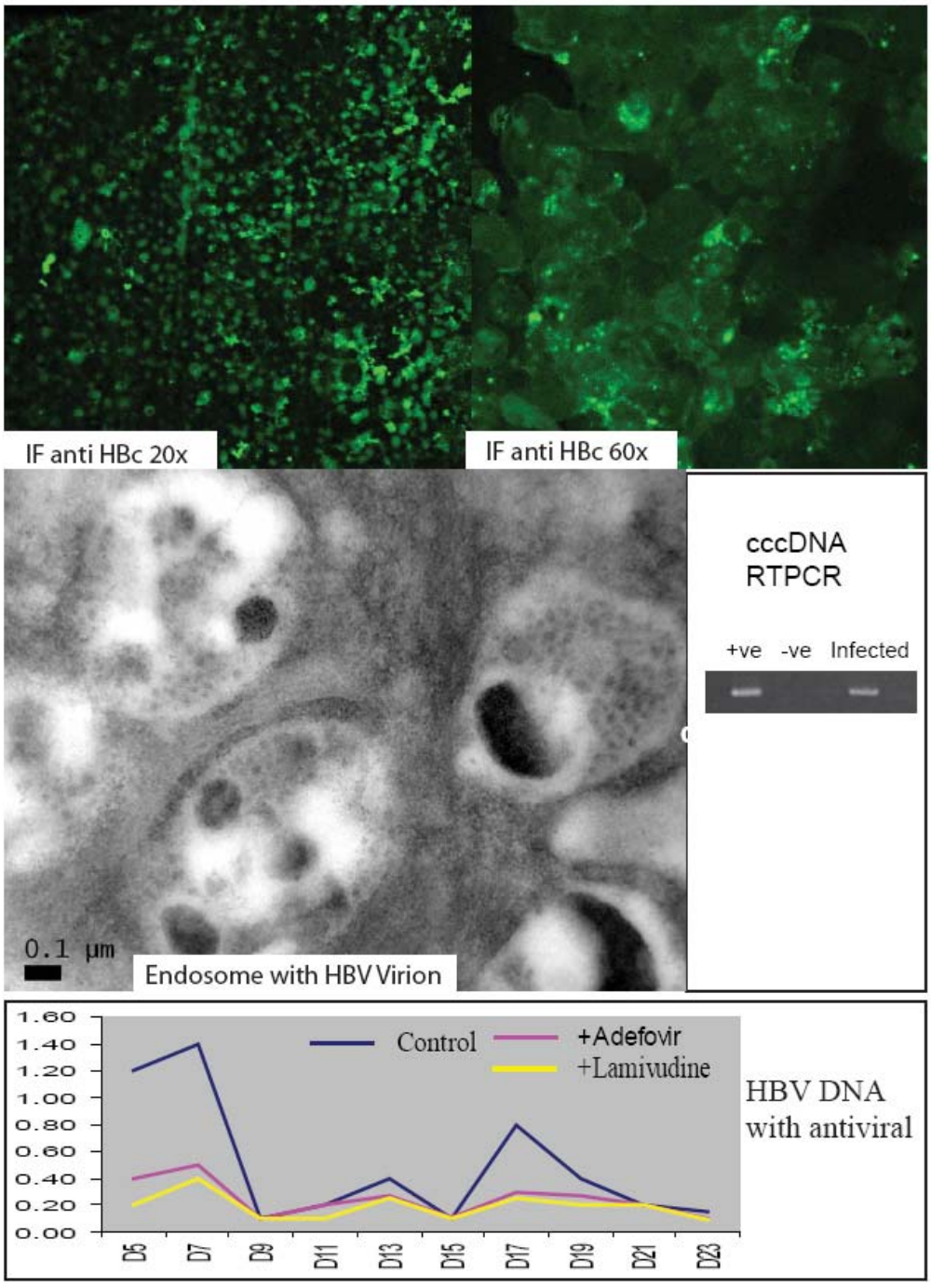


Figure 4.9 Infectibility of Differenitated Hepatocytes with Hepatitis B virus II

Inmmunofluorescence of HBcAg shows positivity in majority of differentiated hepatocytes with staining in cytoplasm. EM shows HBV virions in endosomes. Positive cccDNA and inhibition with antivirals lamivudine and adefovir confirm in situ infection and replication.

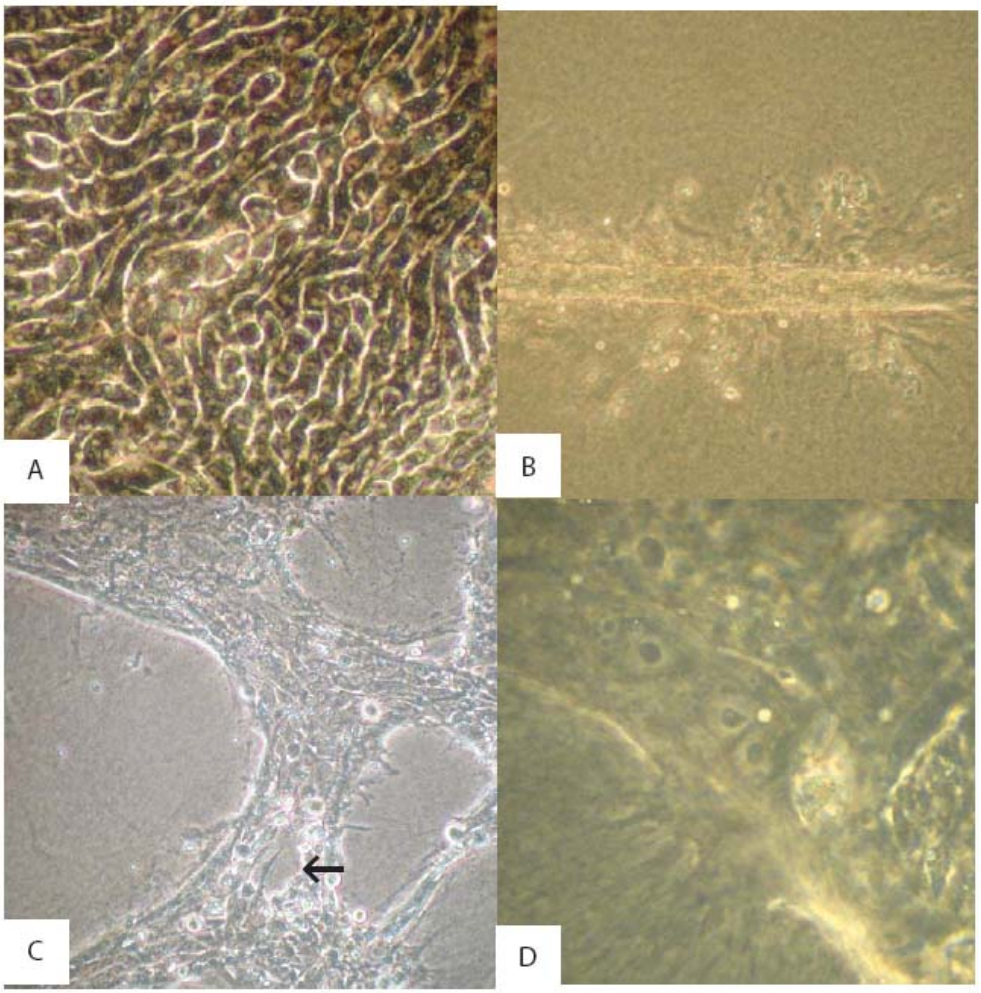
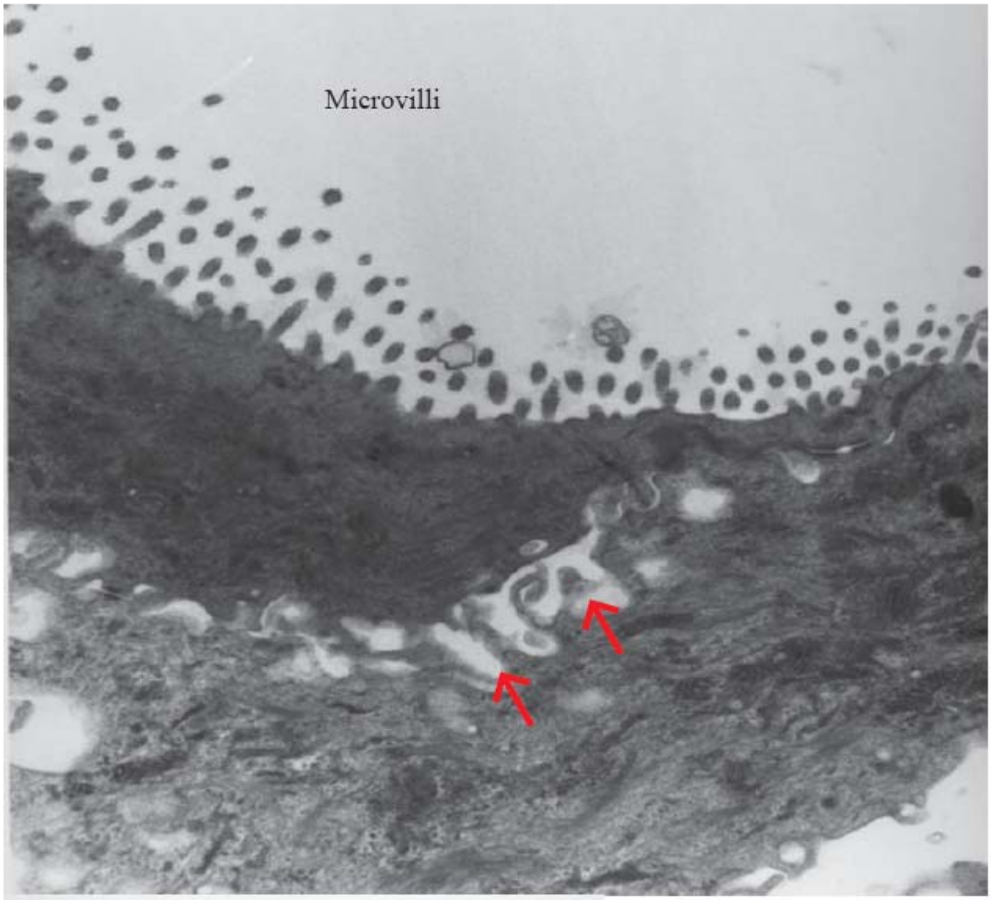


Figure 4.10 Cholangiocyte Differentiation

Culture of progenitor cells in 3D collagen gel result in cells realigning to form longitudinal tracts (A). With pulse FGF, the cells respond to the FGF gradient and form tubules, branching ducts (B)and cysts (C). 3-dimensional columns can be seen with cells stacking on each other around a central lumen (arrow). Cells facing the lumen of the cyst assume a columnar morphology.



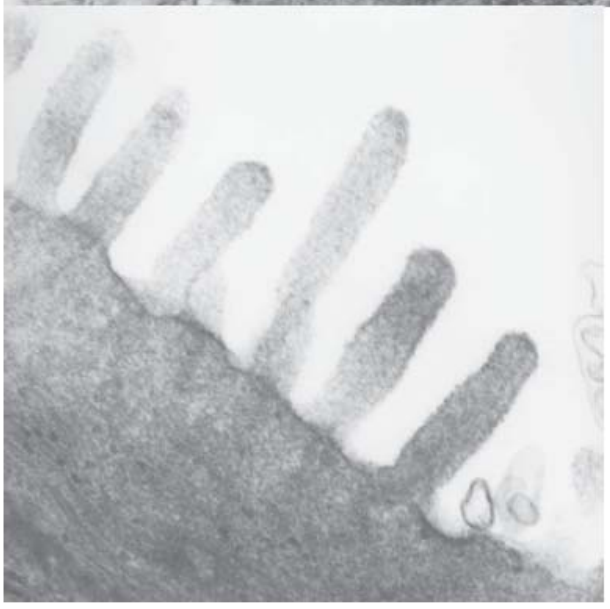


Figure 4.11 Electron Micrograph of Differentiated Cholangiocyte from Fetal Liver Progenitor Cells

Cholangiocyte forms tight junctions with neighbouring cells (arrow) and exhibit characteristic features of microvilli on the apical surface.

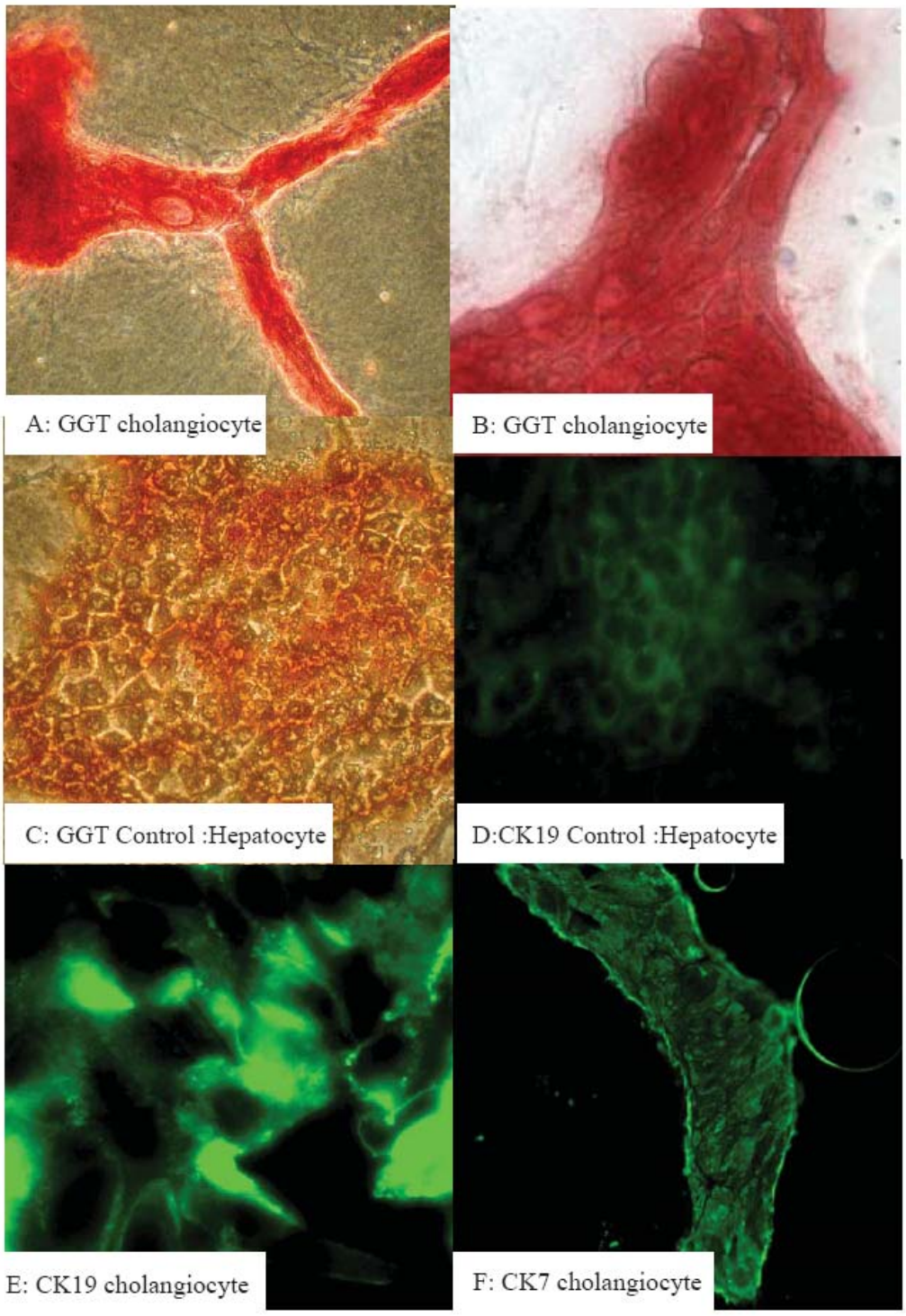


Figure 4.12 Cholangiocyte Differentiation (Histochemistry)

Branching tubes are strongly positive for GGT as compared to control hepatocytes Differenitiated cholangiocytes are strongly positive for bile duct markers, CK19 and CK7.

Chapter 5

Exploring the plasticity

Stem cells are highly plastic and have the ability to cross stochastic lineage fate to form other cell types. The putative embryonic stem cells obviously can differentiate into any of the dermal lineages. Adult stem cells would seemingly have more of a hurdle especially if they are derived from terminally differentiated tissues. However several reports have surfaced that mesenchymal cells such as Multipotential Adult Progenitor Cells[107] or even bone marrow mesenchymal cells[101] can be transdifferentiated into hepatocytes. It is not surprising since every cell continue to hold on to the same 46 chromosomes and complete set of genes, it is conceivable that tissue specific stem cells may not have locked down their specification fate. By reprogramming the relevant genes through environmental manipulation, it is possible to push the cell into other lineages. Fetal liver progenitor cell have seemingly mixed endodermal-mesenchymal markers and have yet to undergo liver specification. We thus attempted to differentiate these cells into mesenchymal lineages of bone, cartilage, fat and endothelial cells.

All differentiation protocols were performed in triplicate with P1–P4 and P8–P12 progenitor cell colonies. Percoll gradient separation was used to isolate human fetal MSCs (34) and parenchymal primary fetal hepatocytes (35) for use as controls. Protocols were adapted from publication on mesenchymal transdifferentiation[101] and successful differentiation were based on at least 2 confirmatory markers with mesenchymal stem cells from fetal liver as positive control. (Table 6)

5.1 Fat/ Adipocyte Induction

Fetal liver progenitor cells were transferred onto fibronectin plates and cultured using fat differentiation medium containing the key components of 3-isobutyl-1-methylxanthine, hydrocortisone, indomethacin, and horse serum. Cells increase in size and by D9, 40% of cells demonstrate accumulation of coalescing vacuoles that stain positive for fat (Oil red O). This was in contrast with differentiated hepatocytes which do not survive in fat induction medium. Derived fat-like cells also express peroxisome proliferator-activated receptor γ2 (PPAR γ2) mRNA which were negative in fetal liver progenitor cells and differentiated hepatocytes. (Figure 5.1)

5.2 Bone/Osteogenic Induction

Cultured in fibronectin plates with DMEM(High glucose), dexamethasone, β-glycerol phosphate and ascorbic acid, 10% FBS and antibiotics, cells become elongated and formed a complex meshwork with irregular processes. By D14, individual cell morphology was no longer discernible and instead, large amounts of cellular matrix were noted between the cells with areas of deposits which stained positive for calcium using von Kossa stain. RNA harvested from these cells also express strong osteopontin mRNA. Osteopontin mRNA was absent in differentiated hepatocyte although it was weakly positive in progenitor cells as had been previously reported[46]. (Figure 5.2)

5.3 Cartilage/ Chondrogenic Induction

The principle of cartilage induction hinged on creation of mass of cells in suspension. The cells in the middle of the mass would be in relative hypoxic conditions and stimulated by proline, transforming growth factor-β3 and ITS (insulin-transferrin-

selenium), would be induced to become cartilage cells expressing proteoglycans as well as cartilage specific type II collagen. 3 weeks of culture in polypropylene tube was needed for cells to organize in a ringed sphere. Extracellular matrix in the core was positive for glycosaminoglycans, which stains pinkish red with Safranin and is positive for cartilage-specific collagen II using immunofluorescence. The negative control differentiated hepatocytes do not survive in cartilage differentiating medium (Figure 5.3)

5.4 Endothelium Induction

Cultured on fibronectin plates with endothelium induction medium containing platelet-derived growth factor (PDGF)-CC[135], fetal liver progenitor cells became spindle-shaped with formation of linear channels between cells. After 9 days of induction, 38% of cells are positive for von Willebrand factor (immunofluorescence) and 28% of cells express CD31. Both of these factors are specific for endothelium and are not found in both progenitor cells and differentiated hepatocytes. (Figure 5.4)

5.5 Ectodermal differentiation

Ectodermal differentiation was attempted using published protocols from MAPCs[107]. However, despite repeated attempts, fetal liver progenitor cells do not survive in cultures beyond 3-4 days and neuronal transdifferentiation was unsuccessful.

5.6 Interpretation:

Human fetal liver progenitor cells can clearly behave like mesenchymal stem cells and, with appropriate stimulation, give rise to each of the mesenchymal lineage-like cells. It is important to note that positivity of 2 markers does not define a cell type and

in many aspects, the transdifferentiated cells have not undergone detailed functional analysis of mesenchymal lineages. However, it does appear fetal liver progenitor cells is fairly plastic and are relatively easy to differentiate into mesenchymal lineages-like cells. Ectodermal lineage transdifferentiation however appears much more difficult. Whether this is due to technical limitation or physiologically, the threshold for ectodermal transdifferentiation is much higher is not known. What can be concluded is that fetal liver progenitor cells demonstrate multipotency and plasticity in that they can differentiate into at least 2 dermal lineages with appropriate stimuli.

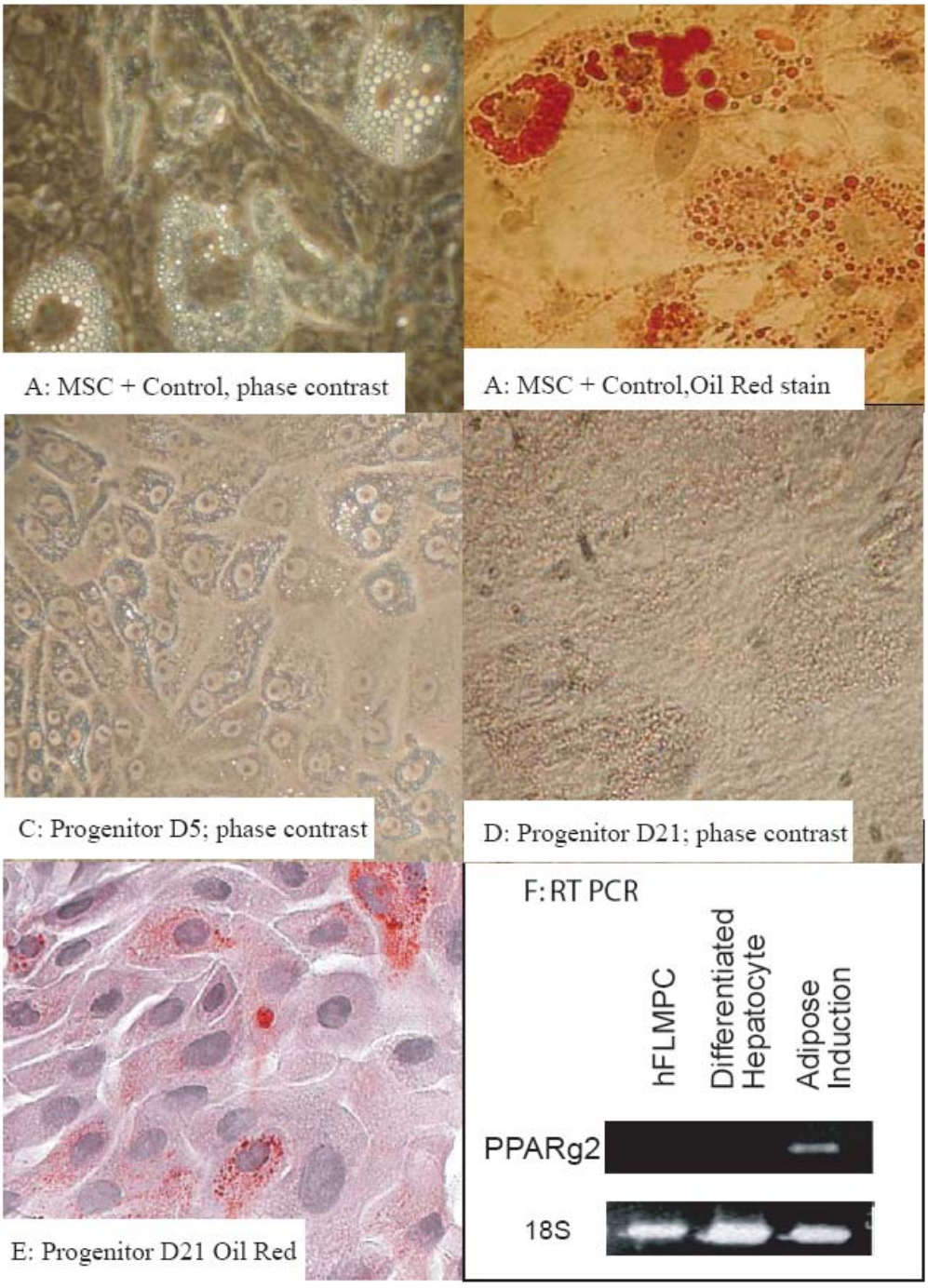


Figure 5.1 Adipocyte Induction

Polygonal shaped cells derived from progenitors accumalate lipid droplets and stain positive for Oil red-O. RTPCR for PPARgamma 2 is also positive suggesting a transition to adipocyte-like phenotype.

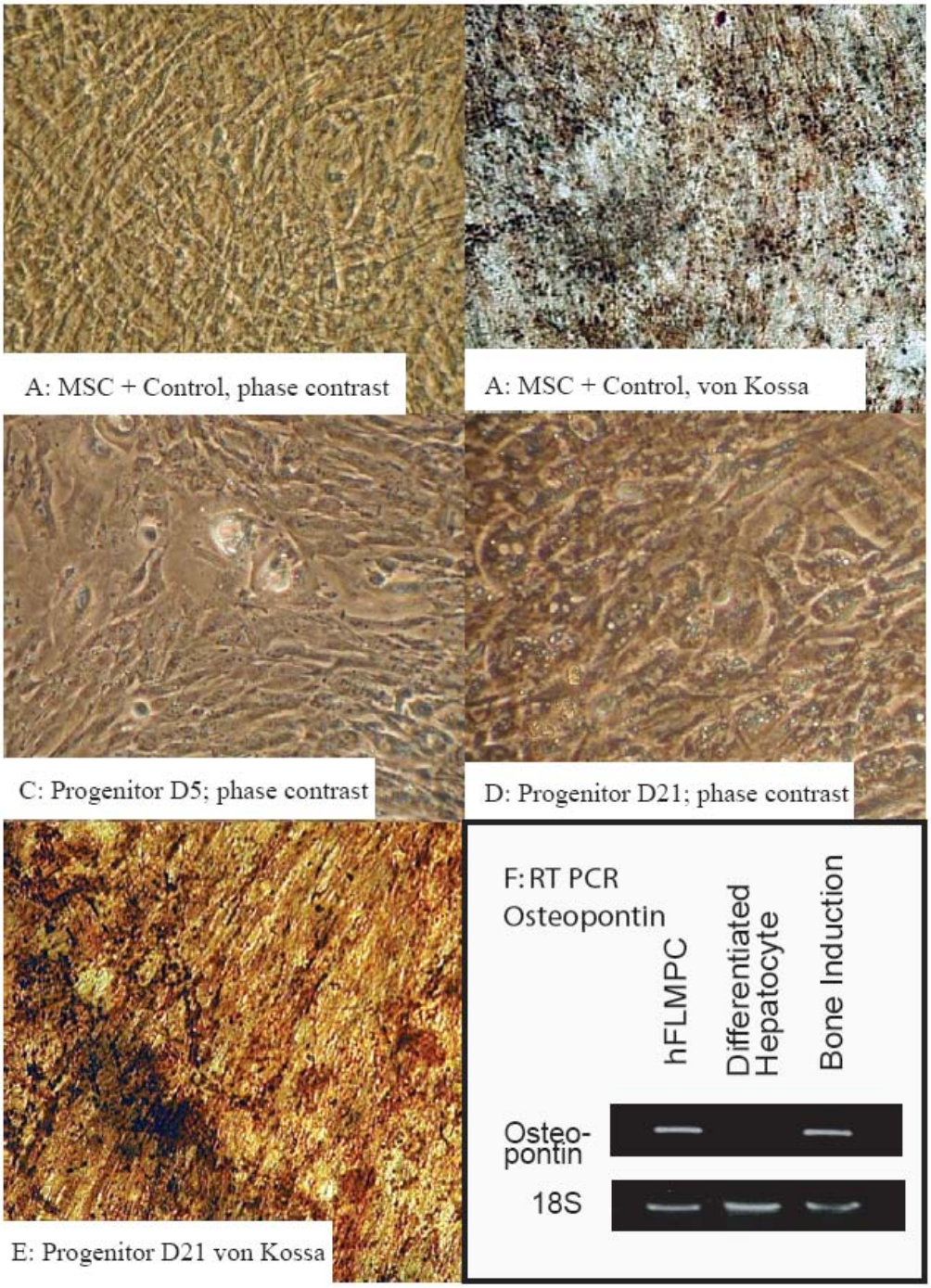


Figure 5.2 Osteocyte Induction

Mesenchymal Stem Cell as positive control (A-B). In bone induction, liver progenitor cells lose their morphology and form a meshwork of interconnected cells (C-D) which deposit calcium into extracellular matrix. Calcium is can be detected by Von Kossa stains (E). Osteopontin is marker for osteoblast but is seen in progenitor cells but not in differentiated hepatocytes. (F)

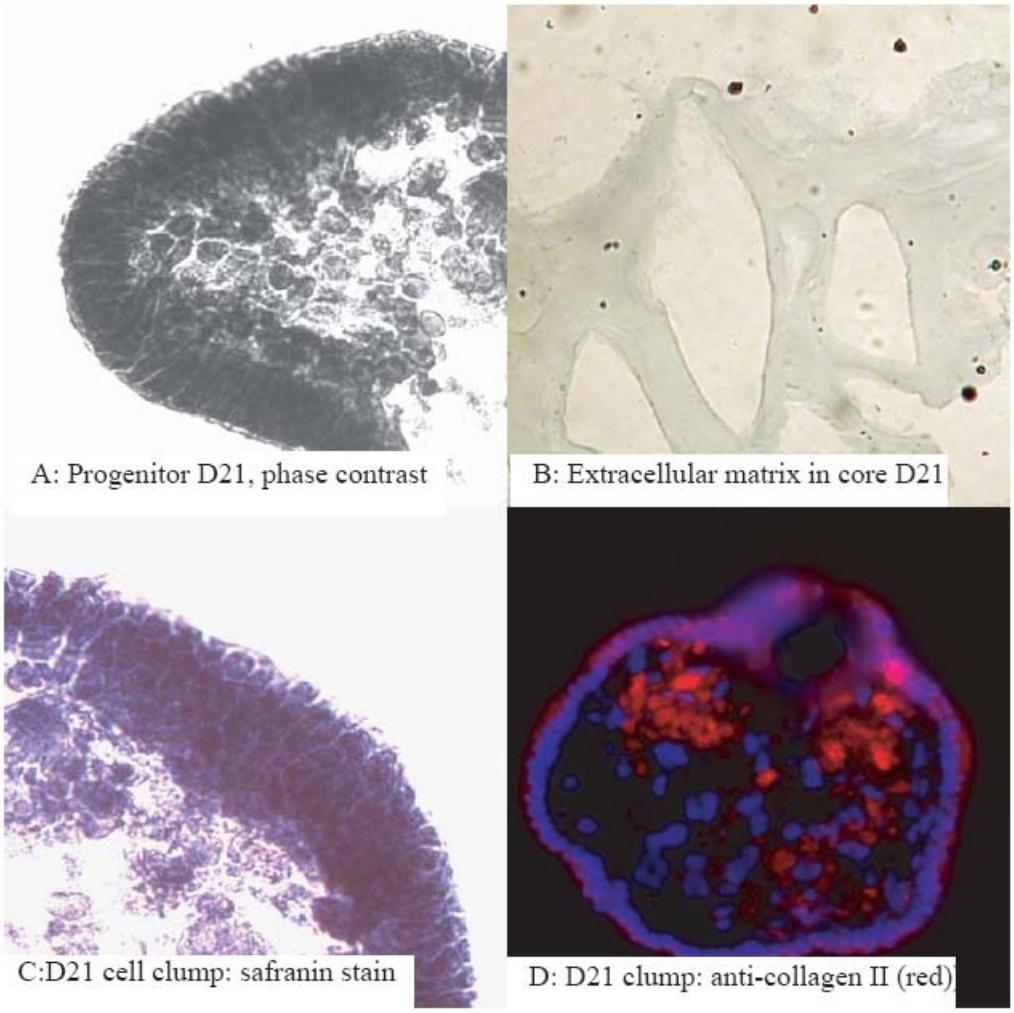


Figure 5.3 Cartilage Induction

Cells were cultured in clump in suspension drop. TGF alpha and low oxygen in the core of the cell clump is conducive for cartilage differentiation. At D21, soft extracellular matrix is seen in core (B). They stain postive for glycosaminoglycans (safranin stain)(C) and collagen II - a collagen isomer that is unique to cartilage.

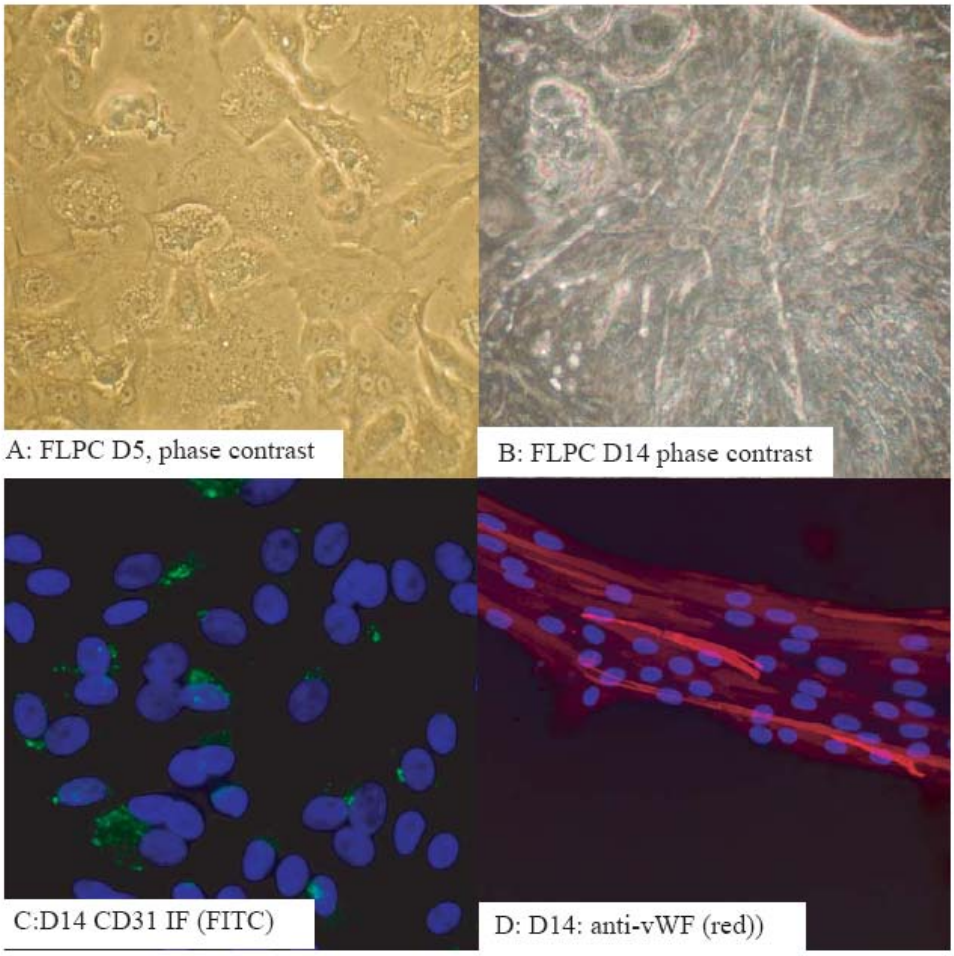


Figure 5.4 Endothelium Induction

Cultured with PDGF-CC endothelial differentiation medium, progenitor cells formed longitudinal bundles and developed lumen between cells reminsicent of capillaries by D14. Immunostaining with CD31 yielded positivity in 28% of cells (conjugated FITC anitbody) and 40% positivity for von WillieBrand factor. Both CD31 and vWF are markers of endothelial lineage.

Table 6. Protocols for Mesenchymal Lineage Transition

Bone	Cartilage	Fat	Endothelial	Nerve
DMEM-LG	DMEM HG	DMEM-HG	DMEM-HG	DMEM-HG
FCS	FCS	Horse serum	FBS	
glyceroP04	Pyruvate Proline	Methyl-isobutyl xanthine		Bmercapto-ethanol
	ITS+	Insulin		
Vit C	Vit K	Indomethacin		
dexa	Dexa	dexa		
	TGFB3		PDGF CC	

CHAPTER 6

Demonstrating *In vivo* Transplantatability

6.1 *In vivo* Function

The key test of practical usefulness of a progenitor/ stem cells lies in demonstrating ability to undergo in-vivo repopulation, perform repair and regenerative functions and if possible, show survival benefit. Can fetal liver progenitor/ stem cell be transplanted into animal models where it can survive, engraft, integrate into target tissue, differentiate and function like normal differentiated cell? A pilot study was undertaken to establish the optimal conditions for transplantation. (Table 7)

To achieve repopulation of human cells in xenotransplant models, 4 critical issues were studied:

1) Immunopermissive animals models
2) Ability to deliver cells to target organ
3) Regenerative stimulus for cells to grow
4) Competitive advantage for progenitor cells to grow

6.1.1 Immunopermissive model

6.1.1.1 Nude Mice model

Nude mice with deleted B cells and T cells were first used in transplantation experiment. 1 million human progenitor cells were injected intrasplenically after treatment with Fas ligand[136]. Liver injury and growth stimulus was induced using 2/3 partial hepatectomy model. Mouse serum was examined for human specific albumin at D12 and at D28 when mice were sacrificed. Immunohistochemistry and H&E

stains were then performed looking for human specific albumin staining cells in liver and spleen. Human specific albumin was negative in mouse serum at both time points. On H&E histology, clusters of abnormal looking dead cells were seen strewn randomly throughout the mouse liver but no positive human albumin staining was noted. These clusters were surrounded by fibrous strands and mononuclear cells and were presumed to be due to imunnological rejection mediated by Natural Killer(NK) cells. (Figure 6.1)

6.1.1.2 Rag2 double knockout mice

An alternative model of (C57BL_6J _ C57BL_10SgSnAi)-[KO]gc-[KO]Rag2 which ware lacking T,B and NK cells and specially bred for transplantation purposes were then used for repopulation studies and were found to be more tolerant of xenotransplantation[137]. (see details below)

6.1.2 Ability to deliver cells to target organ

Hepatocyte transplantation has been performed via intra-portal route or intrasplenic route. The intrasplenic route is technically easier and has been shown that almost all cells will migrate over to the liver by 24 hours[138]. As the portal vein network traverses through the liver cords via the portal tract, transplanted cells enter the hepatic parenchyma by squeezing out from within the endothelial sinusoidal space. Reported success rates published to date have suggested 1-2% repopulation. We thus surmised that the low degree of repopulation is due partly to cells traversing through the hepatic system without homing and instead, getting stuck embolically in organs such as the lung. To increase the yield of transplanted cells entering the hepatic cords, we treated the mouse with modified ischemic-reperfusion conditioning just prior to

transplantation. Ischemia-reperfusion is known to injure the endothelial cells lining the sinusoids[139], opening up gaps for enhanced passage of progenitor cells.

In a pilot trial, 2 Rag-2 mice were treated with ischemic-reperfusion prior to transplantation with 2 mice as controls. Live surgery was performed and the pedicle of median and left anterolateral segments were dissected, exposed and clamped with vascular clip for 20 min and released for reperfusion of 10 min before progenitor cells were injected into the spleen. After 14 days, the left lobe of the liver was then assessed for human cells using anti-human albumin by immunofluorescence/ immunohistochemistry. The animals with ischemia reperfusion showed 12-16% increase in number of human cells on mouse histology using identification with human specific albumin.

6.1.3 Regenerative stimulus for cells to grow

Growth stimulus was provided to the progenitor cells by injury induction to the native liver after transplantation of human fetal liver cells. Comparison was made between classical two third partial hepatectomy and intraperitoneal injection of carbon tetrachloride. In partial hepatectomy, 70% of liver mass i.e. the median and left lateral lobes are removed while in carbon tetrachloride, injection was given intraperitoneally for 3 weeks in 2 mice each[140]. Repopulation of human progenitor cells in mouse was not significantly different between the two techniques. Decision was made to use the carbon tetrachloride model as it simulated a real life chemical injury model and the advantage in repopulation from ischemia reperfusion is not lost when the median lobe and left lobe are removed during the 2/3 partial hepatectomy. Carbon tetrachloride also had the advantage that injury is usually at zone 3 of the

hepatocyte lobules, transplanted cells at zone 1 will be spared from direct toxicity yet receive a strong regenerative stimulus.

6.1.4 Competitive advantage for progenitor cells to grow

To give competitive advantage to our cells, we treated mice with 2 intraperitoneal injections of retrorsine 2 weeks apart to block replication of native hepatocytes[140]. This would theoretically block the natural repair and regenerative pathways in injured liver, giving a chance for the transplanted cells to proliferate to repair the liver. Pilot dose titration experiment was carried out initially with 6 Rag -/- mice with each mice at different doses of retrorsine ranging from 50-80 mg/kg at 10mg increments. Mice that received 70 ug and 80ug/kg dose died and 60ug/kg was determined to be the optimal dose for inhibition of native hepatocyte replication.

6.2 Transplantation

A total of 38 male Rag-/- double knockout mice at 6 weeks were treated with 2 doses of intraperitoneal retrorsine 2 weeks apart. Transplanted mice were administered with 4 million human fetal liver progenitor cells enriched in primary fetal liver cultures via the intrasplenic route after ischemia-reperfusion. Control animals were given sham surgery but infused with equivalent 0.2 mls phosphate buffered saline intrasplenically. One week after transplantation, animals were injected with weekly doses of carbon tetrachloride weekly for 3 weeks. Mice were then sacrificed at week 4 to determine if human progenitor cells persisted and followed up at week 12, 24 and 36 to track the repopulation levels. A total of 10 mice died before their designated timepoints. These were due to deaths post retrorsine (5 mice), post transplantation due to technical complications such as splenic laceration or uncontrolled bleeding. (Figure 6.2)

6.3 Localising the engrafted human cells

6.3.1 Haematoxylin and eosin staining

H&E staining showed the preservation of normal liver architecture in all mice and degree of injury and fibrosis in both transplanted and control mice were not easily discernible. At high magnification of mouse liver sections which had received transplants, clusters of dead cells could be seen stuck in portal veins. In addition, there were sporadic clusters of larger and more eosinophilic cells around the portal veins tributary where the cells had been introduced into the liver in association with hemorrhagic areas. (Figure 6.3)

6.3.2 Human specific albumin to identify human hepatocytes

Serial sections showing the presence of human cell clusters were stained with human specific albumin antibodies using immunofluorescence label. Using serial sections for localisation, the same large cells seen in H&E slides were confirmed to express human specific albumin. With immunohistochemistry, these cells become even more easily distinguishable with larger nucleus, cytoplasm and irregular shape. Other than the occasional cell clusters with >100 cells, isolated sprinkling of human specific albumin labeling cells can be seen around portal vein tributary. Using image analyzer and random counting of 10 high powered field, the degree of repopulation was estimated to be 0.8–1.7% in these mice. (Figure 6.3-6.4)

This was further corroborated by presence of human specific albumin mRNA in the transplanted mice while no human specific albumin staining cells, human albumin in the serum or human albumin mRNA were detectable by RT_PCR from the liver

lysate of control mice. These data indicate that human fetal liver progenitor cells that were intrinsically albumin negative, can survive and differentiate into functioning hepatocytes in an *in vivo* injury model. (Figure 6.4)

6.3.4 Confirmation of co-labeling with human hepatocyte-paraffin antigen

To further confirm that these were indeed human cells, a second human specific marker (human –specific Hep-Par) was used to stain these cells and showed that the same cells that were human albumin positive, also stained positive for human Hep-Par by co-labeling experiments. (Figure 6.4)

6.3.5 Identifying Human Chromosomes As Ultimate Proof Of Human Origin

Brulport et al[141] had reported that *in vivo* transdifferentiation results must be interpreted in caution as besides transdifferentiation, lateral gene transfer of human genes into mouse liver due to phagocytosis of dead human cells or through fusion with mouse hepatocytes may result in identification of human genes or product in mouse cells. To exclude lateral gene transfer, in situ hybridization was performed with human centromere probe which picked up only human chromosomes. Cells that stained positive for human specific albumin and hep-Par did stain positive for human specific centromere. (Figure 6.5)

6.4 Excluding Fusion in human derived cells in mouse liver

To exclude the possibility of fusion, we combined in situ hybridization with human –specific centromere probe labeled with alexa 488 green and mouse centromere probe labeled with alexa 594. If fusion events had occurred, we would be able to see cells that had both human and mouse chromosome and would thus have red and green

labels within the same nucleus. In all the 100 nucleus that were analysed, human and mice chromosomes were exclusive and there was not a single nucleus that had both human and mice centromere together within the same nucleus. Although this does not exclude fusion completely, fusion does not appear to be the predominant mechanism by which human progenitor cells repopulate the injured mouse liver. (Figure 6.5)

6.5 Functions of in-vivo human cells

The aim of transplantation lies in the hope that transplanted cells can take over the functions of the injured liver and support the injured animal. It is thus imperative to establish principle of proof that differentiated cells from the transplanted fetal liver progenitor cells are functional and can integrate into the host tissue organ.

6.5.1 Production of albumin into serum

Albumin production into the serum is a core function of hepatocytes and plays the role of carrier proteins for both nutrients and toxins as well as maintenance of oncotic pressure. Using the same human specific albumin antibody for ELISA, we were able to detect human specific albumin in the serum of transplanted mice. In control mice, none of the albumin in serum was of human origin. (Figure 6.4)

6.5.2 Production and Storage of glycogen

Periodic Acid Schiff stain was performed to demonstrate functional production of glycogen in human derived cells. Human hepatocytes stain more darkly compared to the mouse hepatocyte for glycogen. Localising human hepatocytes with human specific albumin IHC, serial sections were tested for glycogen and showed that the

same large cells staining positive for human albumin were strongly positive for glycogen similar to adult hepatocytes controls. (Figure 6.6)

6.5.3 Integration with native mouse hepatocytes

Functional hepatocytes integrate with neighboring cells by forming tight junctions at canaliculus which can be delineated with ZO-1, a tight junction protein. Co-labeling study was performed using human specific Alexa 594(red) antibodies and non specific ZO-1 antibody labeled with Alexa 488 (green). (mouse specific ZO-1 antibodies were not available). Composite pictures showed that there were cells showing human specific ZO-1 localising adjacent to cells expressing mouse ZO-1 suggesting the formation of shared tight junctions between hepatocytes of human and mouse origin. (Figure 6.7)

6.6 How long can human derived cells survive *in vivo*?

To test the durability of these cells and whether they continue to expand *in vivo* as well as explore the safety issue from oncogenic point of view, mice were transplanted using the same protocol and followed up in cohorts up to 3 months, 6 months and 9 months. Repopulation was quantitated by human specific albumin producing cells. Repopulation was patchy but repopulation indices and both human specific serum albumin concentration and mRNA RTPCR quantification showed a peak at 3 months in all animals. This was corroborated by presence of Ki67 positivity in human clusters up to 3 months. 1 mice continued to show robust human cells engraftment with large clusters of human cells seen on histology, high albumin mRNA on RT PCR and high serum human albumin. (Figure 6.8) The persistence of engraftment was noted to depend on whether fibrosis persisted in the animals. Animals with continued fibrosis

saw robust proliferation of clusters of human cells while animals with escape and reversal of fibrosis resulted in loss of human cell clusters, likely due to loss of growth stimulus.

6.7 Do fetal liver progenitor cells differentiate into bile duct cells *in vivo*?

As carbon tetrachloride injury model is predominantly a hepatocyte injury model, bile duct regeneration is not a significant reponse. Using Fluorescent insitu hybridization for human centromere probe, occasional human chromosomes within bile ductules was noted, suggesting that fetal liver progenitor cells were able to differentiate into biliary cholangiocyte *in vivo*. (Figure 6.9)

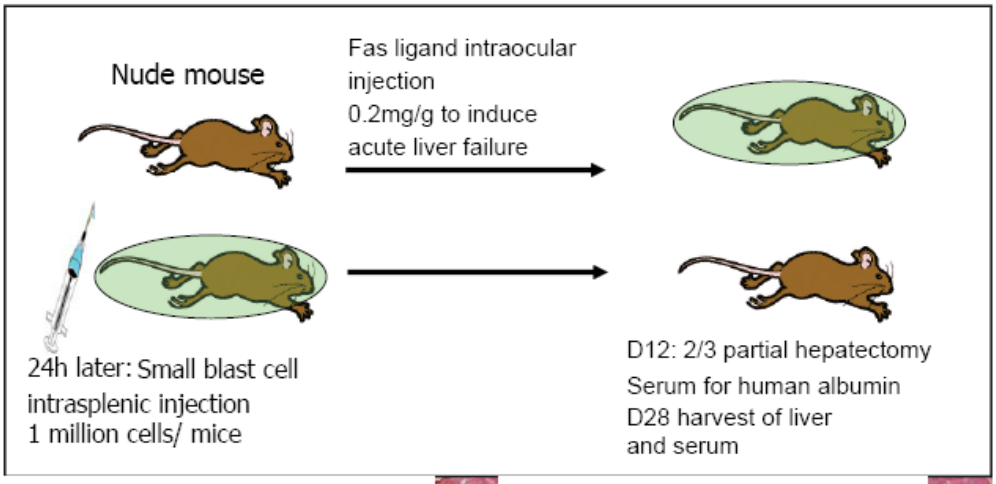
6.8 Testing oncogenic potential of human liver progenitor cell

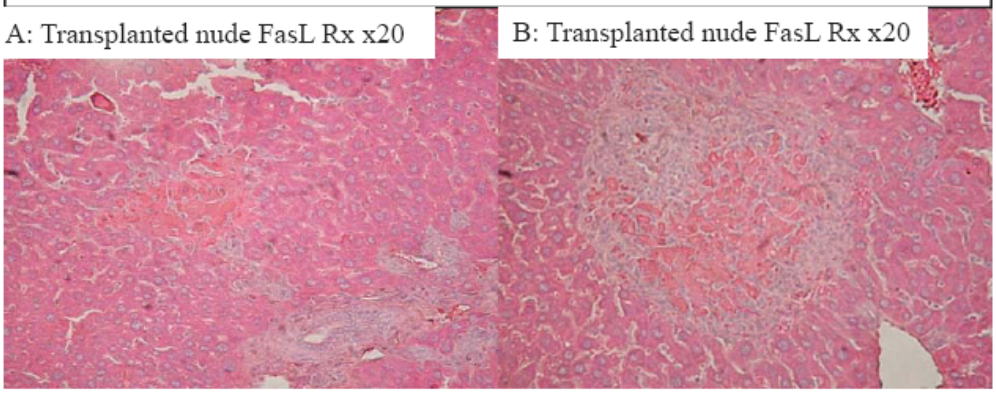
Oncogenicity remains one of the hurdles towards use of progenitor cells in clinical treatment. To test if transplanted human progenitor cells may undergo transformation, clusters of human hepatocytes derived from 9 month samples post transplantation were analysed for oncogenic potential. Haematoxylin and eosin sections were analysed by 2 senior gastrointestinal pathologists and staining were performed for alphafetoprotein, Ki67 and CD34. While cellular disarray were noted, there were no evidence of irregular mitosis, dyskaryosis and AFP, KI67 and abnormal patterns of CD34 were all negative. To prove that that there were no nuclear aneuploidy, in situ hybridization was performed with chromosome 7 and 17 centromere probe, the two commonest chromosome implicated in HCC aneuploidy[142]. In all the nucleus examined, no abnormal numbers of chromosome 7 and 17 were detected. We concluded that at least up to 9 months post-transplant, there was no evidence of

oncogenic change or aneuploidy in transplanted human fetal liver progenitor cells. (Figure 6.10)

6.9 Interpretation

An immunologically permissive model with ischemia-reperfusion conditioning to increase delivery of cells to the injured organ, continued regenerative stimulus and competitive advantage are key factors that affect repopulation. Rag 2 KO mice were used for these experiments as they were bred especially for human cell transplants and were supposedly more immunologically permissible and hardier than NOD/SCID mice. Human fetal liver progenitor cells are able to repopulate livers of murine model of liver injury. They survive and differentiate into functional liver cells, integrating with mouse hepatocytes as well as into the biliary tree. They express albumin, Hep-par, store glycogen and persist up to 9 months if there is continued regenerative stimulus with no evidence of fusion. Human fetal liver progenitor cells do not appear to form tumours with nuclear aneuploidy up to 9 months post transplant.





	D12	D28
Serum for ELISA –human albumin	Negative	Negative
Spleen		Negative for Engraftment
Liver human albumin IHC And H&E		Clusters of dead cells with immune reaction

Figure 6.1 *In vivo* Transplantation in Nude Mice with Fas Ligand Injury

Acute liver failure was induced by Fas ligand injection intraperitoneally and 1 million human fetal liver progenitor cells was injected intrasplenically. However no human albumin was detectable at D12 and D28 in mouse serum, liver or spleen. On H&E, clusters of dead cells with surrounding immune reaction is seen suggesting failure of repopulation due to immune rejection.

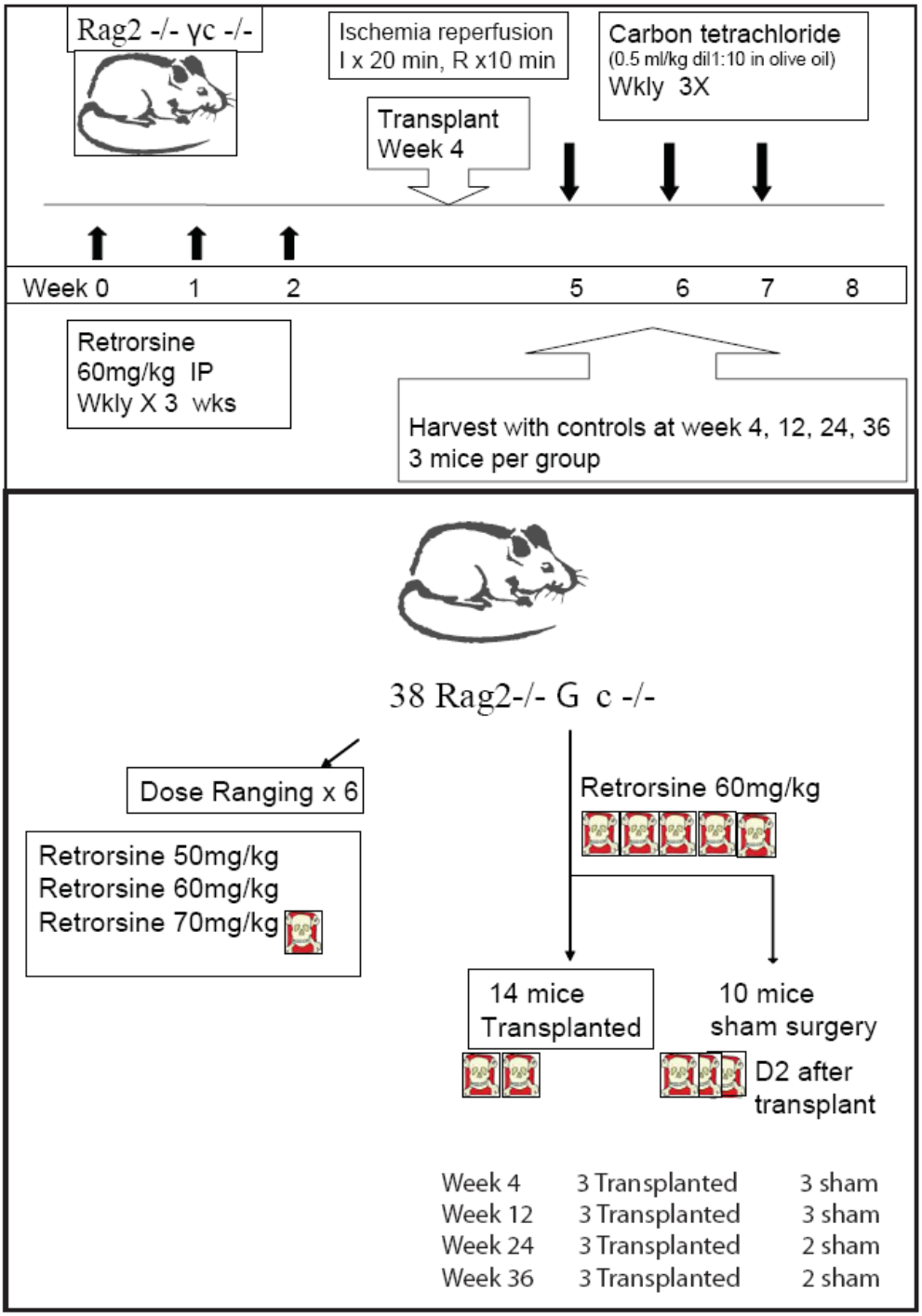


Figure 6.2 ***In vivo*** **Transplantation in Rag2 GC KO**

Dose ranging showed that 60mg/kg retrorsine was optimal with minimal death. 32 mice were treated with retrorsine and underwent transplantation or sham. Animals were sacrificed at 1 month to determine principle of proof of repopulation and repeated for 3, 6 and 9 month cohorts to trace degree of repopulation.

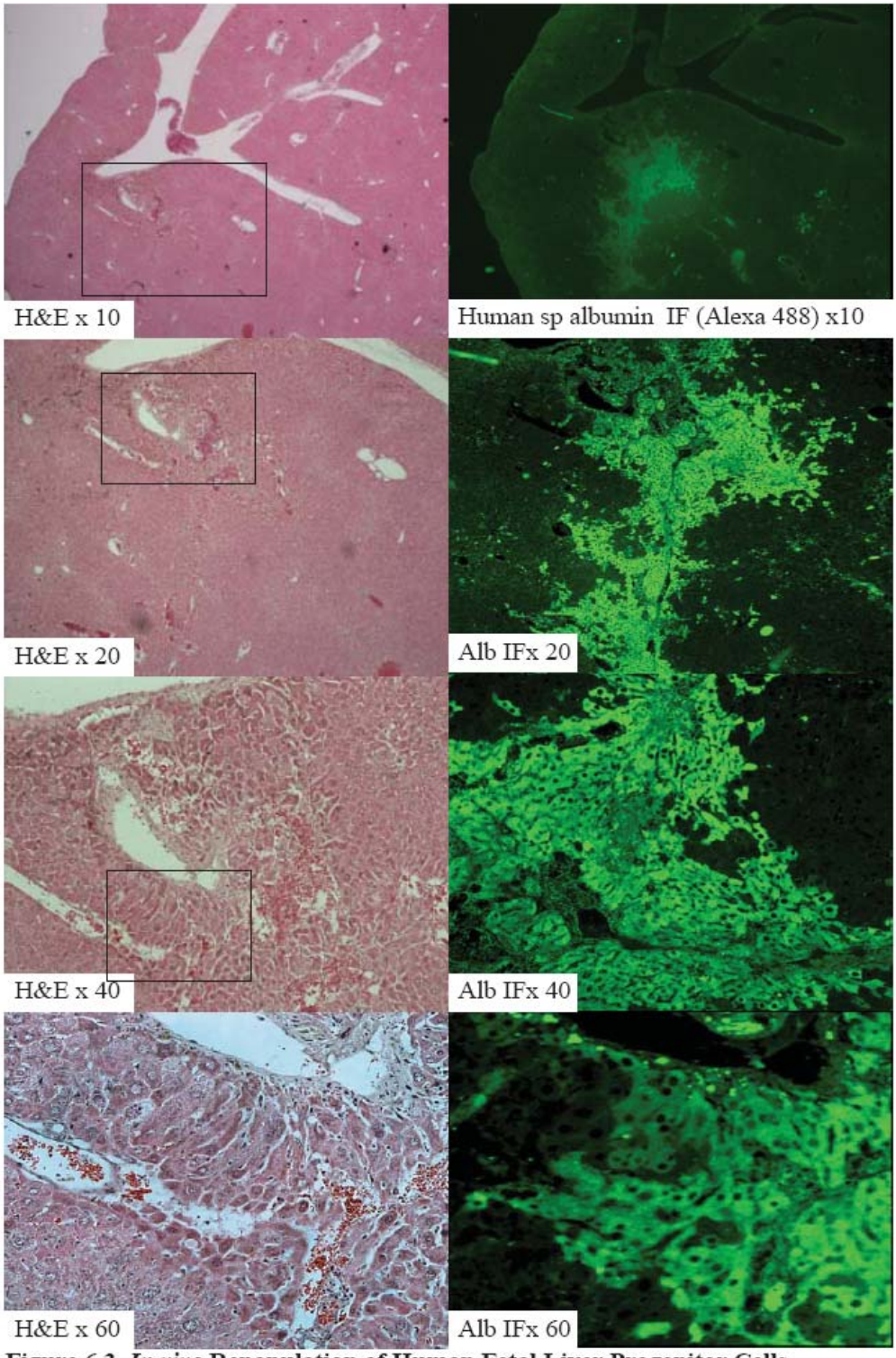


Figure 6.3 ***In vivo*** **Repopulation of Human Fetal Liver Progenitor Cells.**

Paired serial sections of transplated mouse liver week 4 post transplant showing H&E and human specific albumin IF on serial sections. Human cell clusters are usually seen next to portal tracts and are recognisable even on H&E.

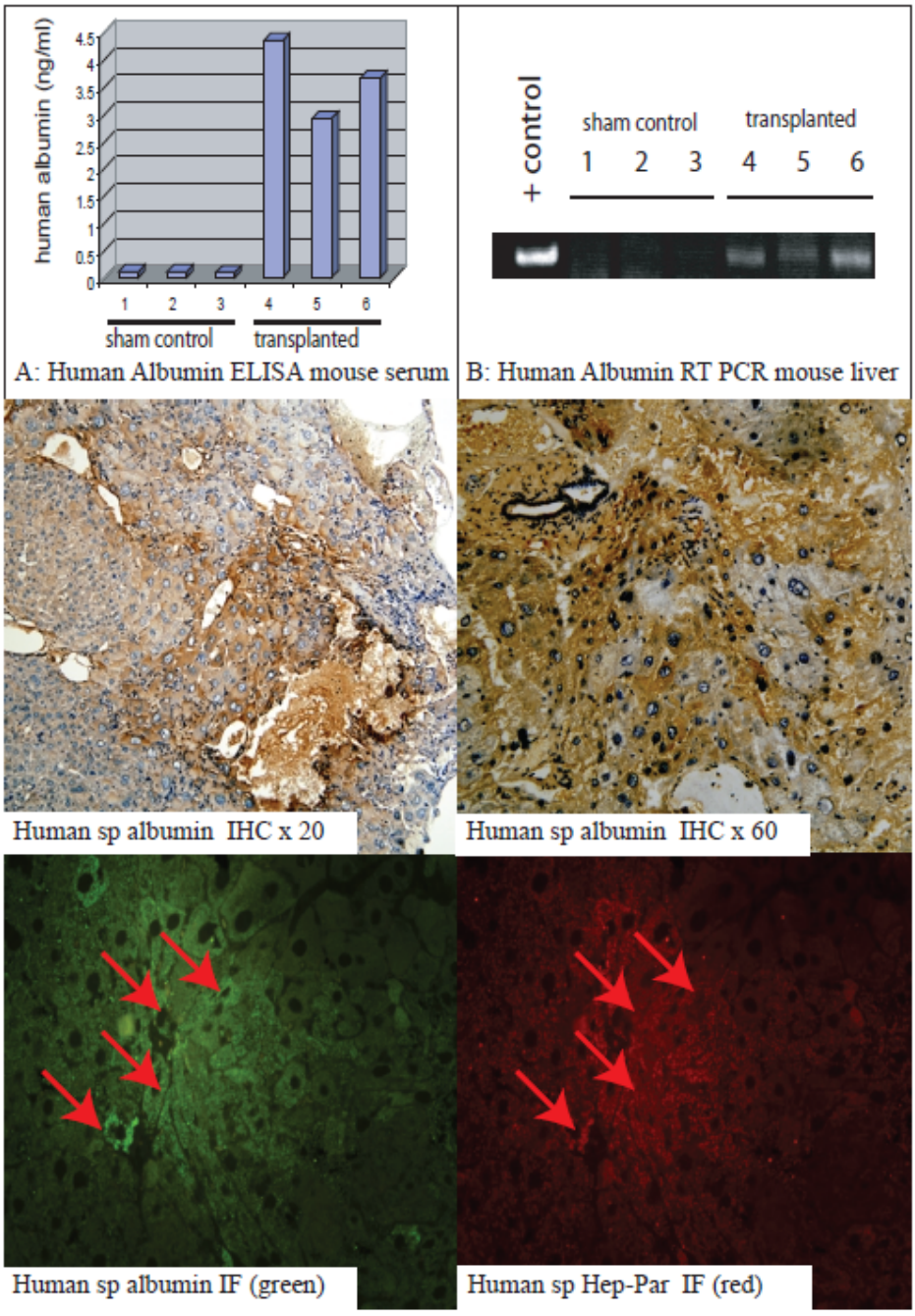


Figure 6.4. *In Vivo* Functions of Human Fetal Liver Progenitor Cells.

Transplated animals at 30 days post transplantation showed human specific albumin transcripts mRNA by RTPCR as well as in mouse serum by ELISA. These were both negative in control mice. Human cells are postive for both human albumin and human specific Hep-Par.

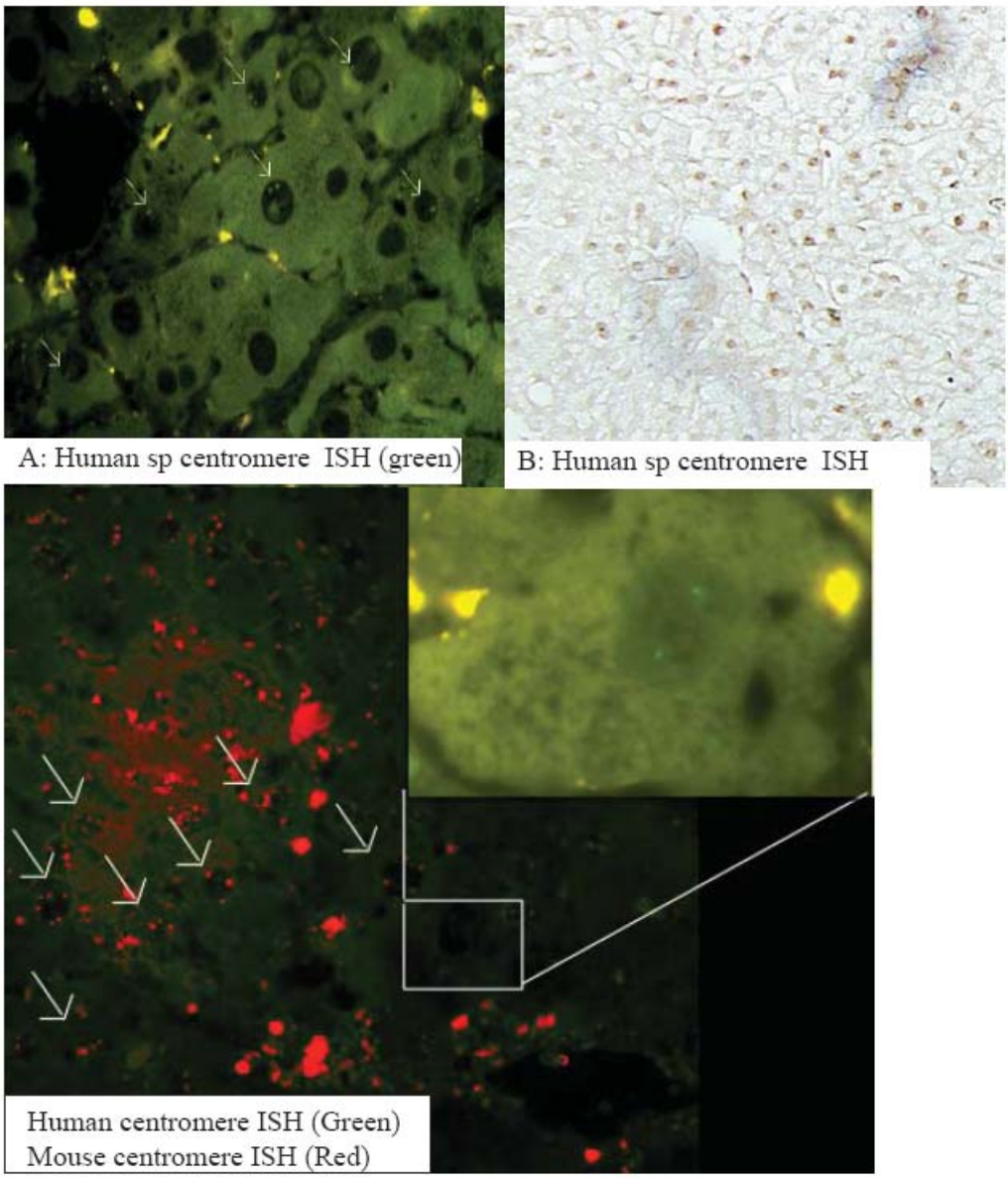


Figure 6.5 *In situ* hybridisation of Engrafted Cells

In-situ hybridisation was performed to confirm orgin of observed human cells in transplanted liver. Probing with human specific centromere confirmed human albumin producing cells were of human origin using IF and DAB (A, B). To confirm the absence of fusion events, hybridisation was performed with human centromere (green) and mouse centromere (red) probes. In all the nucleus analsyed, no nucleus were seen containing both human and mouse chromosomes.

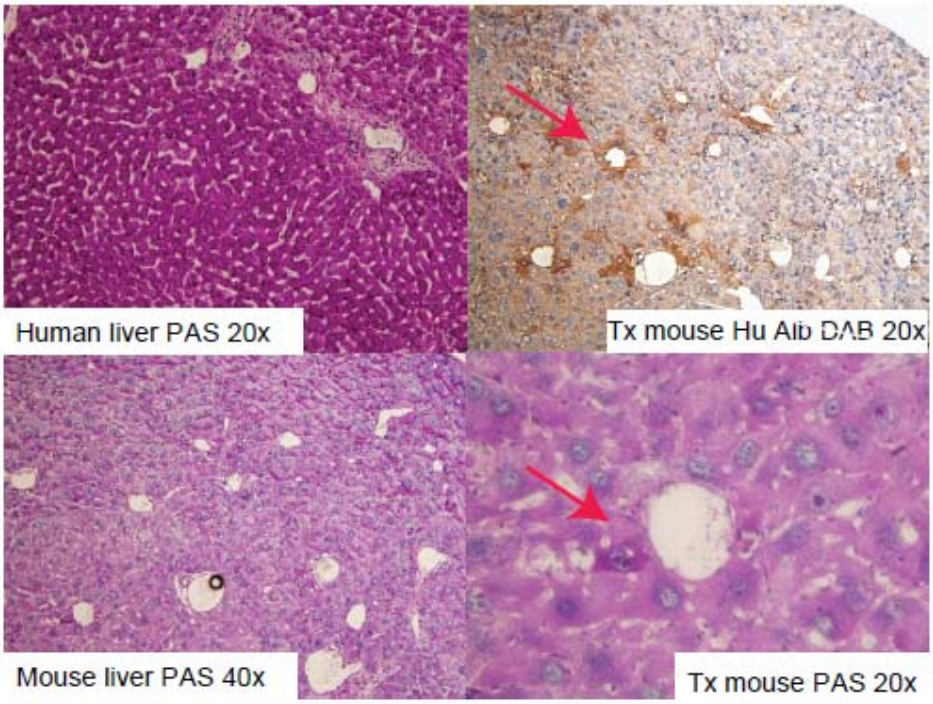


Figure 6.6 Glycogen Storage in Transplanted Cells

Human hepatocytes stain darker for glycogen compared to mouse hepatocytes. Human cells were found around portal tracts and were identified using human specific albumin IHC. Corresonding cells in serial section stained strongly for glycogen, indicating the engrafted cell was functioning in vivo.

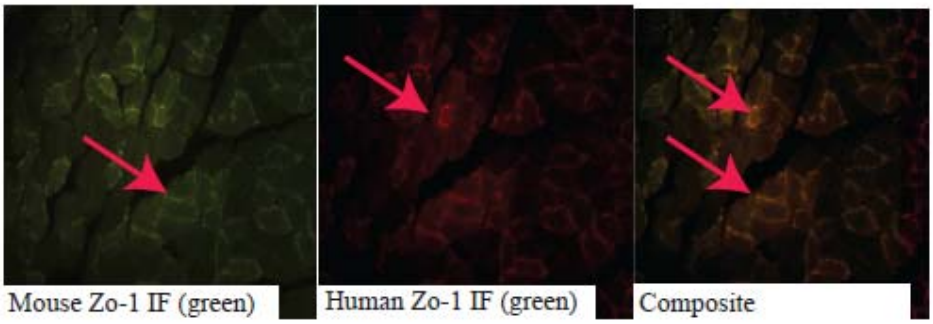


Figure 6.7 Integration of Tight Junctions between Mouse and Human Hepatocytes

Mouse ZO-1 antibody is non-specific and stain both human and mouse ZO-1 but human ZO-1 antibody is specific for human only. On composite image, cells expressing human ZO-1 are adjacent to mouse ZO-1 suggesting the formation of tight junctions and integration of human hepatocytes with mouse hepatocytes.

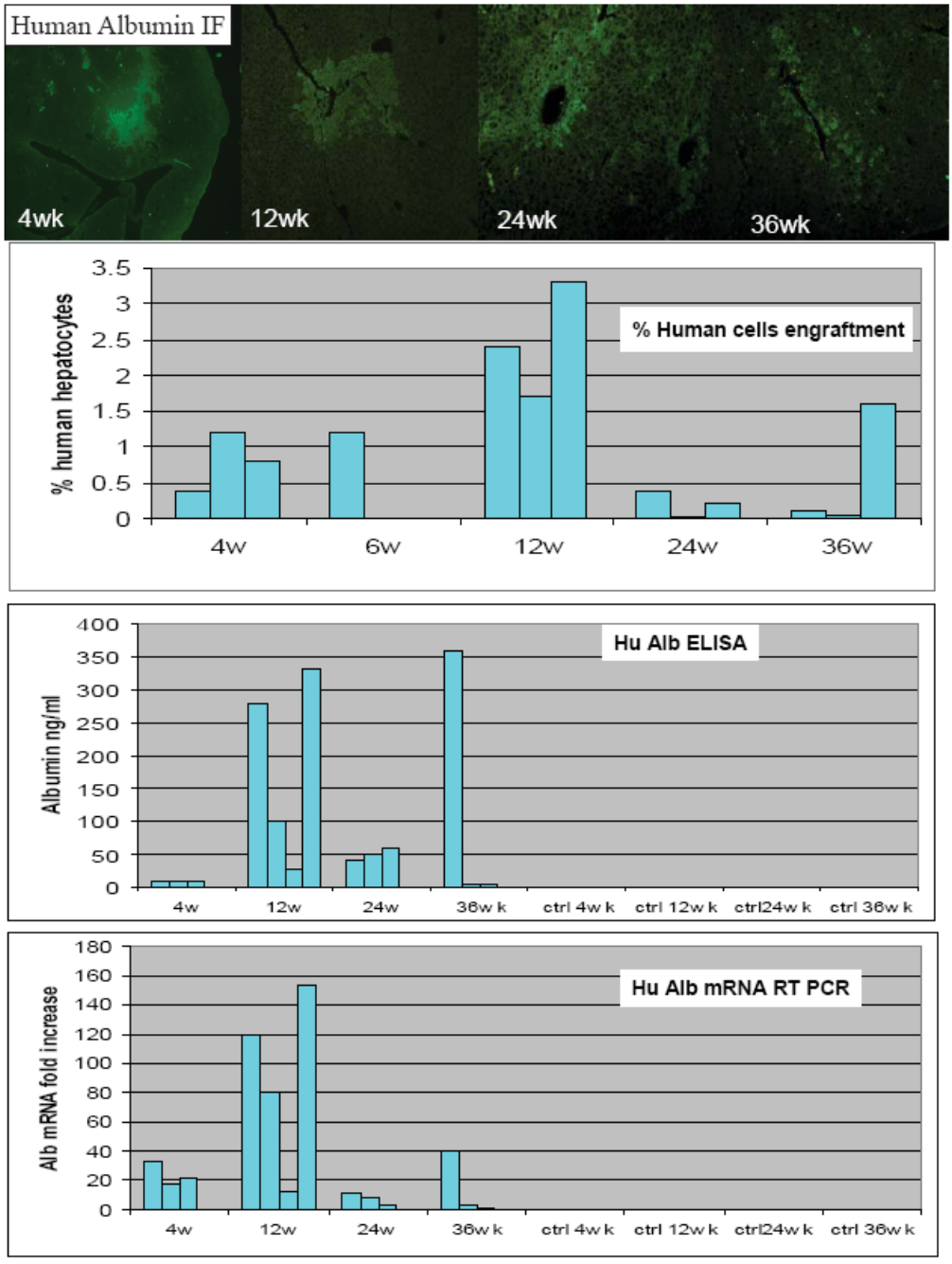


Figure 6.8 Temporal Analysis of Functional Engraftment of Human Hepatocytes

Quantitaion of % engraftment and albumin production by ELISA (serum) and RTPCR (liver lystae) are analysed at 1,3,6 and 9 months post-transplant. Peak engraftment of up to 3.5% occurrred at 3 months and dropped significantly after although 1 animal continued to show robust human cell engraftment up to 9 month. Albumin ELISA and mRNA RT PCR corroborated with engraftment % trend.

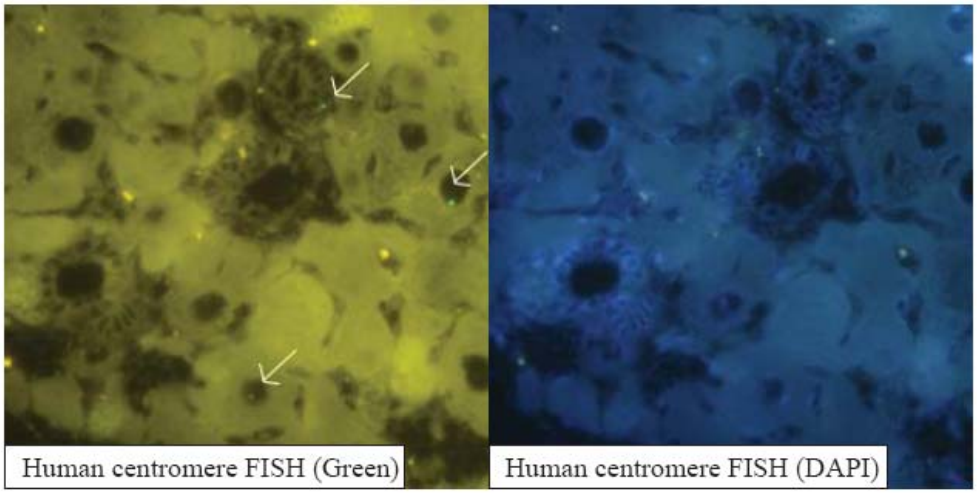


Figure 6.9 *In situ* Hybridisation of Human Centromere at Bile Ducts

Although CCL4 injury is primarily hepatocytic, rare bile duct cell is noted to arise from human cell lineage, staining positive for human centromere.

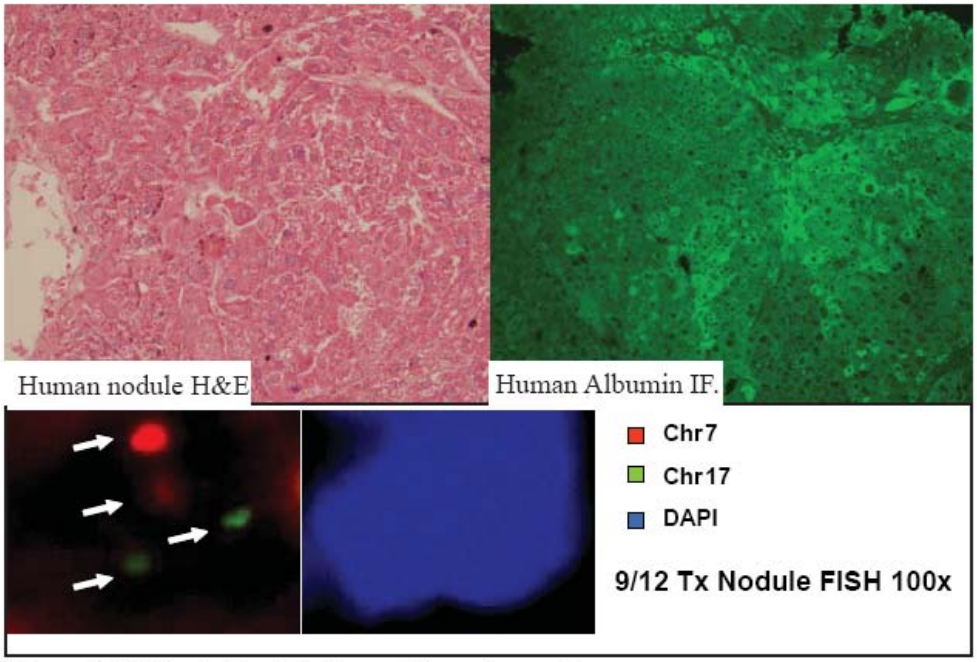


Figure 6.10 Excluding Malignant Transformation

A nodule cluster of human cells from 9 month transplanted animal was analysed for malignant transformation using FISH for chromosome 7 and 17, the two most commonly reported chromosome in liver cancer aneuploidy. None of the nucleus examined showed chromosomal aneuploidy of chromosome 7 and 17.

Table 7 Pilot Trial of optimal conditions for *in vivo* transplantation

Animal Model	# of mice	Repopulation
Nude Mice	2 transplanted: 1 control	0%
Rag γc double KO	2 transplanted: 1 control	0.93- 1.14%
Delivery of Cells		
Intra-portal	1	0.87%
Intra-splenic	1	0.922%
Ischemia- reperfusion + Intra- splenic	1	1.14%
Regenerative Stimulus		
Partial Hepatectomy	1	0.98%
Carbon Tetrachloride	1	0.91%
Retrorsine preconditioning		% died within 2D
60ug/kg intraperitoneal	2	0%
70 ug/kg intraperitoneal	2	50%
80ug/kg intraperitoneal	2	100%

CHAPTER 7

Discussion

Human fetal liver progenitor cells holds tremendous promise in that they represent a entity that will allow us to understand normal precursor-progeny development of hepatocytes and biliary cells.

They appear to be a distinct population in human fetal liver and can be enriched and isolated using a novel technique that capitalized on unique characteristics of their resistance to geneticin and requirement for feeder layers. Interestingly, they have mixed mesenchymal-epithelial properties and show multipotency in being able to differentiate into progenies of both these lineages.

7.1 Where do fetal progenitor cells come from?

The fetal liver contains a plethora of cells at mid-trimester. It is the nesting ground of hematopoietic stem cells, mesenchymal stromal cells, hepatoblast, maturing hepatocytes, biliary cholangiocytes as well as liver non-parenchymal cells such as Kupffer cells, myofibroblast and endothelial cells. Prospective origins of our fetal liver progenitor cells would include

1. Dedifferentiated cell from hepatoblast/hepatocytes
2. Hematopoietic stem cell origin
3. Mesenchymal stem cell origin

7.1.1 Human fetal liver progenitor cells are not dedifferentiated hepatocytes

To prove that the progenitor cells are a natural entity within the fetal liver and not a culture artifact due to dedifferentiation of mature hepatocytes in culture, immunohistochemistry was performed on fresh fetal liver across various gestations staining for EPCAM and CD44. Cells of similar phenotype were seen at the ductal plates in liver specimens of less than 14/52 gestation. By about 16 weeks, these cells start radiating out from portal tracts, suggesting that these cells exist de novo in fetal livers.

To exclude the possibility of hepatocytes dedifferentiating into progenitor cells, we performed lineage tracing by tagging the hepatocytes in primary fetal liver culture. Percoll density fractionation was used to isolate hepatocyte subfraction which was determined to be 89% pure using immunofluorescence with human albumin. These cells were transfected with GFP-labeled lentiviral vectors driven by the EF1alpha promoter [143-144]. Transfection efficiency was almost 90%. Primary hepatic cultures were then reconstituted by adding labeled hepatocytes with unlabelled non-parenchymal fraction from the same specimen into coculture. The same enrichment protocol was used and progenitor cells isolated using the same technique described. Progenitor cells colonies were then checked to see if they were GFP producing. GFP positivity would imply that they were derived from GFP labeled hepatocytes that had dedifferentiated in cultures. In all 10 colonies studied, none of them were GFP expressing making it unlikely by chance, for progenitor cells to be derived from dedifferentiating hepatocytes. (Figure 7.1)

7.1.2 Human fetal liver progenitor cells are not hematopoietic stem cells derived.

Human progenitor cells can be identified on fetal liver histology and was noted in ductal plates and portal tracts. These were anatomically separate from hematopoietic stem cells seen in vascular sinusoids making them unlikely to be of hematopoietic origin. CD45 and Sca-1 antibody staining (markers for hematopoietic origin)[145] were performed and progenitor cells were uniformly CD45 and Sca-1 negative.

7.1.3 Human fetal liver progenitor cells are not mesenchymal stem cell derived

Using a similar lineage tracing technique, we isolated mesenchymal stem cell fraction using Percoll. Purity was 85% using Cd44 immunofluorescence count and mesenchymal stem cells were transfected with GFP lentivirus up to 90% efficiency. Similar constitution was performed with unsorted culture from the same specimen. Of 8 colonies isolated using the same technique described, none of the progenitor cell colonies were positive for GFP. (Figure 7.2) These progenitor cells were also negative for mesenchymal markers such as CD73, CD105 and CD31[145]. Taken together, it is thus very unlikely that fetal liver progenitor cells had arisen from mesenchymal stem cells.

7.2 A meso-endodermal cell that undergoes epithelial mesenchymal transition

Human fetal liver progenitor cells co-express epithelial and mesenchymal markers and are able to transit between the two lineages, differentiating into liver cells and mesenchymal lineages. Could the mixed epithelial and mesenchymal phenotype and multipotency in differentiation been due to impure culture of both epithelial progenitor cells and mesenchymal support cells? Co-localisation studies have shown that the same cell co-expresses both epithelial and mesenchymal features

simultaneously. (Figure 4.3) Differentiation experiments had been performed from colonies that were clonally derived and clonality of colonies derived using the method had been validated with Humara assay, In addition, cultures were propagated in multiple passages and observed under microscope to be uniform, homogenous and of high quality in morphology before being subjected to differentiation protocol. NIH 3T3 feeder cells have been irradiated or mitomycin treated and in repeated cultures that numbers in hundreds of combined passages, escape with overgrowth of NIH3T3 has never been observed. In addition, all these observations and experiments including dual lineage immunophenotype and potency in differentiation have been successfully replicated and reported subsequently in 2008 by other researchers[43-44, 146], corroborating our data.

Human hepatocytes have been traditionally thought to be endodermally derived. Yet elsewhere in the animal kingdom, 80% of the turtle liver is apparently derived from the mesenchyme[147]. Additionally, multiple studies have shown that MSCs or mesoderm-related multipotent adult progenitor cells in both mice and humans can differentiate into hepatocytes[101, 107]. Mesenchymal–epithelial transitional cells are not uncommon in fetal development[148] and have been demonstrated in both rodent neonatal[149] and fetal livers[150], as well as human fetal livers, in which they have been reported to support hematopoiesis[127]. It has been hypothesized that mesoderm and endoderm arise from a common bipotential mesendoderm precursor[151]. Tremblay and Zaret[152] demonstrated that cells from the ventral midline of the endoderm lip, probably originating from the mesendodermal prechordal plate, give rise to part of the liver bud. Human Fetal liver progenitor cells appear to have mesendodermal origin, but further fate-map analysis and characterisation of liver specification profiles will

determine whether they arise from the cells described by Tremblay and Zaret[152] or are from a distinct progenitor niche. We thus propose that human fetal liver progenitor cells are mesenchymal–epithelial transitional cells and suggest that they have a mesendodermal origin.

7.3 What happens to these cells in adult liver

Schmelzer et al[43-44] has subsequently reported similar cells that they have isolated from both fetal and adult liver. These cells have the same immunophenotype and appear to be the progenitor cells that reside in the canals of Hering in adult liver. This would suggest that this population of progenitor cells exist from fetal liver and are maintained through adult life as a quiescent niche, to be activated only when there is significant injury to the liver to help repair and regenerate the liver.

7.4 Future Directions

7.4.1 A critical analysis of obstacles to use of stem cells in therapeutic applications

Significant challenges still exist before stem cells can be translated into clinical applications to treat patients. These challenges include the following:

7.4.1.1 Continual sufficient supply of hepatocytes either from progenitor cells or transdifferentiated from multipotent cells.

Human derived cells are needed in large numbers for purposes of transplant. It is projected that a normal sized adult will require at least 200 million cells to have a practical sizeable functional benefit. Though stem cells are self replicative, they need to be scaled up significantly in short period of time.

7.4.1.2 Understanding of regulatory mechanisms that control lineage transdifferentiation and hepatocyte specification.

The key regulatory genes that specify mesenchymal to epithelial transition have not been identified. The sonic hedgehog pathway, βcatenin, c-met and E-cadherin pathways[153],[11] have all been reported to be involved in stem cell homeostasis, maintenance and differentiation. Understanding the role of these markers may yield targets to further augment the differentiation pathway so as to increase the efficiency of hepatocyte differentiation.

7.4.1.3 Meaningful and practically useful physiological function from *ex vivo* hepatocytes for the purpose intended.

Current assessment of hepatocyte function such as albumin, indocyanine green, urea synthesis and cytochrome P450 have been mostly qualitative. Many of the hepatocytic functions are also not specific to the liver[154]. For cellular transplants for liver failure, cells will need to demonstrate adequate clotting factors secretion and ammonia metabolism. For drug testing, cytochrome P450 functional assays and drug metabolism and clearance capacity will need to be established.

7.4.1.5 Efficacy in survival outcomes in transplantation or dialysis.

Repopulation is the surrogate marker of *in vivo* engraftment for most animal studies. While these show principle of proof success, demonstration of therapeutic efficacy such as improvement in survival or liver function parameters such as bilirubin, prothrombin time and ammonia are needed[154]. Large animal studies of cellular transplant or artificial assisted liver devices are also lacking due to the deficiencies in animal models of liver injury that closely reflect the clinical diseases seen in patients.

7.4.1.6 Safety from avoidance of animal products contact (GMP grade) as well as no increased risk of oncogenesis.

Most ES cell lines were reported to be contaminated with animal peptides when grown on feeder layers. To move cells nearer to clinical application, Good Medical Practice (GMP) protocols need to be developed to avoid contamination. Careful testing of cytogenetics of derived cells will need to be done to screen for oncogenic potential as well as long term study of transplantation of progenitor cells are needed.

7.4.1.7 Ability to cryopreserve these cells with high viability for use when needed

For therapeutic applications such as transplants to be useful, the ability to cryopreserve large number of cells so that they can be effectively used when needed has to be demonstrated. While hematopoietic cells and murine fetal hepatocytes have shown resilience during cryopreservation, human hepatocytes have shown significant deterioration during cryopreservation and large scale storage remains a challenge. Vitrification of ES cells has been shown to be a superior method of cryopreservation but large scale vitrification of human hepatocyte colonies for the purpose of transplant has not been studied.[155]

7.4.1.8 No rejection of engrafted cells.

In the clinical setting, while it will be ideal to grow one's own autologous cells, the pace of acute liver failure will likely necessitate the use of banked similar blood group cells. Rejection continues to pose a challenge to the feasibility of cellular transplant.

To attain the goal of using these cells for therapeutic applications, the following future directions are being pursued:

7.4.2 Optimising cultures to avoid feeder layers and animal products

For the purpose of transplantation, cells need to be free from animal products to avoid risks of zoonosis. Our current culture protocols uses NIH 3T3 feeder cells which are mouse embryonic fibroblasts as well as several culture products that are animal derived. We have replaced collagen, laminin and FGF with human recombinant versions and also replaced bovine serum with human serum with no observable deleterious effect on culture of these cells.

To overcome the need for feeder layers, progenitor cells were isolated using magnetic flow sorting using EPCAM antibodies conjugated with magnetic beads. Two rounds of magnetic sort yields 95% pure population of progenitor cells that are identical to the cells we have described. These cells are cultured on laminin at high density in transwell membrane or special silicon nitride membranes (in collaboration with Prof Hanry Yu) using human fetal liver fibroblasts as feeder layers. Preliminary results have shown successful maintenance of these cells using a combination of collagen, laminin as well as hyaluronic acid. Cell-cell contact appears to be critical for maintenance of these cells.

7.4.3 Study on genetic determinants of stem cell regulation and differentiation

Preliminary results with collaborators have implicated the role of sonic hedgehog pathways in the signaling of stem cell maintenance as well as differentiation and epithelial mesenchymal transition[34]. Microarray analysis will be performed on

progenitor cells as well as differentiated cells to look for unique genetic signatures that would identify new surface markers to isolate these cells as well as unravel genes involved in stem cell regulation and differentiation.

7.4.4 Establishment of therapeutic effect

To document principle of proof of fetal progenitor cells in being able to have a therapeutic effect, experiments are currently underway using DDC diet and galactosamine to create acute and chronic liver failure models of irreversible progressive liver injury. Progenitor cells will be transplanted to determine their potential to reverse the effects.

7.4.5 Serial Transplantation of Human Progenitor cells.

The ultimate proof for a cell to be considered a stem cell is their ability for single cell serial transplantation in animal models. This has been achieved for bone marrow hematopoietic stem cell. The true stem cell will have to be clonally derived under time-lapse microscope tracing. Clonal colonies will need to be marked with lentivirus before transplantation into animal model of liver injury. They will then need to be re-harvested, isolated and retransplanted into another animal to give rise to all lineages of the liver to fulfil this criteria of a stem cell.

7.4.5 Risks of Malignancy

The more pluripotent a stem cell is, the higher the risk of malignancy. Just as embryonic stem cells or induced pluripotent stem cells harbour the highest risk of teratoma, fetal stem cells being highly plastic, will always need to pass the most stringest tests on theirs risks of malignancy before human trials can be attempted.

Cells will need to be transplanted in the subcutaneous tissue of SCID mice and followed up long term for up to 1 year to document their risks of malignancy. Similar cytogenetics testing will need to be donew on long term cultures of these cells.

7.5 Conclusion:

We have isolated clonal human fetal liver progenitor cells that are capable of giving rise to lineages of the adult liver. Systematically characterizing their phenotype and ideal niche culture environment, we were able to capitalise on their relative resistance to geneticin and isolate them using a novel chemical-mechanical technique. High density feeder layers, laminin matrix signaling and fibroblast growth factors are needed to maintain their 'stem cell" state and to keep them proliferating. These cells behave more like human stem cells rather than mouse progenitor cells. Human fetal liver progenitor cells appear to have durable proliferative capability and can be maintained in culture long term with preservation of telomere lengths. They demonstrate multipotent differentiation potential and can be induced to differentiate into functional hepatocytes and biliary duct cells *in vitro*, even after being kept in culture for 6 months. Differentiated hepatocytes can secrete albumin into supernatant, store glycogen, metabolise drugs through inducible cytochrome P450 enzymes and be infected with Hepatitis B viruses. They can undergo epithelial-mesenchymal transition and be differentiated into mesenchymal cell lineages including cartilage, bone, adipocytes and endothelial cells. Transplanted directly into injured livers of immuno-permissive murine models and given a competitive advantage and growth stimulus, they can differentiate into hepatocytes and bile duct cells. These differentiated hepatocytes survive up to 9 months post transplant. They integrate with

native mouse liver and are functional, expressing human proteins with no evidence of transformation.

The successful isolation of these cells has tremendous implications. The ability to infect these cells with Hepatitis B infection would provide valuable in-vitro models for studying Hepatitis B infection as well as development of new drugs against Hepatitis B. More importantly, fetal liver progenitor cells provide natural unadulterated cell model to understanding the critical factors and mechanisms that determine their stemness, proliferation as well as differentiation and lineage transition. This knowledge can be then applied to other stem cell source in our attempts to derive large numbers of functional hepatocytes. This will move us nearer to the eventual goal of developing progenitor/ stem cell therapies for patients with liver failure.

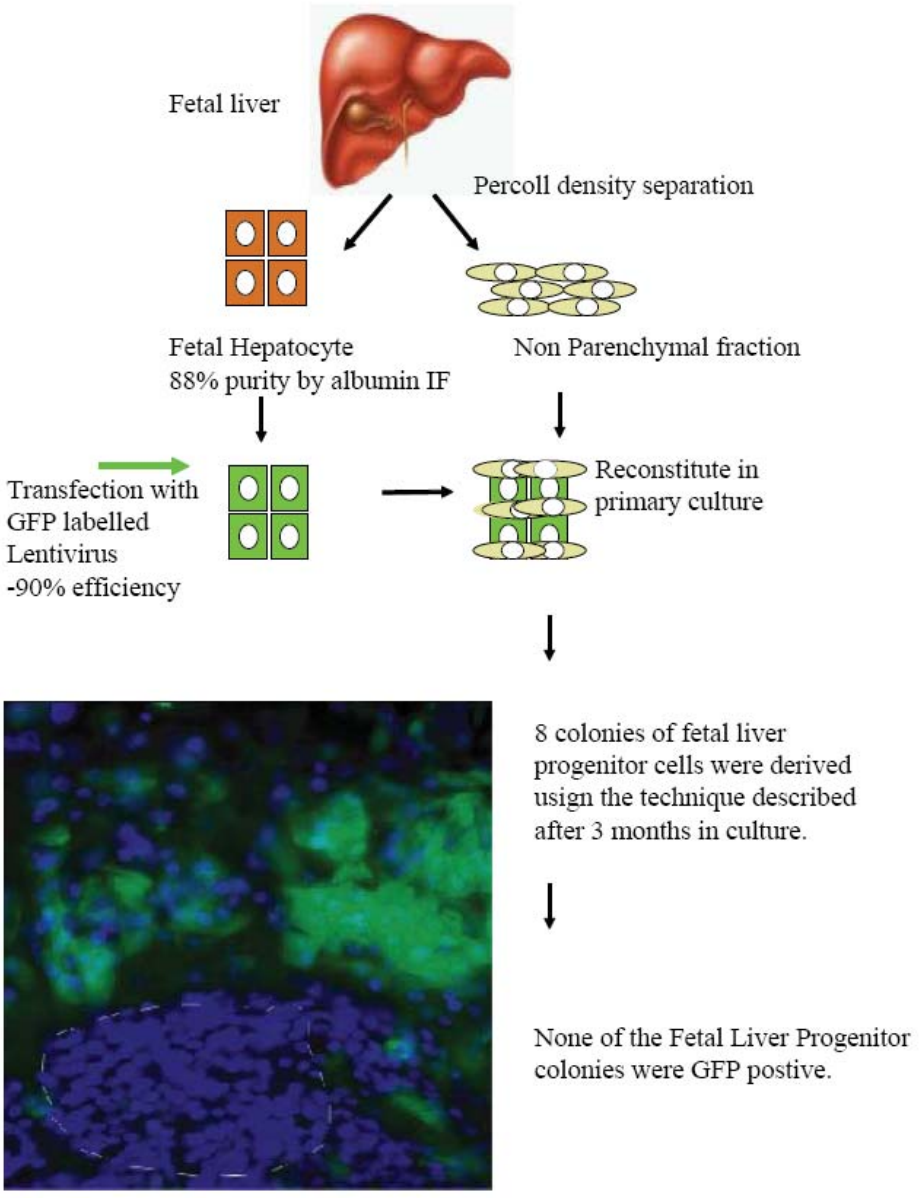


Figure 7.1 Lineage Tracing of Fetal Liver Progenitor Cells: FLPC are not dervied from Dedifferentiating Fetal Hepatocytes

Fetal hepatocytes were isolated up to 90% purity and labelled with GFP before reconstituting with non-hepatocyte subfraction . Progenitor colonies were dervied 3 months later and GFP stautus determined. GFP positivity of any colonies would indicate derivation from fetal hepatocytes. Negativity of GFP does not exclude this possibility completely but would make it very unlikely that progenitor cells are derived from hepatocytes dedifferentiating in culture.

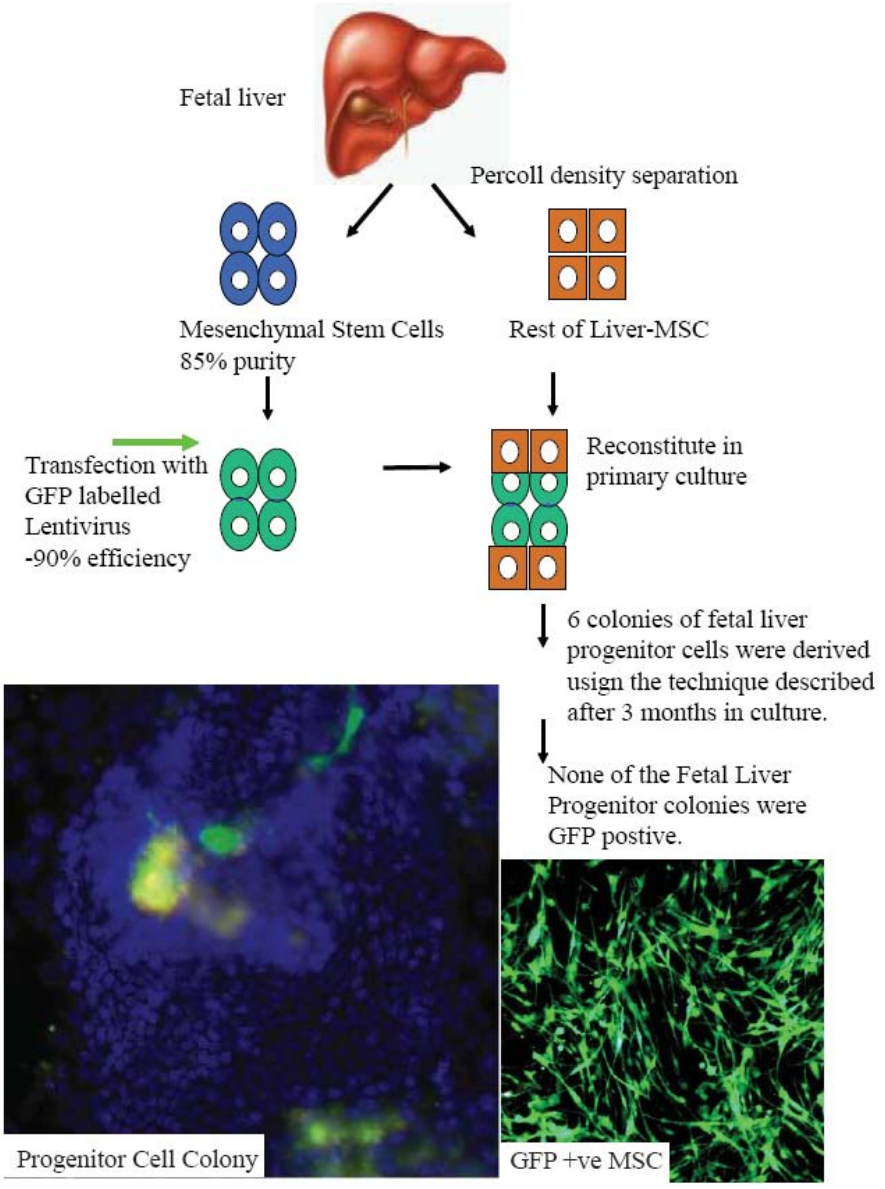


Figure 7.2 Lineage Tracing of Fetal Liver Progenitor Cells: FLPC are not dervied from Mesenchymal Stem Cells

Mesenchymal SCs were isolated at 85% purity and labelled with GFP before reconstitution in fetal liver cultures and corresponding progenitor cells were isolated at 3 months. GFP positivity of any progenitor colonies would indicate derivation from MSCs. Negativity of GFP does not exclude this possibility completely but would make it very unlikley that progenitor cells were derived from mesenchymal stem cells.

CHAPTER 8

Detailed Materials and Methods

8.1 Cell Culture

8.1.1 Source and isolation of human fetal liver progenitor cells

Human fetal livers were obtained from the Central laboratory for Human Embryology at the University of Washington as well as Dept of Obstetrics and Gynecology at National University Hospital in accordance with protocol approved by the institutional review board of both Universities. Primary fetal hepatocyte cultures were raised and kept in culture using the Fetal Hepatocyte medium (CSHFM) as previously described.[50] Cultures were maintained for at least three months to allow enrichment of small blast cells before they were passaged with 1mg/ml collagenase(Boehringer Ingelheim, Ingelheim,Germany) and 10U/ml dispase (BD Biosciences, Bedford, MA). 50ng/ml geneticin (Gibco/Invitrogen, Carlsbad, Ca) was added to the P1 culture media immediately after passage for 6 days. Colonies of progenitor cells were then mechanically isolated under microscopic visual guidance and transferred onto feeder culture plates.

8..1.2 Determining ideal Culture Conditions

To test the ideal conditions for fetal progenitor cultures, three elements including culture medium, extracellular matrix substrate as well as feeder layers were studied. Plates were coated with laminin (5ug/ml), collagen (1:5), fibronectin (5ug/ml), gelatin (5ug/ml) and hyaluronic acid (Suplasyn, Bioniche Life Sciences Inc., London, Ontario, Canada), washed and air-dried before use.

8.1.3 Culture and cloning

Fetal liver progenitor cells were cultured on irradiated NIH 3T3 feeder layers (180 000 cells/ml) coated on collagen(Vitrogen; Collagen Corp., Fremont, CA) /Laminin (Invitrogen, Carlsbard, CA) substrate and maintained with 50% CSHFM minus dexamethasone, 20% FBS and 30% conditioned medium which was the medium supernatant used for primary hepatocyte culture. This was further supplemented with bFGF (10 ng/ml) and complex fatty acids (5 mls/l) (both from Sigma Aldrich, St. Louis, MO). Medium was changed daily and cultures maintained in incubator at 37'C at 6% CO2. Cloning was performed with dissociation by collagenase 1mg/ml and serial dilution. Daughter colonies were derived from single or at most 2 cell clusters on feeder layers as witnessed under inverse microscope. Colonies were then passaged every 10 days using a cut and paste technique similar to that used in human ES cell cultures protocols.[156]

8.1.4 *In Vitro* Differentiation Protocol

All differentiation protocols were performed in triplicate with P1-P4 and P8-P12 progenitor colonies. Percoll gradient separation was used to isolate human fetal mesenchymal stem cells[157] and parenchymal primary fetal hepatocytes[158] for use as comparison controls. Basic culture medium was purchased from Gibco/Invitrogen (Carlsbard, CA) and supplements were from Sigma Aldrich (St Louis, MO) unless otherwise specified.

8.1.5 Hepatocyte Differentiation

Cells were plated onto collagen coated plates at density of $1X10^4$ per cm2. Cultures were treated with standard hepatocyte culture medium CSHFM. Oncostatin M

(OSM) (10 ng/mL; R&D Systems, Minneapolis, MN) was added from D3 to further augment liver specific markers

8.1.6 Bile duct Differentiation

Cells were cultured in bilayer 3D fibrillar collagen gel as described.[159] Collagen was prepared using 8 parts collagen:1 part 1M NaOH and 1part 10X PBS, neutralized to pH7.4 and allowed to gel at 37'C in room air. 2 days after cell colonies have attached and spread out as a monolayer, a second layer of gel was added to form a sandwich gel culture. Medium for bile duct differentiation was constituted with Williams E with 50% conditioned medium and supplemented with 100nmol/l insulin, 5μmol/l hydrocortisone, 10ng/ml EGF (BD Biosciences,Bedford, MA), antibiotics and daily pulse of 20ng/ml FGF. Medium was changed every 3 days.

8.1.7 Fat differentiation

To induce fat differentiation, cells were cultured on collagen at a density of $1X10^4$ cells/cm2. Adipogenic medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (Low glucose) supplemented with 0.5 mmol/L 3-isobutyl-1-methylxanthine, 1 μmol/L hydrocortisone, 0.1 mmol/L indomethacin, and 10% horse serum and was changed twice weekly. Adipogenesis was determined by Oil Red O staining and RT PCR for human PPARγ2.

8.1.8 Osteogenic Differentiation.

Osteogenic differentiation was induced with DMEM(High glucose) supplemented with 0.1 μmol/L dexamethasone, 10 mmol/L β-glycerol phosphate, and 0.2 mmol/L ascorbic acid, 10% FBS and antibiotics. Cells were cultured on fibronectin with

medium change every 3 days for period of 28days. Osteogenesis was assessed by osteopontin RT PCR and von Kossa staining for calcium deposit.

8.1.9 Chondrogenic Differentiation.

Cells were centrifuged at 1000 rpm, 5 minutes, to form a pelleted micromass and cultured in suspension in polypropylene tube. Chondrogenic medium consisted of DMEM supplemented with 0.1µmol/L dexamethasone, 50µg/mL ascorbic acid, 100µg/mL sodium pyruvate (Gibco/Invitrogen, Carlsbard, CA), 40µg/mL proline (Sigma-Aldrich), 10 ng/mL transforming growth factor-β3, and 50 mg/mL ITS+ (insulin, transferrin, selenium) premix (BD Biosciences, Bedford, MA). Medium changes were performed twice weekly, and chondrogenesis was assessed by histochemical staining for proteoglycans as well as immunohistochemical identification of cartilage specific type II collagen.

8.1.10 Endothelial Differentiation

Endothelial differentiation was induced with DMEM (High glucose) supplemented with 10% FBS, antibiotics and PDGF-CC[91] (10 ng/ml) in fibronectin substrate. Medium was changed every 2 days and cells tested for presence of CD31 and vWF by immunofluorescence after 9 days of induction.

8.2 Characterisation of Fetal Liver Progenitor cells.

8.2.1 Immunohistochemistry and Immunofluorescence

Progenitor cell colonies were treated with 0.25% trypsin to remove feeder layers, harvested and frozen in OCT (Electron Microscopy Sciences, Fort Washington, PA). All other monolayer cells were cultured in lab-tek slide chambers and processed fresh.

Sections for immunofluorescence were fixed with 3.5% cold paraformaldehyde for 5 mins, -20'C methanol for 2 mins followed by 0.5mM glycine to reduce paraformaldehyde fluorescence. Slides for immunohistochemistry were fixed with 1:1 volume mixture of -20'C methanol/acetone followed by 0.3% H2O2 to block endogenous peroxidase. Sections were then blocked with appropriate serum (2% to 10%) for 30 minutes. Primary antibodies against human antigens were applied to slides for 1 hour at room temperature and they include anti-albumin (ICN Pharmaceuticals, Aurora, OH), α-fetoprotein, CK7, vWF (Dako Cytomation, Inc., Carpinteria, CA), α1-antitrypsin (α1AT), vimentin (Sigma Aldrich Co. St. Louis, MO), CD44h (Hybridoma bank, University of Iowa), cmet, ckit(R&D Systems, Inc., Minneapolis, MN) , CD34, CD31-phycoertythin conjugated, CD105 and CD90 (Thy-1) (BD BioSciences/Pharmingen, Franklin Lakes,NJ), EPCAM (Oncogene Research Products,Boston, MA) SSEA-4, cytokeratin 18 (ICN Pharmaceuticals), CK19 (Amersham, Buckinghamshire, England). For immunohistochemistry, Horseradish peroxidase–conjugated anti-mouse, anti-rabbit and anti goat antibodies (all from Vector laboratories, Burlingame, CA) served as secondary antibodies with positive detection based on reaction with 3,3-diaminobenzidine tetrahydrochloride. Fluorescent detection was by Alexa Fluor® 488 or Alexa Fluor® 594 conjugated secondary antibodies (Molecular probes/Invitrogen, Carlsbad,CA). Nuclei were cross stained with 4',6-Diamidino-2-phenylindole (DAPI). Specificity was confirmed using immunoglobulin G–specific controls. Cell morphology were studied with Nikon Inverse phase contrast microscope and immunofluorescence was captured by Nikon Eclipse 600 immunofluorescent microscope or confocal Leica TCS SP/NT on an DMIRBE inverted microscope. Percentage of positive cells was determined by

manual counting of positive immunofluorescent cells to DAPI positive nucleus in 3 separate random fields (at 40X magnification objective) on the Nikon microscope.

8.2.2 Histochemistry

Periodate-Schiff technique was performed according to that described by McManus.[160] GGT assay was performed according to the method of Rutenburg.[134] Bony calcification was detected by von Kossa stain while cartilage specific proteoglycans were stained with sofranin-O and fast green stain.

8.2.3 Transmission Electron Microscopy

Specimen preparation for electron microscopy used standard techniques. Cells grown in standard culture dishes were fixed with Karnofsky's fixative and postfixed in 1% osmium tetroxide before dehydration and embedding in Epon.

8.2.4 Enzyme-Linked Immunosorbent Assay

Human albumin concentrations from culture media were measured by the sandwich enzyme-linked immunosorbent assay as previously described.[50] The lower limit of sensitivity of the human albumin assay was 15 pg/mL and was linear up to 1,000 μg/mL. The capture antibody (goat anti-human antibody; 4 μg/mL), the detection antibody (peroxidase-conjugated goat immunoglobulin G fraction to human albumin; 3 μg/mL), and the purified human albumin standards were purchased from ICN Pharmaceuticals (Durham, NC).

8.2.5 RTPCR

Using Trizol reagent (Ambion, Austin, TX), total RNA was isolated from P3-P5 colonies of hFLMPC, partially differentiated hepatocytes at D3 of differentiation protocol, differentiated hepatocytes at D20 of differentiation protocol and cells treated with 21 days of adipose or bone induction. One μg of RNA was reverse transcribed (Ambion Retroscript kit), and RT-PCR was used to determine expression of several genes using specific primer pairs (see table 2). In all cases, PCR cycle analysis was first performed to determine the linear range of each amplicon for a given primer pair. Expression levels of each gene were normalized to that of β-actin or 18S ribosomal subunit as described in the Quantum RNA kit (Ambion).

8.2.6 Western Blot

Protein cell lysates and immunoblotting were performed as previously described.[161] Antibodies used were similar to those used for immunostaining as described above. β-actin (Sigma-Aldrich, St Louis MO) served as a loading control. Antigen-antibody complexes were detected with the appropriate horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz,CA). Semi-quantitaion of bands, where relevant, was performed using ImageQuant™ TL software (GE Healthcare Technologies,Waukesha, WI)

8.2.7 Cytochrome P450 Assay

Cytochrome P450 Cy2B6 was induced in cells by adding phenobarbitone 2μM to culture medium for 72 hours. Cy2B6 activity was tested by incubating cells with 5uM Pentoxyresorufin O Dealkylase assay (PROD), with 20uM dicumerol (all from Sigma-Aldrich, St Louis, MO) and DMEM without phenol red for 30 mins. The amount of resorufin fluorescence was read with an excitation wavelength of 530 nm

and an emission wavelength of 590 nm using Packard Fluorocount (Packard Instrument Co., Meriden, CT) using reference standards of 5nM to 1200nM resorufin. (Molecular probes, Carlsbad,CA). PROD activity was then normalized to protein concentration and expressed as per microgram/ml of protein.

8.2.8 Cell Proliferation Assay and Doubling Time

Bromodeoxyuridine (BrdU) (Roche Diagnostics Corp, Indianapolis, IN) was added to cells at final concentration, 10 umol/L for 48 hours. BrdU labeling was then determined by immunohistochemistry using anti BrdU Clone 20U (Dako Cytomation, Inc., Carpinteria,CA)

8.2.9 Cell doubling and growth curve.

Progenitor cells were cultured in 48 well plates with feeder layers. Cells were incubated with 100ul of solution containing 1mg/ml of XTT, 7.5 ug/ml phenazine methosulfate (all from Sigma Aldrich Co. St. Louis, MO) and phenol red free DMEM, every day for 30mins. The assay is based on the conversion of the yellow tetrazolium salt XTT into an orange formazan dye by metabolically active cells and has been previously shown to be linearly related to the number of cells.[162] Activity due to progenitor cells as measured using BIORAD Smartspec 3000 at A450nm (Bio-Rad laboratories., Hercules, CA), was calculated from XTT(hFLPC+ Feeder layer) – XTT(Feeder layer only) from triplicate experiments. Log values were then plotted against time to obtain growth curve from which doubling time was derived

8.2.10 Telomere Length Determination

Telomere length assay was performed on D4 primary fetal hepatocyte, P1, P10 and P20 progenitor cells using TELOTAGGG telomere length assay (Roche Diagnostics Corp, Indianapolis, IN) according to manufacturer's instructions.

8.2.11 Cell labeling for origin of fetal liver progenitor cells

Cells were transfected with GFP using lentiviral vectors of up to 90% efficiency. To determine origin of fetal progenitor cells, fetal liver was dissociated and parenchymal and mesenchymal stem cell fractions were separated using percoll density centrifugation.[157-158] Purity of parenchymal and mesenchymal cells was determined by random sampling and counting albumin and CD44 immunofluorescence respectively. Purity was in excess of 90% in both fractions. GFP transfected parenchymal fraction were then cocultured back with unlabelled non-parenchymal fraction and GFP transfected mesenchymal stem cells were similarly cross cultured with unlabelled primary fetal hepatocytes. Progenitor cells were derived 3 months later and expression of GFP was determined in these cells.

8.2.12 Determination of cell clonality

Clonality of progenitor cells was determined using the Humara Assay as described based on concept of random X chromosome inactivation.[132] PCR was first performed to select progenitor cultures that were from females heterozygous for Humara allele. Clones were then derived and DNA isolated from P3-5 colonies. 1ug of DNA were digested with Hha I (Promega Corp. Madison, WI) in accordance to manufacturer's instructions. Nested PCR was then performed as previously reported.[163] PCR products were ran on 6% polyacrylamide gels and visualized with ethidium bromide to determine if samples had non-random X inactivation, and hence were clonal.

8.3 *In vivo* Transplantation

4- to 6-week-old $Rag2^{-/-}\gamma c^{-/-}$ mice[137](C57BL/6J × C57BL/10SgSnAi)-[KO]gc-[KO]Rag2; Taconic Farms) were used for *in vivo* transplantation of hFLMPCs and maintained in accordance with animal care protocols. All mice were treated with weekly retrorsine (60 mg/kg i.p.) for 3 weeks to inhibit replication of native hepatocytes. Three mice then underwent intrasplenic injection with enriched fetal liver progenitor cells (3×10^6 cells); the remaining three mice underwent a sham operation (laparotomy). i.p. carbon tetrachloride (0.5 ml/kg diluted 1:10 in olive oil) was injected 1 week later and repeated weekly for 2 more weeks in all mice, and all were harvested 4 weeks after transplantation. Expression of human albumin was determined by serum ELISA, RT-PCR.

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Isolation of multipotent progenitor cells from human fetal liver capable of differentiating into liver and mesenchymal lineages

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Little is known about the differentiation capabilities of nonhematopoietic cells of the human fetal liver. We report the isolation and characterization of a human fetal liver multipotent progenitor cell (hFLMPC) population capable of differentiating into liver and mesenchymal cell lineages. Human fetal livers (74–108 days of gestation) were dissociated and maintained in culture. We treated the colonies with geneticin and mechanically isolated hFLMPCs, which were kept in an undifferentiated state by culturing on feeder layers. We derived daughter colonies by serial dilution, verifying monoclonality using the Humara assay. hFLMPCs, which have been maintained in culture for up to 100 population doublings, have a high self-renewal capability with a doubling time of 46 h. The immunophenotype is: CD34+, CD90+, c-kit+, EPCAM+, c-met+, SSEA-4+, CK18+, CK19+, albumin−, α-fetoprotein−, CD44h+, and vimentin+. Passage 1 (P1) and P10 cells have identical morphology, immunophenotype, telomere length, and differentiation capacity. Placed in appropriate media, hFLMPCs differentiate into hepatocytes and bile duct cells, as well as into fat, bone, cartilage, and endothelial cells. Our results suggest that hFLMPCs are mesenchymal–epithelial transitional cells, probably derived from mesendoderm. hFLMPCs survive and differentiate into functional hepatocytes *in vivo* when transplanted into animal models of liver disease. hFLMPCs are a valuable tool for the study of human liver development, liver injury, and hepatic repopulation.

epithelial–mesenchymal transition | liver differentiation | liver progenitor cell

The potential applicability of the transplantation of hepatocytes or liver progenitor cells for the treatment of liver diseases has received much attention. Central to the success of this approach is an understanding of hepatic cell lineages and the main steps that give rise to hepatocytes during embryonic development. Liver progenitor cells persist in the adult rodent liver in the canals of Hering (1, 2). These progenitor cells give rise to oval cells, which can generate both hepatocytes and biliary epithelial cells when hepatocytes are unable to mount a proliferative response to injury (3–5).

In rodents, there has been considerable success in isolating precursor cells from the fetal liver and liver epithelial cells or oval cells from adult liver (5–7). Suzuki *et al.* (8, 9) isolated a mouse fetal liver stem cell (c-met+/CD49F+/CD29+/CD45−/CDTER119−) that not only differentiated into hepatocytes and bile duct cells but also was capable of differentiating into intestinal and pancreatic epithelial cells. In humans, isolation of liver stem cell lines has proven more difficult, and to date no unmodified human embryonic liver progenitor cell lines have been reported (10).

Given the diversity of potential hepatoblast precursors described in the mouse, we hypothesized that the human fetal liver might contain intermediate cells in the differentiation pathway from endoderm to hepatoblast. We report here the isolation and characterization of a stable population of human liver progenitor cells from the human fetal liver that does not express liver-specific genes but is able to differentiate into functional hepatocytes and bile duct cells. Of great interest is that these cells, which we named human fetal liver multipotent progenitor cells (hFLMPCs), have features of mesenchymal–epithelial transition; retain multipotent capability to differentiate into fat, cartilage, bone, and endothelial cells; and have repopulation capacity in a mouse model of liver injury.

Results

Derivation of hFLMPCs. In our previous work with primary human fetal hepatocyte cultures, we observed a subpopulation of small blast-like cells that were highly proliferative and expressed markers of liver progenitor cells (11). To isolate and characterize hFLMPCs, we used seven liver specimens obtained from legally aborted first- and second-trimester fetuses between 74 and 108 days of gestation. Using the method described (11), we maintained cells from these livers in primary culture for 3 months to allow enrichment of blast-like cells until they formed a uniform layer over the multilayered cell cultures. Calibrated doses of geneticin were used on passaged cells to selectively arrest fibroblast growth, allowing the proliferating progenitor cell colonies to be mechanically isolated onto feeder layers. We were able to derive hFLMPC colonies from five of seven fetal liver specimens (see Table 1, which is published as supporting information on the PNAS web site). Clones were further derived by serial dilution and maintained for up to 6 months (100 population doublings, 20 passages).

Characterization of hFLMPCs. hFLMPCs grow in a 3D fashion similar to human ES cells (Fig. 1*A*). They are small cells averaging 10 μm in diameter with scanty cytoplasm (Fig. 1*B*). We confirmed that the hFLMPC colonies we derived were clonal by using the Humara X assay. Using an inactivation ratio <0.4 (12–14), all three colonies derived from a heterozygous female specimen (no. 18849) showed nonrandom skewed inactivation of the unmethylated Humara X allele (Fig. 1*C*), confirming clonality.

hFLMPCs Have Self-Renewal Properties Without Evidence of Telomere Shortening. One of the key characteristics of a stem cell population is its ability to self-regenerate. hFLMPCs have an estimated doubling time of 46 h by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) assay (Fig. 1*D*) and demonstrate high BrdU incorporation (see Fig. 7, which is published as supporting information on the PNAS web site). The morphology, immunophenotype, and differentiation potential are identical among hFLMPCs from different passages (data not shown). Cells from P1, P5, and P20 cultures had similar average telomere length that did not shorten, even after >100

Conflict of interest statement: No conflicts declared.

Abbreviations: αFP, α-fetoprotein; HNF, hepatocyte nuclear factor; hFLMPC, human fetal liver multipotent progenitor cell; MSC, mesenchymal stem cell; P*n*, passage *n*; D*n*, day *n*.

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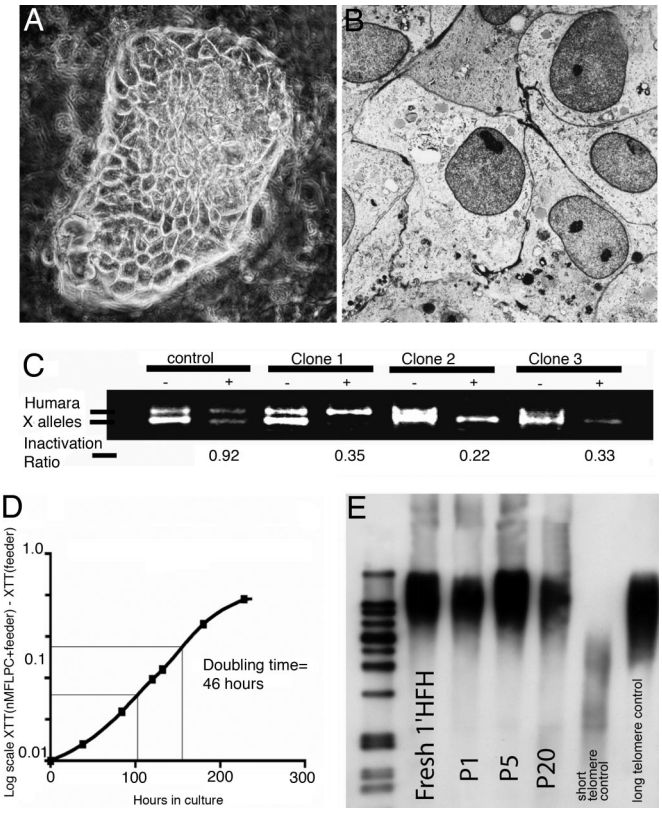

Fig. 1. hFLMPC cultures. (*A*) Phase-contrast micrograph. hFLMPCs grow in heaped-up clusters on NIH 3T3 feeder layers. (*B*) Electron micrograph. hFLMPCs have a high nuclear to cytoplasm ratio with scanty organelles in the cytoplasm and absent bile canaliculi. (*C*) Humara X nested PCR for clonality. Specimen no. 18849 was heterozygous for the Humara X allele (control lanes). DNA extracted from clones 1–3 derived from no. 18849 was subjected to digestion by HhaI (+) or without HhaI (−). The allele inactivation ratios (density ratio of least-intense band to more-intense band, before compared with after digestion) were <0.4 for all three clones, indicating clonality. (*D*) Growth curve of hFLMPCs as determined by XTT activity, revealing a doubling time of 46 h. Proliferation decreases after 10 days as the irradiated feeder layer starts to degenerate. (*E*) Telomere length assay by Southern blotting. Fresh fetal hepatocytes. P1, P5, and P20 hFLMPCs show no apparent shortening of average telomere length, even after 100 population doublings.

population doublings (Fig. 1*E*), suggesting that their self-regenerative potential is durable.

Immunophenotype of hFLMPCs. To determine the immunophenotype of hFLMPCs, we used immunofluorescence or immunohistochemistry, with confirmation by either RT-PCR (when confirming negativity) or immunoblotting (when confirming positivity). hFLMPCs are positive for CD34 (Fig. 2*A*), CD90 (thy-1), c-kit, and SSEA-4 (see Fig. 7). These markers are consistent with a stem-cell signature (15) and have been reported in mouse liver progenitor cells (16). hFLMPCs are also positive for epithelial markers such as EPCAM (Fig. 2*B*), CK18 (Fig. 2*C*), and CK19 (Fig. 2*D*). CK18 and -19 are characteristic markers of hepatocytes and bile duct cells, respectively. Interestingly, hFLMPCs are also positive for the mesenchymal markers CD44h (Fig. 2*E*) and vimentin but are negative for other mesenchymal cell markers, including CD105, CD73, and αSMA (data not shown). Hematopoietic markers such as CD45 and AC133 are also negative (data not shown). hFLMPCs are positive for c-met (Fig. 2*F*) but did not express liver-specific markers such as albumin (Fig. 2*G*), α-fetoprotein (αFP; Fig. 2*H*), or the "liver specification" transcription factors hepatocyte nuclear factor (HNF)1α, HNF3β, and HNF4α (Fig. 4*B*). hFLMPCs are thus a progenitor population within the fetal liver, expressing mixed mesodermal and endodermal markers.

Are hFLMPCs Derived from Other Cells in Fetal Liver Culture? To determine the origin of hFLMPCs, we performed lineage tracing by selective labeling of subpopulation fractions within initial fetal hepatocyte cultures and subsequently deriving hFLMPC colonies (see Fig. 8, which is published as supporting information on the PNAS web site). In cultures in which parenchymal fetal hepatocytes had been GFP-labeled, none of eight derived hFLMPC colonies were GFP-positive. Similarly, in cultures in which mesenchymal stem cell (MSC) fractions were GFP-labeled, none of the six derived hFLMPC colonies were GFP-positive. These results suggest that hFLMPCs are a distinct population in the fetal liver and are unlikely to arise from dedifferentiating parenchymal hepatocytes or transdifferentiating MSCs.

hFLMPCs Are Progenitor Cells for Hepatocytes and Bile Duct Cells. When grown on collagen plates, hFLMPCs flatten into a monolayer, progressively increase in size, and assume a polygonal morphology characteristic of hepatocytes (Fig. 3*A*). Differentiated cells start expressing albumin on day 3 (D3), with 96% of cells becoming strongly positive by D21 (Fig. 3*B*). αFP expression transiently appears at D3–D4 but is no longer detectable by D21 (see Fig. 9, which is published as supporting information on the PNAS web site). These trends were corroborated by immunoblot and RT-PCR (Fig. 4). Correspondingly, albumin in the culture medium was undetectable by ELISA at D0 but increased to 180 ng/ml at D5 and 1.2 μg/ml at D15 (Fig. 3*C*). Differentiated cells store glycogen within the cytoplasm (Fig. 3*D*), and electron microscopic analysis reveals a cytoplasmic organization characteristic of hepatocytes and the formation of bile canaliculi between neighboring cells (Fig. 3*E*). These cells also express α-1-antitrypsin (Fig. 4; also see Fig. 9), and liver-specific cytochrome P450 enzymes inducible by phenobarbital (see Fig. 9). Cytochrome 3A4 production increased 1.6× (immunoblot density quantitation), whereas cytochrome 2B6 activity [pentoxyresorufin-*O*-dependentylase (PROD) assay] was up-regulated 1.4× after phenobarbital treatment. The liver-specific transcription factors HNF1α, HNF3β, and HNF4α, which are not detectable in hFLMPCs, are expressed in differentiated hepatocytes (Fig. 4). Taken together, our data indicate that hFLMPCs can differentiate into functional hepatocytes. These hepatocytes decrease in proliferation upon differentiation but can be maintained in continuous culture up to 6 weeks.

When grown on 3D collagen sandwich gels, hFLMPCs rearrange into cystic and tubular structures by D5 (Fig. 3*F* and see Fig. 9), with cells lining the lumen assuming a columnar shape with tight junctions. Electron microscopy demonstrates long parallel microvilli on the luminal surface of these cells, typical of biliary epithelial cells (Fig. 3*G*). These cells are uniformly positive for markers of biliary cells, including GGT (Fig. 3*H*), CK7 (Fig. 3*I*), and CK19 (Fig. 3*J*).

Expression of Both Mesenchymal and Epithelial Markers in hFLMPCs. hFLMPCs express both epithelial and mesenchymal cell markers. We used immunofluorescence colabeling to determine whether these makers are coexpressed in the same cells, and if so, whether these markers change when hFLMPCs differentiate into hepatocytes. hFLMPCs coexpress both epithelial and mesenchymal markers within the same cell (see Fig. 10, which is published as supporting information on the PNAS web site), and as the expression of liver-specific proteins like albumin increases, mesenchymal markers such as vimentin and CD44h are down-regulated and disappear altogether with complete hepatocyte differentiation. This phenotypic change is characteristic of mes-

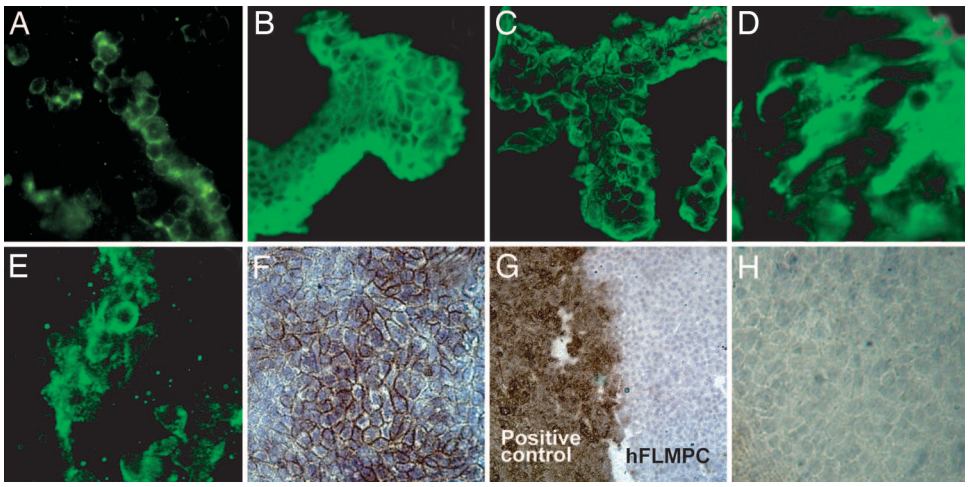

Fig. 2. Characterization of hFLMPCs. (*A–E*) Immunofluorescence and (*F–H*) immunohistochemistry. hFLMPCs are positive for stem cell markers such as CD34 (*A*); epithelial markers such as EPCAM (*B*), CK18 (*C*), and CK19 (*D*); and mesenchymal markers such as CD44h (*E*). They are also positive for c-met (*F*) but do not express liver-specific markers such as albumin (*G*) or αFP (*H*).

enchymal–epithelial transition and suggests that hFLMPCs represent transitional cells between mesenchymal and endodermal lineages.

hFLMPCs Have the Developmental Capacity of MSCs. In view of the expression of mesenchymal markers by hFLMPCs, we investigated whether these cells have the capacity to develop into mesenchymal lineages. In adipose induction media, cells increase in size, and 40% accumulate coalescing vacuoles that stain positive for fat (Oil red O), as opposed to differentiated hepatocytes, which do not survive in fat induction medium. These fat-like cells also express peroxisome proliferator-activated receptor γ2, in contrast to hFLMPCs and differentiated hepatocytes, which do not (Fig. 5 *A* and *B*). In contrast, differentiated hepatocytes do not survive in fat induction medium. Maintained in bone induction medium, cells become elongated with irregular processes, deposit calcium (von Kossa stain), and express osteopontin mRNA (Fig. 5 *C* and *D*). In cartilage induction media, cell clusters form a matrix core containing glycosaminoglycans, which stain positive for safranin O and cartilage-specific collagen type II (Fig. 5 *E* and *F*). In endothelium induction medium containing platelet-derived growth factor (PDGF)-CC (17), hFLMPCs become spindle-shaped and express the endothelial markers CD31 (28%; data not shown) and von Willebrand factor (38%; Fig. 5 *G* and *H*). hFLMPCs can clearly behave like MSCs and, with appropriate stimulation, can give rise to each of the mesenchymal lineage-like cells.

hFLMPCs Can Give Rise to Functional Hepatocytes *in Vivo*. To determine whether hFLMPCs can function as hepatocytes *in vivo*, we transplanted enriched hFLMPCs into immunotolerant Rag2$^{-/-}$ γc$^{-/-}$ mice (18) by using a modified retrorsine/carbon tetra-

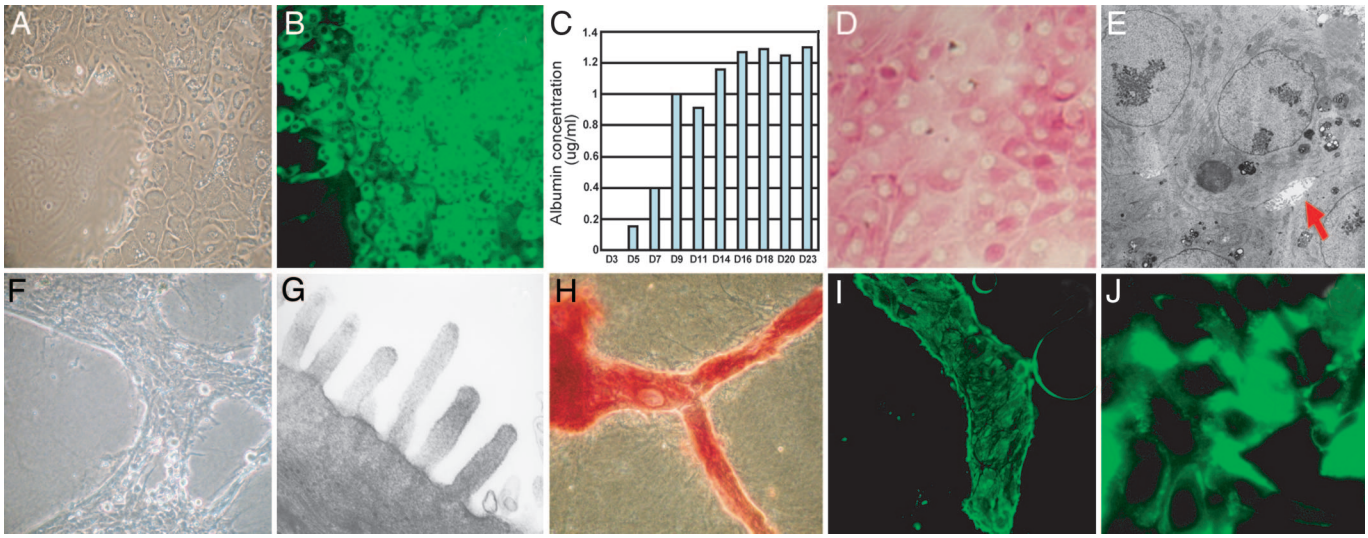

Fig. 3. hFLMPCs are progenitors of hepatocytes and bile duct cells. (*A*) Phase-contrast micrograph. When cultured without feeder layers, hFLMPCs flatten and increase in size to become polygonal cells characteristic of hepatocytes. (*B*) Immunofluorescence. Ninety-five percent of cells at D21 of hepatocyte differentiation are positive for albumin. (*C*) ELISA for human albumin in media increases progressively from D3. (*D*) Differentiated hepatocytes express glycogen deposits (PAS stain). (*E*) Electron micrograph. Differentiated hepatocytes show characteristic formation of bile canaliculi (red arrow). (*F*) Phase-contrast micrographs. When cultured in collagen sandwich gels, hFLMPCs assume a cystic and tubular ductal morphology. (*G*) Electron micrograph. Parallel villi characteristic of biliary epithelial cells are shown. These cells were positive for GGT (*H*, histochemistry), CK7 (*I*, immunofluorescence), and CK19 (*J*, immunofluorescence).

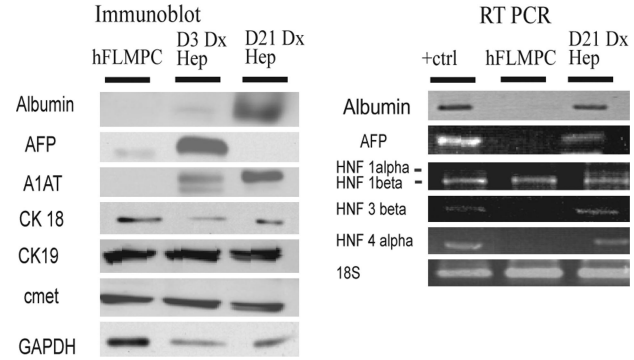

Fig. 4. Expression patterns of hFLMPCs and differentiated hepatocytes. The immunophenotypes of hFLMPCs and differentiated hepatocytes (D3 and D21) were confirmed with immunoblotting (*A*) and RT-PCR (*B*). Albumin, αFP, and α-1-antitrypsin protein are not expressed in hFLMPCs but appear with hepatocyte differentiation. This expression pattern for albumin and αFP is further confirmed with RT-PCR. Similarly, HNF transcripts, including HNF1α, HNF3β, and HNF4α, were absent in hFLMPCs but are present in differentiated hepatocytes. Dx Hep, differentiated hepatocytes; +ctrl, positive control.

chloride model (19). Three of three mice killed 30 days after transplantation had human-specific albumin in the serum (Fig. 6*A*) and expressed human-specific albumin in the liver (Fig. 6*B*). Liver sections of transplanted mice demonstrated clusters of morphologically larger cells (Fig. 6*C*), which stain positive for human-specific albumin (Fig. 6*D*). The degree of repopulation is estimated to be 0.8–1.7% in these mice. These data indicate that hFLMPCs can differentiate into functioning hepatocytes and integrate into the liver parenchyma in an *in vivo* injury model.

Discussion

We report the isolation and characterization of cells from the human fetal liver (hFLMPCs) that are capable of differentiating into hepatocytes and bile duct cells as well as into mesenchymal lineages, including adipose tissue, bone, cartilage, and endothelium. hFLMPCs have a high self-renewal capacity and showed no telomere shortening after 100 population doublings. These cells are discernible as a distinct population at the onset of primary cultures and are highly proliferative (11). Based on our data from mixing experiments with GFP-labeled hepatocytes or MSCs, it is unlikely that hFLMPCs are derived from either of these cell types in culture. Thus hFLMPCs are a unique cell type with stem cell characteristics. To our knowledge, the establishment of clonal cells from human liver that can be maintained in long-term culture in an undifferentiated state and in which directed differentiation to liver and mesenchymal lineages can be controlled at will, has not been previously described.

Although hFLMPCs readily differentiate into hepatocytes and bile duct cells, we did not detect expression of albumin, αFP, or the transcription factors HNF1α, HNF3β, and HNF4α in their undifferentiated state, indicating that these cells are either prehepatoblasts or liver progenitor cells from a parallel differentiation pathway. hFLMPCs coexpress epithelial and mesenchymal markers, and in view of their ability to generate both liver cells and mesenchymal lineages, we consider hFLMPCs to be mesenchymal–epithelial transitional cells and suggest that they have a mesendodermal origin.

It has been hypothesized that mesoderm and endoderm arise from a common bipotential mesendoderm precursor (20). Tremblay and Zaret (21) demonstrated that cells from the ventral midline of the endoderm lip, probably originating from the mesendodermal prechordal plate, give rise to part of the liver bud. Elsewhere in the animal kingdom, 80% of the turtle liver is apparently derived from the mesenchyme (22). Additionally,

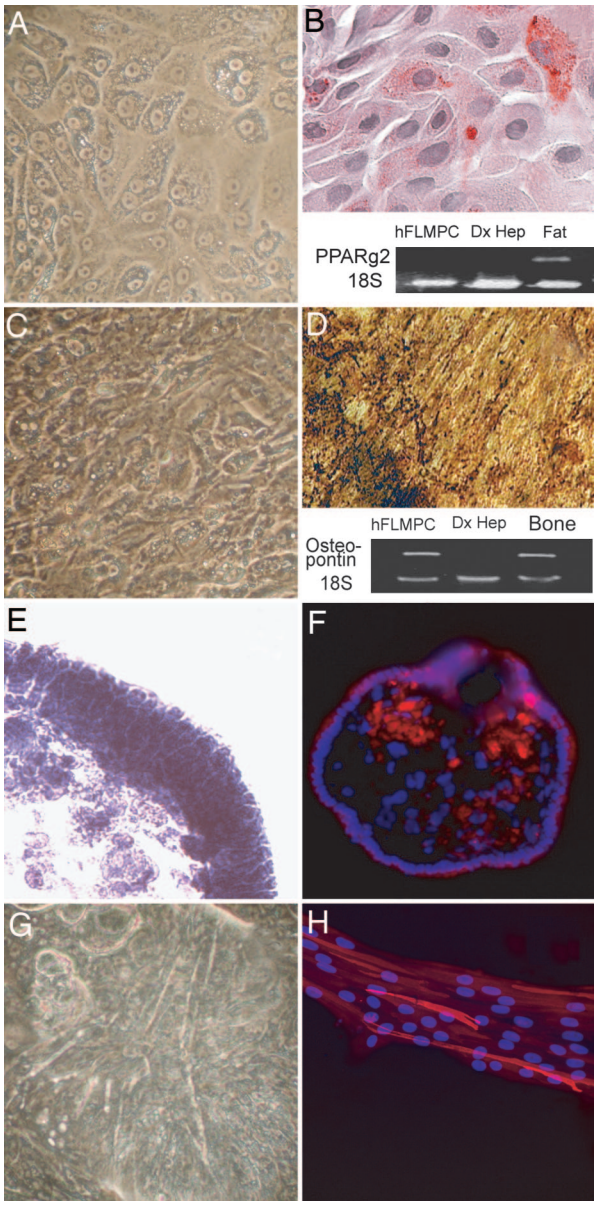

Fig. 5. hFLMPCs have the developmental capacity of MSCs. (*A* and *B*) Adipose tissue induction. hFLMPCs increased in size (*A*) and accumulated vacuoles that stained positive for fat (*B*, Oil red O stain, 40% of cells) and expressed peroxisome proliferator-activated receptor-γ2 (RT-PCR). (*C* and *D*) Bone induction. hFLMPCs assume a longitudinal and interwoven conformation (*C*); deposit calcium into the extracellular matrix, which stains black with von Kossa stain (*D*); and maintain expression of osteopontin (RT-PCR). (*E* and *F*) Cartilage induction. hFLMPC cultures in suspension form a ringed cluster with extracellular matrix in the middle that stains pinkish red with Safranin (*E*) and is positive for collagen II (*F*, red immunofluorescence with blue DAPI nuclear stain). (*G* and *H*) Endothelium induction. (*G*) hFLMPCs become spindle-shaped with formation of linear channels between cells. (*H*) Thirty-eight percent of cells are positive for von Willebrand factor (red immunofluorescence with blue DAPI nuclear stain).

multiple studies have shown that MSCs or mesoderm-related multipotent adult progenitor cells in both mice and humans can differentiate into hepatocytes (23, 24). hFLMPCs appear to have mesendodermal origin, but further fate-map analysis and characterization of liver specification profiles will determine whether they arise from the cells described by Tremblay and Zaret (21) or are from a distinct progenitor niche. Mesenchymal–epithelial

DEVELOPMENTAL BIOLOGY

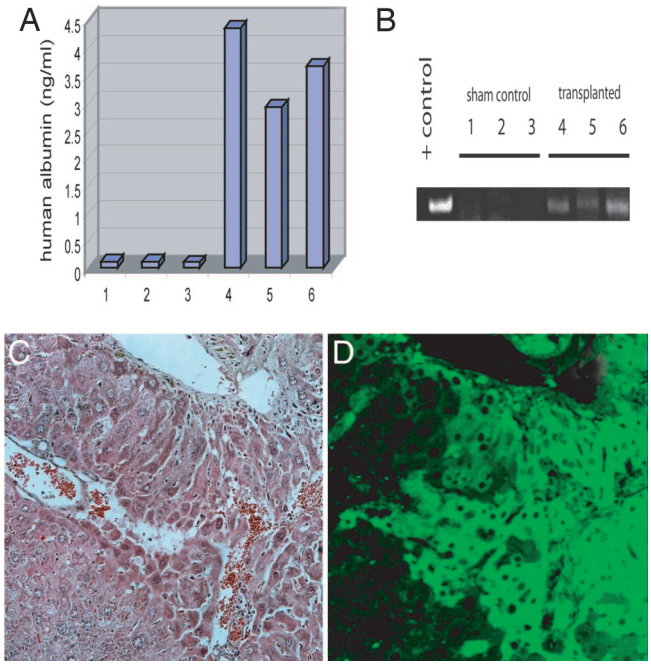

Fig. 6. *In vivo* transplantation of hFLMPCs into Rag2$^{-/-}\gamma c^{-/-}$ mice. (*A*) ELISA for human-specific albumin is positive in serum from mice transplanted with hFLMPCs but not in that of control (sham-operated) mice. (*B*) RT-PCR for human albumin. Livers from transplanted mice express human-specific albumin, in contrast to control mice. This hematoxylin/eosin-stained liver section from a mouse transplanted with hFLMPCs shows a cluster of morphologically different hepatocyte-like cells (*C*), which stain positive for human-specific albumin by immunofluorescence (*D*). The repopulation ratio is estimated to be 0.8%, 1.4%, and 1.7% for the three transplanted mice.

transitional cells are not uncommon in fetal development (25) and have been demonstrated in both rodent neonatal (26) and fetal livers (27), as well as human fetal livers, in which they have been reported to support hematopoiesis (28).

hFLMPCs are clearly different from MSCs or multipotent adult progenitor cells, as described by Jiang *et al.* (29). Their morphology, inherent epithelial markers, and potential to spontaneously become hepatocytes set them apart from MSCs transdifferentiating into epithelial lineages. In our liver differentiation protocols, MSCs from the same fetal livers were used as controls, and they did not become hepatocytes or bile duct cells. Interestingly, hFLMPCs share some morphologic and immunophenotypic characteristics of epithelial cells isolated from patients with subacute hepatic failure (30) and other hepatic diseases (31). They also appear to be similar to bipotential mouse embryonic liver (BMEL) progenitor cells isolated from mouse fetal liver (32). hFLMPCs are derived differently, however, and appear to have a mixed mesendodermal origin rather than the hepatoblast origin of BMEL cells.

In conclusion, we believe we have isolated and characterized human fetal liver progenitor cells with multipotent differentiation potential. hFLMPCs appear to have durable proliferative capability and can be induced to differentiate into functional hepatocytes and biliary duct cells *in vitro*, even after being kept in culture for 6 months. They can differentiate into functional hepatocytes *in vivo*, suggesting liver repopulation potential. We are currently investigating the critical factors that determine their differentiation, proliferation, and lineage transition, as well as the optimal conditions for maintaining differentiated hepatocytes in culture for longer periods of time. We are also exploring the use of this approach to isolate similar cell populations from other embryonic organs, such as pancreas and kidney. We anticipate that hFLMPCs will be a valuable tool to study differentiation pathways in the human liver and may have important therapeutic applications in patients with liver failure.

Materials and Methods

Materials. Basic culture media were purchased from Gibco/Invitrogen; supplements and chemicals were from Sigma Aldrich, unless otherwise specified.

Source and Isolation of hFLMPCs. Human fetal livers were obtained from the Central Laboratory for Human Embryology at the University of Washington, in accordance with a protocol approved by the Institutional Review Board. Primary fetal hepatocyte cultures were raised and maintained in human fetal hepatocyte medium, as described (11).

Cultures were maintained for at least 3 months to allow enrichment of small blast cells before passage with 1 mg/ml collagenase (Boehringer Ingelheim) and 10 units/ml dispase (BD Biosciences, Franklin Lakes, NJ). Geneticin (50 ng/ml; Gibco/Invitrogen) was added to culture media immediately after passage and maintained for 6 days. Colonies of hFLMPCs were then mechanically isolated with microscopic guidance and transferred onto hFLMPC culture plates.

Culture and Cloning. hFLMPCs were cultured on irradiated NIH 3T3 feeder layers (180,000 cells/ml; American Type Culture Collection, Manassas, VA) in plates coated with collagen (Vitrogen, Collagen Corp.)/laminin substrate, maintained with 50% human fetal hepatocyte medium without dexamethasone, 20% FBS, and 30% conditioned medium. Conditioned medium was taken from primary fetal hepatocyte cultures (at least 3 months old) every 48 h and filtered before use. The medium was further supplemented with basic FGF (10 ng/ml) and complex fatty acids (5 ml/liter). Media were changed daily and cultures maintained at 37°C with 6% CO_2. Cloning was performed by serial dilution, and daughter colonies were derived from single- or two-cell clusters on feeder layers, as witnessed under an inverse microscope. Colonies were then passaged every 10 days by using a cut-and-paste technique, as used in human ES cell culture protocols (33).

***In Vitro* Differentiation Protocols.** All differentiation protocols were performed in triplicate with P1–P4 and P8–P12 hFLMPC colonies. Percoll gradient separation was used to isolate human fetal MSCs (34) and parenchymal primary fetal hepatocytes (35) for use as controls. Details of the differentiation protocols are outlined in *Supporting Text*, which is published as supporting information on the PNAS web site.

Characterization of Cells. Immunofluorescence, immunohistochemistry, histochemistry, immunoblotting, RT-PCR, electron microscopy, XTT assay (36), BrdU labeling, and cytochrome P450 assays were based on standard protocols (see *Supporting Text*).

Telomere Length Determination. Telomere length was determined in D4 primary fetal hepatocytes and P1, P10, and P20 hFLMPCs by using the TELOTAGGG telomere length assay (Roche Diagnostics) according to the manufacturer's instructions.

Cell Labeling to Determine Origin of hFLMPCs. To determine the origin of hFLMPCs, parenchymal and MSC fractions from fetal liver were separated by using Percoll density centrifugation (34, 35). The purity of parenchymal and MSC fractions was determined by immunofluorescent expression of albumin or CD44h in random samples of cell fractions in culture at D1 after separation and was ≈90% for both fractions (data not shown). Parenchymal and MSC subpopulations were separately trans-

fected with GFP-labeled lentiviral vectors driven by the EF1α promoter (37, 38) with up to 90% efficiency. Primary hepatocyte cultures were then reconstituted by mixing labeled and nonlabeled fractions, and the presence of GFP expression in derived hFLMPC colonies was determined 3 months later (see *Supporting Text* and Fig. 8 for details).

Determination of Cell Clonality. Clonality of hFLMPCs was determined based on the concept of random X chromosome inactivation by using the Humara assay, as described (39). Clones were derived from a heterozygous female and DNA isolated from P3–P5 colonies. One microgram of DNA was digested with HhaI (Promega) in accordance with the manufacturer's instructions. Nested PCR was then performed as reported (13). Densitometric analysis of ethidium bromide-labeled bands was performed by using SCION IMAGE 4.03 software (Scion, Frederick, MD), and an allele inactivation ratio of <0.4 was indicative of clonality (12–14).

***In Vivo* Transplantation.** Six 4- to 6-week-old Rag2$^{-/-}\gamma^{-/-}$ mice (18) (C57BL/6J $\times$ C57BL/10SgSnAi)-[KO]gc-[KO]Rag2; Taconic Farms) were used for *in vivo* transplantation of hFLMPCs and maintained in accordance with animal care protocols. All mice were treated with weekly retrorsine (60 mg/kg i.p.) for 3 weeks to inhibit replication of native hepatocytes. Three mice then underwent intrasplenic injection with enriched hFLMPCs (3×10^6 cells); the remaining three mice underwent a sham operation (laparotomy). i.p. carbon tetrachloride (0.5 ml/kg diluted 1:10 in olive oil) was injected 1 week later and repeated weekly for 2 more weeks in all mice, and all were harvested 4 weeks after transplantation. Expression of human albumin was determined by serum ELISA, RT-PCR (see Table 2, which is published as supporting information on the PNAS web site), and immunofluorescence.

We thank Prof. A. Bongso and Miss S. Tan (both at National University of Singapore) and Dr. C. Murry, Dr. M. Laflamme, Dr. T. Parks, Dr. M. Reyes, and J. Golob (all at University of Washington) for their advice and assistance. The monoclonal antibodies H4C4 and H5C5, developed by J. August Thomas and James E. K. Hildreth, and CHC1, developed by R. Holmdahl and Kristofer Rubin, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Development and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). Lentiviral constructs were kindly provided by Dr. Naldi Luigini (Instituto Scientifico, H. San Raffaele, Italy). This work was supported by an International Fellowship Cat IIB from the Agency for Science, Technology, and Research, Singapore (to Y.Y.D.); an American College of Surgeons Resident Research Scholarship (to K.J.R.); and National Institutes of Health Grants CA023226-34 and CA074131-09.

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Appendix 2

Abstract: Oral Presentation AASLD November 2007
Hepatology. 2007 Oct:46(S1):322A

Isolation and Expansion of EPCAM positive Progenitor cells from human Fetal Liver

YY Dan, L Amer, LL Su, PC Wong, SG Lim

Liver progenitor cells hold tremendous promise in their ability to provide a continual source of cells for wide variety of applications including cell therapy. Human liver progenitor cells are EPCAM positive but isolation and culture of these cells have proven difficult. We report the successful isolation and of EPCAM+ progenitor cells from the human fetal liver that can be expanded in in-vitro culture.

Methods:13 and 18 weeks human fetal liver obtained from abortion under the purview of ethics board approval were treated with standard collagenase digestion. Cells were sorted using magnetic beads conjugated with anti-EPCAM antibody. Cultures were then performed on plates comparing various extracellular matrices including laminin, collagen, and matrigel, with and without feeder layers in chamber inserts setup. Cells were repeatedly passaged at confluence and purified with repeated EPCAM magnetic sorting.

Results: EPCAM positive cells were successfully isolated with up to 97% purity by immunofluorescence sampling. They demonstrated 3 distinct phenotype characterised by progenitor (CK19+ CD44+, alb-, AFP-), bipotential progenitors (albumin+/AFP+ /CK19+) as well as a committed hepatoblast (albumin+ /AFP+/CK19-). Laminin and matrigel plates together with conditioned media from EPCAM negative non-parenchymal cell fraction of the same liver were critical for maintaining progenitor phenotype. These cells retained high nuclear-cytoplasmic ratio, had a doubling time of 42hours and could be expanded up to 6 passages in 3 months and could be scaled up with up to 100 fold expansion in numbers. On differentiation protocols, colonies of hepatocyte-like cells that were strongly albumin and glycogen positive were observed. On collagen gels, tubular structures were obtained that were positive for GGT and CK7.

Conclusion: EPCAM positive cells select for progenitor cells that are hepatoblast and biliary progenitors. These cells can be maintained in their progenitor status with high and robust proliferative and expansion potential. Manipulation of culture environment can augment differentiation into both hepatocytic and biliary lineages. Current strategy is aimed at isolating subfraction of these cells. The ability to expand progenitor cells in in-vitro cultures holds promise in providing an additional source for hepatocytes for its many potential applications.

Appendix 3

Abstract: Oral Presentation AASLD November 2008
Hepatology. 2008 Oct:48(S1):340A

Long-Term Repopulation Potential Of Human Fetal Liver Progenitor Cells

YY Dan, J Haque, J Campbell, SG Lim, NFausto

Background and Aim:
Liver stem cells hold great promise for therapeutic cellular transplantation. Human fetal liver multipotent progenitor cells (hFLMPC) are unadulterated progenitor cells that can proliferate and differentiate in vitro to become hepatocytes and bile duct cells. We studied their ability for long-term repopulation in animal models of liver

Methodology
Enriched hFLMPCs were obtained from 2nd trimester human fetal liver. 4 groups (n=3 x 4 vs controls) of Rag -/- γc mice pretreated with retrorsine were transplanted with 3 million progenitor cells and exposed to 3 weeks of intraperitoneal carbon tetrachloride. Mice were sacrificed at 1, 3, 6 and 9 months after transplantation. Human cells were identified with Immunofluorescence, Insitu-Hybridisation and mRNA analysis.

Results:
Quantitation studies showed 0.7% to 2.6% to 0.8% and 0.2% repopulation at 1, 3, 6 and 9 month respectively. This correlated directly with the human albumin mRNA as well as albumin concentration which showed a peak at 3 months and decreased with time. Immunofluorescence and Insitu-hybridisation confirmed the human lineage of hepatocytes and isolated bile duct cells. They are functional, demonstrating tight junction integration with mouse hepatocytes and show no evidence of fusion or malignant transformation. One mouse had large nodule of functional human cells at 9 months.

Conclusion:
Human hepatocytes derived from hFLMPCs are able to survive, engraft, integrate as well as function in permissive mouse liver up to 9 months post transplant providing principle of proof of potential therapeutic benefit. The limitation of significant and highly variable repopulation in a environment without extreme selection pressure continue to pose a challenge to their clinical usefulness.