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Immortalized Human Hepatocytes, an Alternate Model for the Study of the Propagation of HCV *in Vivo* and *in Vitro*

By

Seyed Amir Mohajerani

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Committee Members

Dr. Norman Kneteman, Surgery

Dr. Donna Douglas, Surgery

Dr. Andrew Mason, Medicine

Dr. Thomas A Churchill, Surgery

Dedicated to My wife, parents and family members for their support

ABSTRACT

The chimeric Alb-uPA SCID mouse that has been transplanted with human hepatocytes is a model to facilitate in vivo study of HCV. We explored further development of the model by using repopulation with immortalized human hepatocytes (IHH) in place of primary human hepatocyte (PHH) transplantation to support HCV infection. In vitro HCV studies typically utilize a human hepatoma cell line (Huh7) and rely on transfection with transcribed genomic RNA derived from a unique HCV strain (JFH1). Unfortunately, this system has not been successful in support of infection with serum-derived HCV (HCVser). IHH may offer an alternative since their differentiation status remains close to that of PHH.

IHH transfected with HCV RNA (H77 or JFH1) or infected with HCVser showed stable intracellular and supernatant HCV RNA by real-time RT-PCR. IHH showed intracellular HCV NS3 proteins. HCV transfected or infected IHH secrete infectious HCVcc for *in vivo and vitro.*

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ABBREVIATIONS

HH	Human Hepatocytes
IHH	Immortalized Human Hepatocyte
PHH	Primary Human Hepatocyte
SV40LTAg	Simian Virus 40 Large T Antigen
IFN	Interferon
hTERT	human Telomerase Reverse Transcriptase
HCV	Hepatitis C virus
SCID	Sever Combined Immune deficiency
Alb	Albumin
uPA	Urokinase-Type Plasminogen Activator
HCC	Hepatocellular Carcinoma
PBS	Phosphate Buffered Saline
HRP	Horse Radish Peroxidase
hAAT	Human Alpha-1 Antitrypsin
TBST	Tris-Buffered Saline Tween-20
BSA	Bovine Serum Albumin
ABC	Avidin Biotin Complex
DAB	Diaminobenzidine
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase Chain Reaction
IP	Intra-peritoneal
RT	Reverse Transcriptase
5UTR	5’ Un-transcribed Region
JFH	Japanese fulminant hepatitis
NS3	Non-Structural protein3
GCV	Gancyclovir
HSV-TK	Herpes Simplex virus thymidine kinase

1. GENERAL INTRODUCTION:

1.1. Epidemiology

Hepatitis C virus (HCV) is a hepatotropic pathogen of significant importance to public health. An estimated 170 million people worldwide are chronically infected and at risk of progression to cirrhosis, hepatocellular carcinoma and end-stage liver disease. These sequelae make HCV infection the most common cause of liver transplantation (1). There is no HCV vaccine available and the current treatment which is a combination of PEGylated interferon (IFN)-α and ribavirin is effective in only half of cases and in many patients is poorly tolerated (2). HCV isolates can be classified into seven major genotypes, which vary in sequence by more than 30%. In addition to the distinct prevalence and global spread of the virus, the genotype is an important factor determining disease progression and responses to antiviral therapy.

1.2. HCV Structure:

HCV was first discovered as the causative agent of non-A, non-B hepatitis in 1989 (3), and has since become amenable to different studies. HCV is an enveloped flavivirus, with a positive stranded RNA genome of approximately 9600 nucleotides. It is composed of a single open reading frame, which encodes a polyprotein precursor of approximately 3000 amino acids. The coding region is flanked by 5’ and 3’ non-coding regions, which are important for the regulation of genomic duplication as well as initiation of translation. The virus encodes one

long open reading frame, which generates a polyprotein that is processed into ten individual gene products by host-encoded and virally-encoded proteases (4). The single polyprotein is cleaved by host and viral proteases into individual structural and nonstructural (NS) proteins.

The structural components of the virus which are the core protein (C) and the envelope glycoproteins (E1 and E2) and a growing number of non-structural proteins, including P7, NS2, NS3 and NS5A, have been implicated in the production of infectious virions. Infectious particles are thought to form by budding into the lumen of the endoplasmic reticulum, followed by egress through the cellular secretory pathway. The core protein is an RNA-binding protein that is supposed to form the viral nucleocapsid. It is removed from the polyprotein by a host signal peptidase cleavage at the C-terminus, yielding the immature form of the protein (7), and the signal peptide present at the C-terminus of the core is processed further by a host signal peptide peptidase, yielding the mature form of the protein (8), When expressed in the context of heterologous expression systems or HCV replicons, core is found both attached to the endoplasmic reticulum and at the surface of lipid droplets (9, 10). The core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions (11-13). It has indeed been proposed to be involved in cell signaling, apoptosis, carcinogenesis and lipid metabolism. Core makes HCV capsid protein, E1 and E2 making viral envelope, P7 making a ion channel protein essential for virus infectivity, NS2 making autoprotease of the virus, NS3 making protease /helicase of the virus, inhibit innate immune system by targeting TRIF and RIG-I binding protein, NS4A making a protein co-factore for NS3, NS4B makes protein for membrane rearrangement (membranous web), NS5A making phosphoprotein, associate with human vesicle-associated membrane protein, inhibits INF sensitivity of the virus, and NS5B is a RNA-dependent-RNA-polymerase (9,10). Replication of the HCV genome involves the synthesis of a full-length negative-stranded RNA intermediate, which in turn provides a template for the de novo production of positive-stranded RNA. Both these synthesis steps are mediated by

the viral RNA–dependent RNA polymerase NS5B (4-6). NS5B lacks proofreading abilities, and this leads to a high mutation rate and the generation of numerous quasispecies.

2. HCV MODELS:

Development of prophylaxis and novel therapeutics to treat HCV infection rely on in vitro and appropriate in vivo model systems to study the antiviral activity of novel compounds, as well as the mechanisms of infection and pathogenesis caused by HCV. In the past decade, however, enormous progress in the establishment of in vitro systems and the first chimeric mouse models for HCV infection have been described; these are essential tools to develop more effective antiviral compounds. Here, we discuss these *in vivo* and *vitro* models with their advantages and limitations.

2.1. In Vivo Models:

2.1.1. Chimpanzee Model for HCV Infection

For decade, the chimpanzee model has been the cornerstone of HCV infectivity studies. Chimpanzee studies are highly relevant to study the immunology, pathogenesis, and efficacy of novel therapeutics to treat HCV infections. However, experiments are generally performed with a limited number of outbred animals owing to important ethical constraints, their status as an endangered species, and high costs. HCV virions in patient plasma are heterogeneous, and experimental exploration of virus-host interactions remains extremely complicated. Inoculation of prototype strains in chimpanzees with viral kinetics indistinguishable from polyclonal natural infections is not accessible so far (14, 15).

2.1.2. Small-Animal Models for HCV Infection

Small-animal model with exogenously introduced HCV susceptibility traits could significantly accelerate the preclinical testing of vaccine and drug candidates, as well as facilitate in vivo studies of HCV pathogenesis. The development of a small-rodent model in which HCV replicates and is infectious has proven to be highly complex but necessary due to the chimpanzee model's limitations. Because of the extremely narrow species tropism of HCV, all rodent models require xenografting of human cells and a constitutive lack of immune rejection toward these engrafted cells. Several of these models have been developed in the past decade.

i. Immunotolerized Rat Model

This model is unique in its immunocompetent character, as all other rodent models are genetically immunodeficient. Fetal rats inside the gravid uterus were exposed to human hepatoma cells before the time of their immune maturation. After birth, hepatoma cells transplanted in the spleen of tolerized neonatal rats were not rejected but instead engrafted in the liver, making up 6% of total hepatocytes at 14 weeks age. Intrasplenic inoculation resulted in an infectivity rate of 62% as indicated by HCVNS5A immunostaining of liver slides, but viremia remained low. Interestingly, immune mediated liver damage was demonstrated by serum aminotransferase elevations and mononuclear cell infiltrations in the liver (16-18). Although the presence of functional rat immune cells is a major advantage, a genuine adaptive immune response toward HCV is not expected in this model, because rat–human major histocompatibility complex mismatches likely precludes the recognition of presented HCV antigens. Furthermore, antiviral testing will be difficult with the current marginal degree of chimerism and resulting low viremia levels.

ii. Mouse Model with Heterotopic Human Liver Grafts

In this model human liver fragments transplanted under the kidney capsula or behind the ear pinna of immunodeficient mice remain viable for up to 1 month due to an adequate blood supply in these locations. Thereafter, progressive loss of lobar architecture in the liver fragments leads to fibrosis and ischemia, probably due to an absent portal circulation, which therefore cannot provide essential growth factors, and a lack of bile clearance. Before heterotopic transplantation, liver fragments can be infected ex vivo, resulting in detectable serum HCV RNA levels in 25%-85% of animals with viremia peaking at 6 x 10^4 IU/mL and lasting for about 1 month (19). The limitations mentioned in this model led scientists to improve small animal model to mouse model based on liver repopulation.

iii. Mouse Model Based on Liver Repopulation

The chimeric mouse model is based on repopulation of mouse liver by primary human hepatocytes (PHH) isolated from liver sections, commonly of surgical waste. This model is developed based on substitution of human hepatocytes for mouse hepatocytes that are under survival pressure. The first mouse model invented in this way was introduced in 2001. This mouse model has a uPA transgene which results in subacute liver failure secondary to destruction of mouse hepatocytes. The immunodeficient uPA mouse (20) permits repopulation of the liver with human hepatocytes that can be infected with HBV (21) or HCV (22). The Alb-uPA SCID mouse model is described in detail later in the text. Other mouse models have subsequently been reported such as the *Fah*$^{-/-}$ *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mouse (23). In this mouse strain, the selection pressure in favor of the normal transplanted hepatocytes is due to absence of the enzyme fumaryl acetoacetate hydrolase (FAH), which leads to an accumulation of toxic tyrosine catabolites within mouse hepatocytes (24, 25). They have shown that this immunodeficient but otherwise healthy mouse strain can be readily and reproducibly engrafted with human hepatocytes, irrespective of the age of the

mouse (26) and they support HCV and HBV infection in the humanized part of the liver.

2.2. Limitations of In Vivo Models:

Chimpanzees are the only available immunocompetent in vivo experimental system, but their use is limited due to ethical concerns, restricted availability and prohibitively high costs and practical issues (27). Besides, despite the great similarity between humans and chimpanzees, there are some structural, pathologic and metabolic differences that could significantly impact disease processes and research outcomes (28). The chimeric mouse model also has its limitations which include an ongoing need for high quality human hepatocytes. Providing a sufficient supply of optimal liver donors is one important factor that could limit its application which is described in detail in section (5.1.4). The other limitations of chimeric mouse model are lack of normal immune system which prevents detailed study of immune response to HCV and immune based interventions.

2.3. In Vitro Models:

i .PHH infection by HCVser and HCV RNA clones:

Soon after cloning of the HCV genome in 1989 (3), it was reported by a number of groups that primary human and chimpanzee hepatocytes could be infected with HCV-positive patient serum (29, 30). Human hepatocytes are thought to be the primary target cell supporting HCV replication in vivo. However, establishing and maintaining cultures of PHH that sustain cell culture derived HCV (HCVcc) replication is difficult. Hepatocytes support only low levels of HCV replication, requiring PCR detection of negative strand viral RNA to verify active replication (13, 31-33). Infection of PHH with HCV from serum of infected patient's has provided a few important insights into how the virus may infect the liver (34),

including demonstration of a role for CD81 and LDLr in infection. However experiments with PHH are not easy to replicate by others.

In order to try other cell sources, Lazaro et al. reported the successful serum derived HCV (HCVser) infection of primary human fetal hepatocytes (HFH), with the release of infectious virus in the culture media that was able to infect naı¨ve target cells (35). Lazaro et al. demonstrated HCV replication by quantifying viral genomes and by the detection of viral encoded structural and non-structural proteins. Whether transfected with HCV RNA or infected with HCVser, HFH exhibit distinct fluctuations in the amount(s) of virus released, a trend that has also been observed in infected chimpanzees and in HCVcc infected Huh-7.5 cells (see section iv) (36). Infected HFHs demonstrate punctuate cytoplasmic NS3 staining in distinct areas or foci of infection, in agreement with recent reports of HCVcc infected Huh-7.5 cells (see section iv) (37).

As infection of PHH with HCVser is still highly controversial, initial attempts to propagate molecular clones of HCV in vitro did not lead to successful in vitro replication of viral sequences in PHH either. Scientists tried to transfect the PHH by the construction of so-called consensus genomes, which were based on a master sequence representing the majority of viral genomes in a given sample. In this way, the first full-length functional clones of HCV complementary DNA were constructed from the strain H77 (genotype 1a). Importantly, RNA transcripts from those clones were found to be infectious and to convey disease in chimpanzees after intrahepatic inoculation (14, 38). Based on this principle, several additional consensus genomes were constructed of genotypes 1 and 2, but the replication of these HCV clones in vitro remained ineffective (39). The scientists look for other more effective cell models for HCV cell entry and replication.

ii. HCVpp:

A major advance in investigating HCV entry has been achieved by the development of pseudoparticles (HCVpp), which consist of unmodified HCV envelope glycoproteins assembled onto retroviral core particles (40-42). To understand the process of entry an HCV pseudoparticle (HCVpp) system was contrived (40, 43). Extensive characterization of HCVpp has shown that these mimic the early steps of the HCV life cycle. Indeed, they exhibit a preferential tropism for liver cells and they are neutralized specifically by anti-E2 monoclonal antibodies (mAbs), as well as sera from HCV-infected patients(44, 45). HCVpp is made by transfecting 293T cells with 2 plasmids, one containing an envelope deficient HIV proviral gene, with a luciferase cassette, and the second containing the HCV glycoproteins. The particles produced can then be used to infect naive cells and the level of infectivity can be measured by a luciferase assay. The HCVpp system allowed for the study of early infection events, binding and entry, of the HCV replication cycle. Studying entry with HCVpp has shed light on the role of some cell-surface molecules involved in the early steps of the HCV life cycle.

iii. Tissue Culture Adapted HCV (Sub-) Genomic Replicons:

The problem of ineffective cell culture replication of primary or molecular clones of HCV was first overcome by the group of Bartenschlager (46). Their approach was to create antibiotic-resistant HCV genomes and to select replication-competent viral clones by conveying antibiotic resistance to cells. This was achieved by replacing the structural protein-coding sequences as well as p7 of the consensus genome Con1 by the neomycin resistance gene. In addition, a second internal ribosome entry site (IRES) was introduced to promote translation of the nonstructural protein-coding sequences important for viral replication. Upon transfection of these so-called subgenomic or bicistronic (two cistrons) replicons (a genetic element that can replicate under its own control) in specific cell lines, drug-resistant cell colonies were isolated in which high levels of viral

replication occurred. Subsequent analysis confirmed that these HCV replicons indeed were capable of self-amplification through synthesis of a negative-strand replication intermediate, and could be stably propagated in cell culture for many years (47). HCV replication was supported by several cell types, with the human hepatoma cell line Huh7 being the most permissive. Interestingly, removal of replicon RNA from these cell clones by treatment with IFN (the so-called "cured" Huh7 cell) rendered the cells more permissive to reintroduction of replicons, resulting in higher replication rates in this so-called Huh7.5 cells. The HCV replicon system allowed for the study of HCV RNA replication and antiviral therapies focusing on this replication machinery.

iv. Cell Culture Derived Infectious HCV:

Studies using HCV replicons have provided detailed knowledge on the mechanisms of replication of HCV. However, an apparent shortcoming of these models was that stable cell clones containing self-replicating replicons and expressing all viral proteins remained unable to release infectious HCV particles. The inability to secrete viral particles may be the consequence of adaptive mutations, which are needed to enhance viral replication rates, but at the same time may block viral assembly. Indeed, replicons without adaptive mutations show very low replication rates (48, 49). In 2003 an HCV genotype 2a clone was isolated from a Japanese patient with a rare case of fulminant hepatitis C. This clone was designated as JFH1 (for Japanese fulminant hepatitis 1) and the replicon constructed from this strain was found to replicate in Huh-7 cells (hepatoma cell line) without the need for adaptive mutations(50). Subsequently, it was found that transfection of JFH1 RNA into Huh7 cells resulted in the de novo production of infectious virus (designated HCVcc for cell culture derived HCV) that is capable of infecting naive Huh7 cells (51). Transfection of Huh7 and Huh7.5.1 cells with the in vitro–transcribed full length JFH-1 genome or a recombinant chimeric genome with another genotype 2a isolate, J6, resulted in the secretion of viral particles that were infectious in cultured cells, in chimeric mice,

and in chimpanzees (11, 13, 51). The virus produced in tissue culture was infectious in chimpanzees (36) and in immunodeficient mice with chimeric human livers, and the virus inocula derived from these animals was infectious for naive Huh7 cells (50, 52).

The infectivity of cells could be neutralized with antibodies against the HCV entry receptor CD81, antibodies against E2, or immunoglobulins from chronically infected patients. As previously described for HCVcc infection of hepatoma cell lines (11), anti-CD81 and soluble CD81 are both capable of inhibiting HCVcc infection of PHH. Importantly, the replication of cell-cultured HCV in this system was inhibited by IFN-a as well as by several HCVspecific antiviral compounds (36). Similar to the J6-JFH-1 chimera, in these so-called intergenotypic recombinants, the structural genes (core, E1, and E2), p7, and NS2 of JFH-1 were replaced by genotype-specific sequences which often resulted in lower infectious virion production than wildtype JFH-1(53-55). Most NS proteins of intergenotypic chimeras originate from JFH-1, and therefore these genomes are unlikely to reflect genotype-specific characteristics of replication. However, these intergenotypic chimeras may become critically important in the study of differences in HCV entry or to assess the efficacy of HCV entry inhibitors. Why the JFH-1 isolate is more permissive to replication in cell culture than isolates from other genotypes is still not fully understood.

2.4. Limitation of In Vitro Models:

A detailed understanding of HCV life cycle and mechanisem of entry is fundamental for the development of new therapeutic strategies to fight HCV infection. For doing so, developing an in vitro model which could be infected with HCVser and different genotypes of HCV is crucial, but the current models have their own limitations which are mentioned below.

PHH are the ideal system in which to study HCV infectivity. When cultured in vitro, however, they proliferate poorly and divide only a few times (56). The first approaches to studying HCV infection were based on the inoculation of PHH with HCVser (30, 33, 57). The primary limits of this system were (i) the low level of HCV replication, which required the use of RT-PCR to detect viral RNA in infected cells, (ii) difficulties in discriminating between newly synthesized and input HCV RNA and (iii) the absence or very low levels of infectious virus particle production. In addition, a homogeneous and well-characterized inoculum could not be obtained due to virus heterogeneity in the serum and the association of virus particles with plasma lipoproteins. Nevertheless, inoculation of PHH have already been attempted by several groups (29, 30, 32). Moreover, the level of replication is inconsistent from one experiment to another and is extremely low (58, 59). The lack of reproducibility of this approach has two origins: the variability in infectivity of serum-derived HCV and batch-related permissiveness of PHH. Moreover, PHH neither proliferate nor remain differentiated and die within days when cultured in vitro, thus reducing the window to study HCV infection (60, 61).

The development of the HCVcc system (Huh7/JFH1) was a major accomplishment, permitting important insights into virus–host cell interaction. However this system also has some limitations. First, Huh7 cells contain genetic defects, which do not permit normal functional status compare to PHH. These defects may affect virus composition; therefore, the results obtained in this culture system do not necessarily reflect the in vivo situation properly (62). As a matter of fact, the utilization of the transformed Huh7 cells remains a major problem to study the impact of HCV replication on cell physiology, because Huh7 cells do not contain many features of functional characteristics of normal human hepatocytes.

The second limitation to Huh7/JFH1 system is related to the very unique virus. JFH1 is a very rare and unique type of fulminant hepatitis, while 85% of

HCV infections lead to chronic disease (63). Besides JFH is a cell culture adaptive virus different from non-homogenate HCV in blood, and for replication it requires a highly specific part of the JFH1 replicase complex.

In summary, for unknown reasons, HCVser replicates poorly in PHH and hepatoma cells (Huh7) in vitro, and the current Huh7/JFH model has its own limitation due to deficiencies in cell line and unique virus.

3. IMMORTALIZED HUMAN HEPATOCYTE, A Potential Solution to hepatocyte supply for in vivo and in vitro models:

As discussed earlier, it is necessary to embark on a model closer to the original PHH to help overcome certain limitations of in vivo and in vitro models. The availability of mature human hepatocytes is limiting, because they are usually isolated from donor livers not suitable for transplantation or from segments of resected livers; both sources are unavailable to many researchers. Obtaining high yields of viable human hepatocytes (64) is technically challenging and further complicated by the health status of the donor liver. In addition these cells have extremely limited proliferation capacity in vitro (65, 66). Several cell lines derived from human liver tumors, such as the hepatoma cell line (11, 13, 53, 67-70) have been investigated in this sense (71, 72). But in tumor-derived cell lines, the mutations leading to immortalization are largely unknown and not controllable. In an attempt to control the immortalization process and therefore prevent at least part of the dedifferentiation process, several immortalized cell lines have been developed (1, 13, 71, 73). For successful in vitro immortalization, over expression of cell cycle stimulating genes is generally required (74). Due to the low proliferation capacity of mature hepatocytes (66), strong stimulation of cell cycle progression is necessary for immortalization (75) combined with stabilization of the telomeres (73). Immortalized human hepatocytes (IHH) have

been developed by several groups and have the ability to grow in culture indefinitely, which gives the advantage over PHH which could not be passaged in culture (71, 73, 76, 77).

One advantage of IHH over hepatoma cell lines is lack of tumorogenicity in most cases of in vivo application (73, 76, 77). It is rarely observed that immortalized cells accumulated with chromosome aberrations readily acquire tumorigenicity , and in most cases, activation of ras is needed to acquire tumorigenicity (78, 79). This seems to be an advantage of IHH over hepatoma cell lines since apprehension of tumorigenicity is a critical obstacle to in vivo application of hepatoma cell lines for transplantation (71).

The other advantage of IHH over hepatoma cell lines is their differentiation status. In some previous works with IHH , it's been shown that their differentiation status remains, at least transiently, close to that of PHH, as shown by measurement of normal-hepatocyte-specific markers and karyotype stability (71, 73) and resembled the morphologic and active metabolic functional characteristics of PHH. Immortalized hepatocytes retain some of the differentiated features of normal primary cultured hepatocytes, including the expression of albumin, transferrin, hemopexin, and glucose-6-phosphatase (77).

A number of cellular co-receptors of HCV have been identified. They include glycosaminoglycans, the LDL receptor (LDLR), DC-SIGN and L-SIGN , CD81, SRBI, and claudin-1(13, 80), all of which are down regulated through the process of de-differentiation that happens in hepatoma cell lines (10, 81). Huh and IHH cells secrete triglyceride (TG)-rich lipoproteins, apolipoprotein B and show LDL-receptors (82).

Taken together, these factors suggest IHH may be suitable cell lines for replacement of PHH for repopulation of chimeric mouse for in vivo studies, and for application for in vitro studies of HCVser from different genotypes.

3.1. How to Immortalize Human Hepatocytes:

The immortalization of PHH is an important potential route to overcome the existing problems with human hepatocyte supply. To immortalize primary human cells, immortalizing transgenes should be introduced to the cells (83-87). These transgenes are mostly derived from viral proto-oncogenes, such as simian virus large T antigen (SV40 LTAg) and human papiloma virus (HPV E6/7). Although introducing these transgenes has been shown to induce cell division by promoting cell cycle from G0 to M phase, introducing these cell cycle inducers is not enough for immortalization(75, 88). The division of cells results in shortening of telomers, the end part of the genome. After a certain number of cell divisions a crisis occurs, which results in the rapid senescence and death of the cells in culture. This event reflects a critical shortening of telomere length, and can be overcome by expressing the catalytic component of the enzyme telomerase (hTERT) (89). We elaborate some of the methods to induce immortalization in human hepatocytes in the next sections.

3.1.1. Using SV40

Proliferation of terminally differentiated cells obtained from an organ can be achieved through the introduction of cell cycle inducers such as Simian Virus 40 large T antigen (SV40LTAg) into the cells, which results in the elevation of expression of “transforming” oncoproteins(90), and induces extension of cell lifespan (91) until cells enter crisis (92, 93). There are many reports of human cells immortalized by the transduction of the SV40T gene (75, 94), and in the majority of in vitro immortalizations of primary human cells, the gene encoding SV40T, an inhibitor of the cell cycle inhibitors p53 and the Retinoblastoma (Rb) protein, have been used (71, 94-98).

The function of SV40LTAg is not only to extend proliferative lifespan of human cells but also to establish and maintain the transformed phenotype of cells (99). SV40 binds to the phosphorylated (active) form of Rb (Retinoblastoma tumor suppressor gene) and inactivates the Rb (Fig1). SV40 large T antigen can be divided into an N-terminal Rb binding region and a C-terminal bipartite region that interacts with p53. Viral oncoproteins can displace elongation factor 2 (E2F) from Rb–E2F complexes either by direct competition with E2F or via an indirect mechanism in which other factors participate, and E2F is released from Rb upon large T antigen binding and induces cell cycle promotion.

Although SV40 is a cell cycle inducer, hurdle remained in using only the SV40 mechanism. SV40-mediated cell expansion was unable to avoid replicative cell senescence (92, 100, 101). The reason for that was shortening of telomere which is described next.

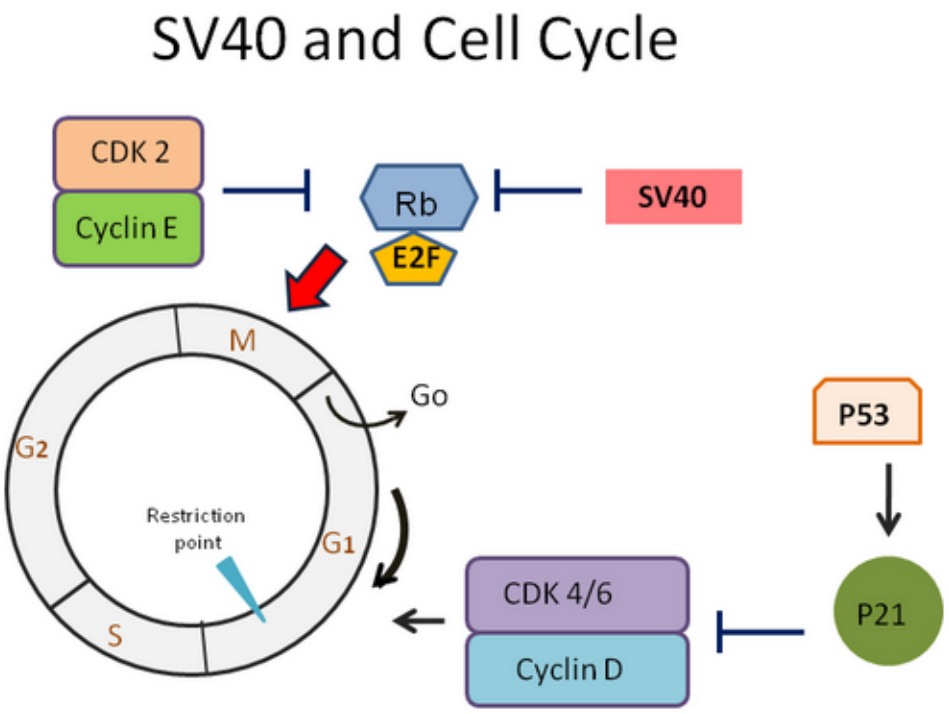


Fig 1. Mechanisem of SV40 cell cycle induction. Simian virus large T antigen (SV40 LT Ag) inactivates Rb tumor suppressor protein and causes the release of E2F transcription factor and progression of the cell cycle from G0 to G1, S, G2, and mitosis (M). The result is progression of cell division. CDK: Cyclin D Kinase, Rb: Retinoblastoma, E2F: Elongation 2 factor, G: growth, S: synthesis. [Sullivan CS, Molecular and Cellular Biology, August 2000, p. 5749-5757, Vol. 20, No. 15]

3.1.2. Using hTERT:

Telomeres are a region of repetitive DNA at the end of a chromosome, are synthesized by the enzyme telomerase, and are responsible for maintaining chromosome length. After each cell division, enzymes that duplicate the DNA chromosome cannot continue their duplication all the way to the end of the chromosome(102). Telomere shortening can induce senescence which blocks cell division (103, 104) (Fig2). Critically short telomeres induce cell crisis characterized by a terminal state of growth arrest (105). Overexpression of the

human telomerase reverse transcriptase (hTERT) stabilizes telomere length, thereby avoiding cellular crisis. As a general principle immortalization by over expression of hTERT only, minimizes the reduction in functionality (106) but by itself is generally insufficient for the immortalization of human primary cells (71, 73, 75, 101) (Fig2).

The absence of telomerase in most somatic cells has been associated with telomere shortening and aging of these cells. In contrast, high levels of telomerase activity are observed in over 90% of human cancer cells(107). Malignant cells which bypass this arrest become immortalized by telomere extension mostly due to the activation of the human telomerase reverse transcriptase enzyme responsible for synthesis of telomeres (108). Telomerase activity is directly correlated with the expression of its active catalytic component, the human telomerase reverse transcriptase (hTERT), which is believed to be controlled primarily at the level of transcription (109). Because hTERT expression is very low in many types of primary human cells, the telomeres of these cells shortens slightly every time a cell divides although in other cellular compartments which require extensive cell division, such as stem cells or other dividing cells, hTERT is expressed at higher levels and telomere shortening is corrected (110).

Telomerase is a ribonucleoprotein polymerase that synthesizes telomeric DNA repeats TTAGGG onto the ends of chromosomes (111). The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat (112, 113). It's been shown that oncogene activation or loss of tumor suppressor function is the probable mechanism by which the strict repression of the telomerase reverse transcriptase component in primary somatic cells is overridden (114, 115). Immortalization of human cells requires the reactivation of the telomere-lengthening enzyme, telomerase (116, 117), as the maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely (118-120).

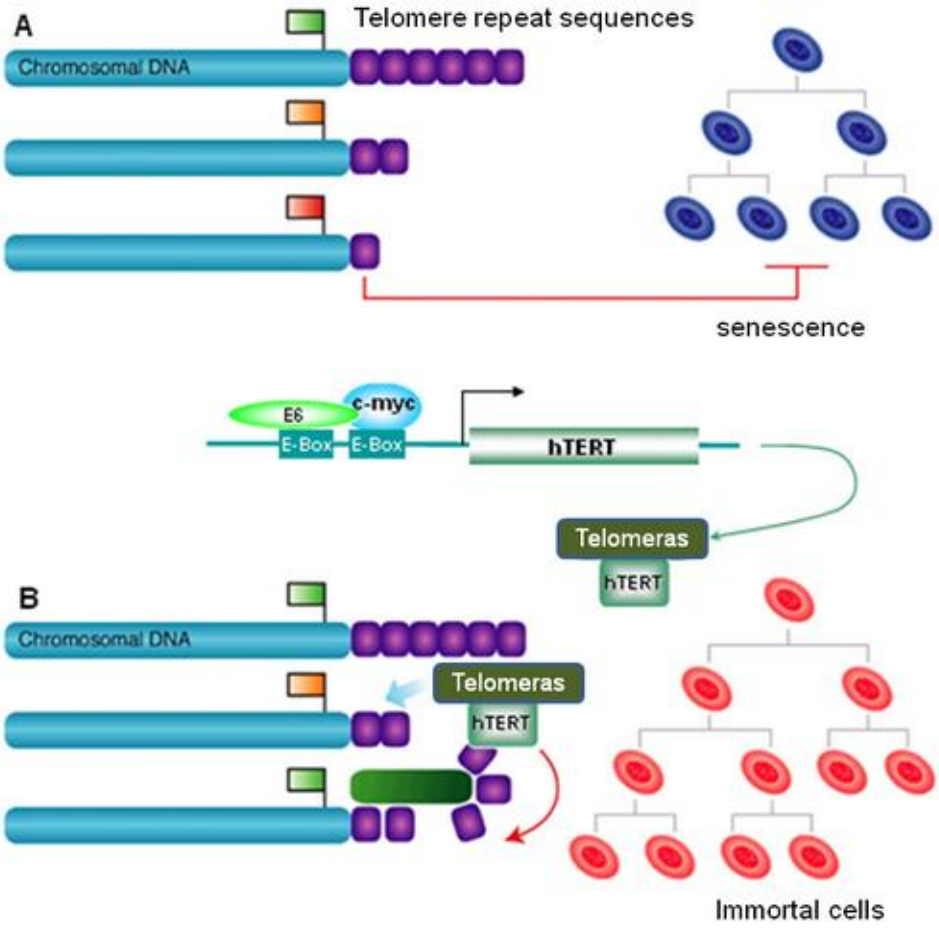


Fig 2. The mechanism of human telomerase reverse transcriptase (hTERT) to induce immortalization after multiple divisions. Telomeres are made up of a specific sequence of DNA bases repeated thousands of time. Each time a cell divides, the telomere part of the genome shortens a little, and after about 50 divisions the telomeres reach to their minimum length and additional cell divisions lead to destruction of chromosome and cell death and senescence(A). hTERT rebuilds shortened telomeres after cell division. hTERT is made by an RNA template of this enzyme for encoding the telomere. The opposite side of DNA is synthesized by a DNA polymerase (B). [Veitonmaki N, *The FASEB Journal*. 2003; 17:764-766].

3.1.3. In Vitro function of IHH:

Culture condition is one major factor for primary cells like PHH to lose their functional status after being cultured. Lack of cell-cell interactions and in vivo space support for the human hepatocytes are probably the main reasons for cells to de-differentiate in culture(121). Freshly isolated free human hepatocytes showed a continuous decrease of albumin secretion within the first week of culture, becoming almost undetectable at 7 days (77). Several studies have showed that IHH show immediately minimal albumin secretion, suggesting that hepatocytes lose some specific functions after immortalization (19, 71, 73). But does the process of de-immortalization return some of the lost function back to IHH cells?

3.2.1. De-Immortalization:

Previous studies have shown that excision of the immortalizing transgene ,integrated intocellular genome, is possible by using a Cre/loxp method in IHH (71). Excision of integrated immortalizing transgenes could induce de-immortalization in IHH which will induce growth arrest in IHH (71, 73). Reverted IHH were capable of regaining some of the aspects of their original function and so to re-differentiate partially back to their original human hepatocyte functions; they were capable of protecting partially hepatectomized animal models from acute liver failure after intrasplenic transplantation (71, 73, 122, 123). However, the requirement for expression of the Cre recombinase to IHH cells in culture in order to trigger irreversible excision of the integrated oncogene necessitates secondary virus-mediated gene transfer in a large number of cells in these studies. This technical requirement has presented a formidable hurdle to make this approach practical for ease of use and so for repeated use in transplantation in animal models such as the Alb-uPA SCID mouse model (71). The stable introduction of an inducible Cre recombinase is a valuable substitute for repeated transfection in these systems as we discuss in the next section.

3.2.2. Cre/Loxp Mechanism:

The site-directed recombinase Cre can be employed to delete or assemble genes in the genome of living cells by using a Cre/loxP system. Cre/loxP site-specific recombination was used to reversibly induce the immortalization of primary human cells. Cre recombinase, which is derived from Escherichia coli P1 phage, can independently cause the following series of reactions: recognition of a loxP sequence consisting of 34 bases (Fig3); excision of the DNA sequence encoded between the loxPs by binding to the site(124-126). By encoding immortalizing genes between a pair of loxP sequences, it is feasible to create reversibly immortalized human cell lines from which such immortalizing genes can later be removed(71, 123, 127, 128). This is an attractive method of producing cells that can be reverted to their original status in large quantities(112, 113). Such a system allows multiplication of the immortalized cells until they reach the desired cell number, at which point the immortalizing genes can be removed by treatment of the cells with Cre recombinase.

The Cre/loxP site-specific recombination system is widely used as a genetic engineering tool due to its well-defined recognition sequence, lack of any necessary co-factors and efficacy in both bacterial and eukaryotic systems. Since its discovery, Cre/loxP has been applied to temporal and spatial gene activation/deactivation, site-specific genomic integration (129, 130) and deletion (131) as well as the construction of libraries and cloning strategies (132-135).

In order to regulate this system scientists have been using chimeric form of Cre recombinase which is explained next.

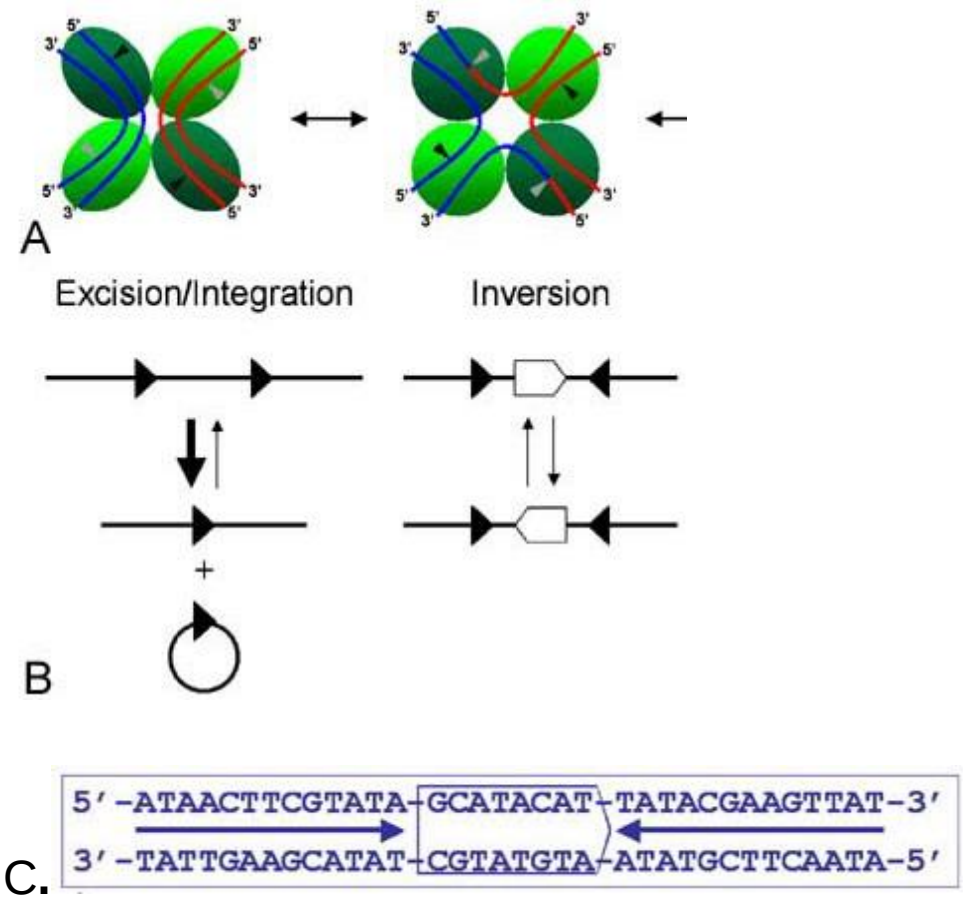


Fig 3. A. Schematic Cre-loxP site-specific recombination. Two Cre subunits bind each loxP site to form a tetrameric structure that stabilizes the synaptic complex. Two opposite subunits cleave and perform the exchange of a first pair of DNA strands to produce a Holliday junction intermediate. The loxP sites are regenerated in the reaction, becoming new substrates for Cre and making possible the reverse reaction. B. Different outcomes of a Cre-mediated site-specific recombination depending on the position and orientation of loxP sites. Recombinase target sites (C). loxP sites contain 13-bp palindromic sequences (indicated by inverted arrows) flanking a 8-bp non-symmetric core sequence (indicated by an open arrow). One recombinase subunit binds each palindromic element, while the spacer sequence provides the site of strand cleavage, exchange and ligation, as well as an orientation of the whole sequence [reprinted from Garcia, AL. Frontiers in Bioscience 11, 1108-1136, January 1, 2006].

3.2.3. Tamoxifen Induced Cre Recombinase:

One significant limitation of the site specific recombinase such as Cre mediated excision of loxp sites is the inability to control the timing of the Cre recombination. Scholars have constructed expression vectors for chimeric Cre recombinases carrying a mutated hormone binding domain either at the C-terminus only (CreMer) or at both the N and C-termini (MerCreMer). Chimeric Cre can regulate the timing and activity of the Cre recombinase (136, 137). These proteins carry a hormone binding domain (HBD) from either the mutated estrogen receptor (138, 139) or the progesterone receptor (140). The chimeric Cre proteins can be activated by culturing transfected cells with 4-hydroxytamoxifen. In the absence of the tamoxifen, the chimeric Cre protein is complexed by the heat shock protein Hsp90, and retained in the cytoplasm (141-143). When the cells are exposed to the tamoxifen, the chimeric Cre protein is released from Hsp90 and translocates to the nucleus where it can recombine the floxed DNA (144-148) (Fig4). It is important to note that, to regulate the activity of HBD-fusion-proteins independently from a naturally occurring hormone, the HBD of a mutated estrogen receptor (Mer) has been used (149, 150). This domain no longer binds estrogen but still binds the estrogen antagonist 4-hydroxytamoxifen (OH-TAM).

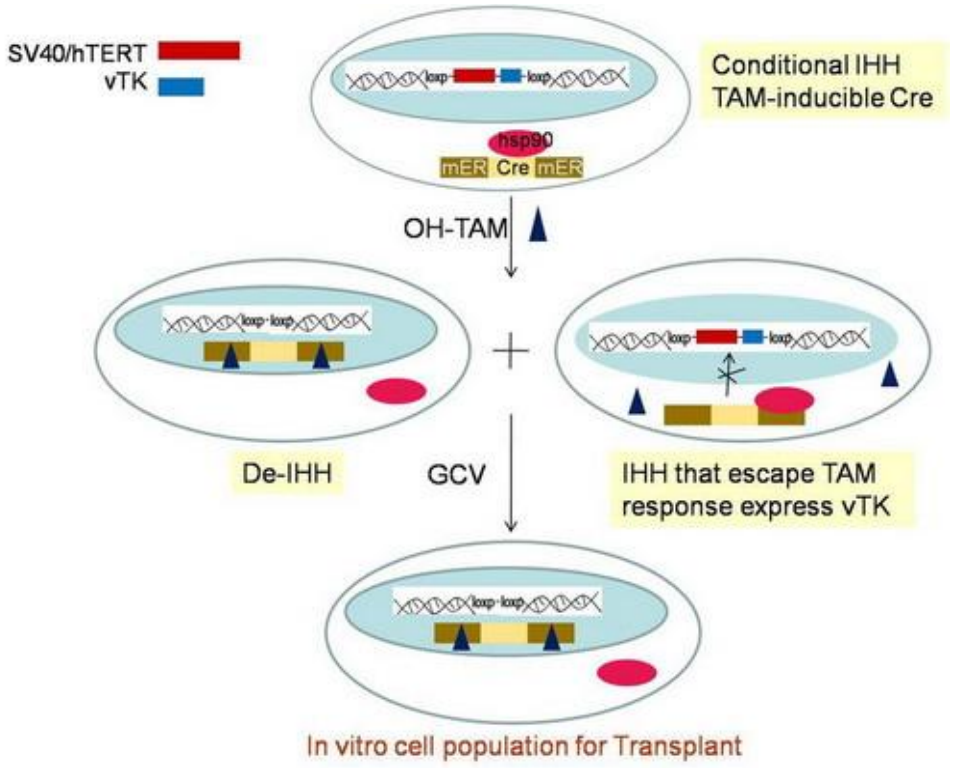


Fig 4. Chimeric Cre nuclear translocation after tamoxifen treatment and GCV killing of escaped cells from Cre/loxp excision of inserted genes . Chimeric Cre is attached to two mutated estrogen receptor (merCremer) in IHH cytoplasm. Immortalizing transgenes (SV40/hTERT, vTK) are located between two loxp sites, which enable attachment of Cre and excision of inserted transgenes floxed by two loxp sites. merCremer is attached to hsp90 which stays in IHH cytoplasm. After exposure of IHH with tamoxifen, tamoxifen attaches to mutated estrogen receptor bound to two truncated domains of Cre protein. Afterward, merCremer is released from hsp90and merCremer is translocated to the nucleus where it binds to loxp sites and excises the immortalizing transgenes. IHH cells that escape from Cre/loxp recombination still contain vTK transgene and could be eliminated with GCV (Fig from Dr.Donna Douglas, Grant proposal to CIHR).

3.2.4. The IHH Model Used in This Study:

We have obtained IHH from Dr. D Trono's lab in Geneva (73, 75). Trono et al made this IHH line by transducing PHH with a Cre-excisable Lentiviral cocktail encoding for SV40 and hTERT (Fig5). These 2 transgenes contribute to the ability of these cells to proliferate in culture indefinitely. Because these genes are contained within loxP consensus sites, exposure of these inserted genes to Cre recombinase resulted in their excision from the genome and subsequent growth arrest (73, 75). These growth arrested IHH showed some aspects of normal hepatocyte functions such as albumin expression, and transplant of IHH saved mice with acetaminophen induced liver failure (73).

In order to solve the problem of requirement for repeated transfection of IHH with a Cre encoding vector in the original IHH line to induce growth arrest (73), our lab has modified the IHH cells so that they express a tamoxifen regulated Cre recombinase. This chimeric protein consists of the Cre recombinase active protein fused to 2 mutated estrogen receptor (mer) ligand binding domains. IHH cells in culture can be treated with tamoxifen to induce growth arrest (Fig4). The two mer can attach to exogenous tamoxifen (not endogenous estrogen) and will then translocate to nucleus where they bind to loxp sites and excise the floxed DNA which contains immortalizing transgenes, SV40 and hTERT.

Another aspect of the IHH model that we used in our studies is that the original construct contains herpes simplex viral thymidine kinase, a suicide gene that induces cell death after exposure to gancyclovir (GCV) (Fig5). This gives us the advantage of negative selection of growth arrested cells. GCV kills the IHH that escape the tamoxifen effect of Cre excision of the transgenes which contains HSV-TK. In fact, after tamoxifen treatment, if the inserted transgene is excised out by Cre/loxp effect then the IHH should be growth arrested and resistant to

GCV killing effect. The cells where the transgene is not excised and still contain the immortalizing transgenes (hTERT, SV40) and HSV-TK could still grow in culture, but will be sensitive to the GCV killing effect.

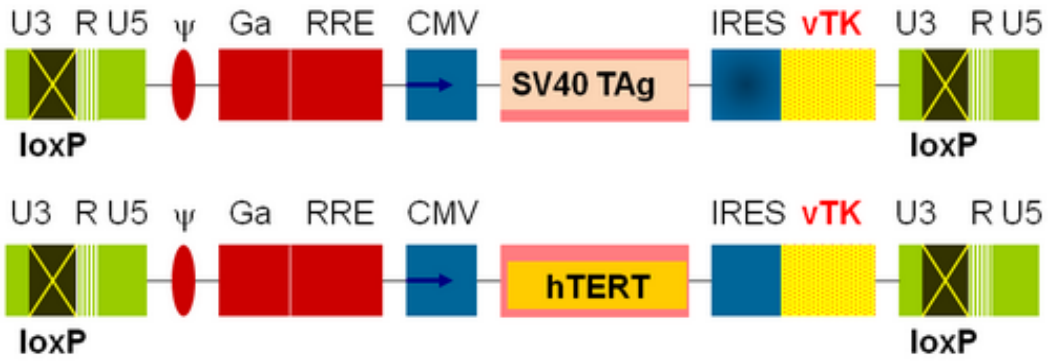


Fig 5. Schematic diagram of the vectors that were designed to make the original IHH pLOX-Ttag-iresTK (upper construct) and pLOX-TERT-iresTK (lower construct) (73). The gene construct for telomerase was identical except for hTERT was inserted in place of SV40 TAg. It also codes for Herpes Symplex viral thymidine kinase. CMV promoter is upstream of the bicistronic coding cassette with hTERT and HSV1-TK. During reverse transcription the U3 region of the 3'LTR, with LoxP, is duplicated; LoxP sites end up flanking the genome of the integrated provirus. Upon expression of Cre, the provirus is excised. The basic elements of HIV-based vectors, i.e., LTRs, SD, SA (splice donor and acceptor, respectively), ψ (packaging signal), Ga (fragment of gag), RRE (Rev-responsive element), have been described elsewhere. CMV: human cytomegalovirus immediate early promoter; SV40 TAg: SV40 large T antigen; IRES: internal ribosomal entry site of the encephalomyocarditis virus; vTK: thymidine kinase of Herpes Simplex Virus type 1 [reprinted from Trono D et al, J Hepatol. 2005 Dec;43(6):1031-7].

4. OBJECTIVES:

These are 2 main objectives for this thesis:

1. To determine if transplanted IHH cells can replace freshly isolated PHHs in the chimeric Alb-uPA SCID mouse model for the in vivo infection of mouse humanized liver by HCV.

2. To determine if IHH cells can provide an improved culture system for studying HCV lifecycle in vitro.

5. IHH FOR IN VIVO MODEL:

5.1. INTRODUCTION

The only natural hosts for HCV are human and chimpanzee. Chimpanzees are endangered animals and studies on them are high cost and have lots of ethical and technical problems. Given the aforementioned limitations, there is a huge need to develop small animal models to overcome our limitations for in vivo models. In addition, the central role of the human liver in the effects and replication of HCV and response to therapy make the chimeric mouse model of particular interest for clinical and research purposes. The small-animal model for the study of HCV infection and HCV-related liver disease was described in different mice models (22). This model allowed scientists to study aspects of HCV life cycle and biology and to test potential new drugs.

5.1.1. Alb-uPA SCID Mouse Model:

The SCID/uPA chimeric mouse model has been shown to be an effective tool in the study and testing of various investigational drug treatments for HCV (151). In addition to supporting HCV and HBV infections, this mouse model has been found to possess hepatic metabolic activity (brought on by the hepatic CYP3A4 enzyme) comparable to that of human subjects (152). Studies have also found that liver enzyme induction by various drugs (which may significantly interfere with the metabolism of drugs) is similar between chimeric SCID/uPA mice and humans (153).

5.1.2. uPA Transgene

The Alb-uPA SCID mouse is the small animal model of choice for many studies. These mice have a SCID background(154-156). The specific gene affected by the SCID mutation is the Protein Kinase DNA Activated Catalytic

Polypeptide (Prkdc) gene (157, 158). The mutation results in a premature insertion of a stop codon at the Tyrosine 4046 position rendering the resultant recombinase enzyme defective (159, 160). The affected DNA recombinase enzyme is responsible for generating the T and B cell receptor diversity necessary for the operation of an effective adaptive immune response. As a result, mice that are homozygous for the SCID mutation have defective T and B cell function. These mice cannot mount a successful cellular or humoral immune response against foreign antigens. In order to support high level human hepatocyte chimerism, the mouse model carries a uPA transgene which targets expression of urokinase type plasminogen activator (uPA) to mouse liver(20, 161, 162). In fact, it has been shown that homozygosity in both the SCID trait and the Alb-uPA trait are necessary for optimal human chimerism in this model (161, 163). The albumin promoter targets the over-production of uPA to hepatocytes (20, 164). SCID/uPA mice carry two tandem copies of the uPA transgene and are also homozygous for the SCID trait. The urokinase plasminogen activator (UPA) transgene, targeted to hepatocytes, leads to extensive destruction of the native mouse liver (165, 166). This provides an optimal environment for engraftment of transplanted hepatocytes given both the physical space available (left over by the destroyed native hepatocytes) and the up regulated endogenous hepatocyte growth signals (167). The uPA transgene confers a clear proliferative advantage to hepatocytes transplanted into uPA mice (167). The optimal timing for hepatocyte transplantation in this model is early after birth (161). This is because, if left alone, the mouse liver will repopulate with endogenous hepatocyte lines that have lost the uPA transgene due to somatic deletion (163). Such hepatocytes clearly have a proliferative advantage over the uPA-expressing hepatocytes that undergo destruction (164). Studies have shown that these uPA-deficient hepatocytes will eventually repopulate the entire mouse liver within 3-6 months (82, 161). Because of this, exogenous hepatocytes have the best chance of successful engraftment and growth when transplanted early. In fact, our laboratory has observed optimal chimerism within the mouse liver when the human hepatocytes are transplanted 5-

10 days after birth (22, 151). This is one reason why mice that survive the prenatal period do not die of liver failure as would be expected (167).

5.1.3. Human Chimerism:

Alb-uPA SCID mice are transplanted with freshly isolated or cryporeserved human hepatocytes to engraft and repopulate the liver of the mouse. The presence and degree of human chimerism achieved in the SCID/uPA mouse model can be determined serologically. Serological methods rely on the detection of markers of functioning human hepatocytes in the serum of transplanted SCID/uPA mice. The two most prominent such markers are human albumin and human alpha-1 anti-trypsin (hAAT). Alpha-1 antitrypsin is a serine proteinase inhibitor that serves as an anti-inflammatory molecule. It has shown to be important in preventing tissue damage and to participate in innate immunity (168, 169). The advantage of biochemical methods over histological ones is the ability to determine the level of human chimerism without the need to sacrifice the mouse. In addition, biochemical methods allow for the serial monitoring of the degree of human chimerism over a period of time and how this changes with certain treatments and manipulations. As such, after treatment with a putative anti-HCV compound, if HCV titers fall and hAAT levels are maintained, this can be ascribed to an anti-viral effect. If both HCV and hAAT levels fall, this may be due to a toxic impact on the human hepatocyte graft, with loss of ability to support the HCV infection.

Both albumin and hAAT levels can be measured in mouse serum with sandwich ELISA assays. Both are secreted exclusively by the human hepatocytes in this model and as such serve as specific indicators of the mass of successfully engrafted and functioning human hepatocytes. The limitations of these measurements however lie in the need for repeat serum sampling and fluctuations due to metabolism and renal system function.

Human albumin is known to have a half-life in serum of around 20 days compared to about 15 hours for hAAT. Therefore, serum albumin levels may not accurately reflect the current mass of functioning human hepatocytes in the model as the albumin from dead hepatocytes will linger for about 20 days. Serum hAAT measurements, on the other hand, offer a much more accurate real-time assessment of the current mass of functioning human hepatocytes when measured weekly. However there is a limitation of hAAT as a marker of human hepatocyte mass after transplantation. hAAT is acute phase reactant and will increase with inflammatory status like infections which could interact with validity of test to be indicator of human engraftment.

5.1.4. Limitations of the SCID/uPA Chimeric Mouse Model

The SCID/uPA chimeric mouse model, however advantageous, suffers from significant practical limitations. Chimeric mice that harbor HCV-permissive tissue can be obtained by transplanting human hepatocytes into mouse recipients with liver injury and severe immunodeficiency (170).Inoculation of HCVcc or sera from HCV-positive patients into these mice leads to a rapid increase in viremia, which is sustained over several weeks to months (22, 36). However there are several limitations to use this mouse model (22, 151, 171). Also there is major variability in human hepatocyte transplantation success (the success of achieving high level human chimerism in mice - as measured by serum human alpha-1 antitrypsin , hAAT) in uPA mice according to donor, recipient and procedure related factors. Given the variability in human chimerism observed in this model, strategies are needed to further enhance the reliability of survival of transplanted human hepatocytes in this model, and in addition to provide an unlimited supply of suitable cells for transplantation.

The success of human hepatocyte transplant and engraftment is impeded by several different factors. Human liver donation is one major obstacle, as finding an optimal human liver is the exception. Such a liver would be non-

cirrhotic, non-fatty and from a relatively young donor. Isolation of human hepatocytes is another bottleneck in making this chimeric model. Digestion time, collagenase quality and purity, and warm and cold ischemic time are all important factors in isolation of human hepatocytes. Using other human hepatocyte cell sources has been only intermittently successful to date. Cryopreserved human hepatocytes are high cost, low yield and success is sporadic, which increases the final cost of each chimeric mouse produced. Using hepatoma cell lines such as Huh or HepG cells has not been a successful option as we have observed tumorogenicity in this mouse model. One possible solution is using IHH which could substitute for the PHH. IHH have the ability to grow in culture indefinitely, thus addressing indefinite supply challenges, and are not tumorogenic.

5.2. HYPOTHESIS:

Selection of growth arrested IHH induced by the Cre-excision of SV40 and hTERT in IHH will substitute for freshly isolated human cells in the chimeric mouse model for HCV.

5.3. METHODS AND MATERIALS

5.3.1. IHH Cell Culture:

The IHH were grown in culture and passaged every week after confluency. Dr Donna Douglas has generated a G418-resistant IHH clone stably expressing an OH-TAM-inducible Cre recombinase protein fused to two mutant murine estrogen-receptor ligand-binding domains (merCremer) .The IHH expressing chimeric Cre recombinase (IHH-mCm) were expanded in DMEM/F-12 medium containing 10% FBS, 1×10^{-6} M dexamethasone, 1×10^{-8} M insulin, 15 mM Hepes (IHH medium) and penicillin streptomycin. For de-immortalization, IHH were incubated with 800 nM 4-HydroxyTamoxifen (4-OHT) for 10 days. For GCV studies , we treated IHH cells with 800 nM 4-OHT for 7 days and then for 5 days with 50 µM of Gancyclovir (GCV) (Roche,P1484-

04) in order to kill the non growth arrested IHH cell – by the mechanism of expression of vTK in the same region of the inserted immortalizing genes.

5.3.2. Expression of Immortalizing Genes

Total DNA was purified with Trizol reagent (Invitrogen, 15596-026, according to manufacturer protocol). PCR amplifications were performed to examine the presence of the transgenes DNA. For PCR reaction 50 µl of reaction mixture containing DNA samples (1:25) and Taq DNA polymerase (Invitrogen, 10342-020) were used. Amplification conditions were: 94°C for 5 min; 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 3 min. Primers used were: forward primer 5'-CAGGCATAGAGTGTCTGC-3' and reverse primer 5'-CAACAGCCTGTTGGCATATG-3' for SV40TAg (422 bp product), forward primer 5'-CGCAGCCACTACCGC-3' and reverse primer 5'-AAGGCCAGCACGTTC-3' for hTERT (250 bp product), and forward primer 5'-GCGCTCTAGATGGCTTCGTACCCCTGC-3' and reverse primer 5'-CGCGTCTAGATCAGTTAGCCTCCCCCAT-3' FOR HSV-TK (1100 bp product run on 2% agarose gel by using 100 bp DNA ladder). PCR conditions for HSV-TK were 95º C for 2 min; 34 cycle of 95ºC for 30 sec, 57ºC for 30 seconds, 72ºC for 80 second, and 72ºC for 10 min.

5.3.3. Immunoblot Analysis of merCremer Expression

IHH-PoP10 clone (original IHH cells provided by Dr, Trono) were transfected with pcDNA3neo-merCremer (provided by Dr. Marek Michalak, University of Alberta) for the expression of merCremer using Lipofectamine^{TM} 2000 (Invitrogen) transfection reagent, and 72 hour after transfection, selection with 5µg/ml G418 was initiated. The merCremer cassette confers G418 resistance, and after high dose treatment of G418, clones 4, 9, 14, 15, 19 and 20 of IHH-merCremer were selected. For detection of expression of merCremer in G418 resistant clones, western blot analysis was used with anti-α-estrogen receptor antibody. Plates were washed once with Phosphate buffered saline

(PBS), lysed in RIPA buffer [50 mM Tris-Cl (PH= 7.4),1% NP40, 0.25% v/v sodium deoxycholate, 0.1% w/v sodium dodecylsulphate, 150 mM NaCl, 1mM EGTA]. Protein content of each sample was measured by micro-BCA-Protein-Standard kit. After that 2xSDS sample buffer were added to the samples, and then 1.5 µl of B-Mercapto-ethanol were added to 100 µl 1xSDS sample. Next, 20 µg total protein from each sample was loaded on acrylamide gel. Proteins were separated on 8% acrylamide gel by gel electrophoresis, and then were electrophoretically transferred to a nitrocellulose (BioRad) membrane. The membranes were blocked in 5% milk-PBS-TWEEN overnight and the next day after wash with PBS-TWEEN, incubated with the primary antibodies [rabbit anti-alpha-estrogen receptor monoclonal antibody ,Santa Cruz, 1:1000 dilution], Then after 3 times wash with PBS-TWEEN, they were incubated with secondary antibodies [Horse-radish-peroxidase-conjugated anti-rabbit IgG antibodies (Santa Cruz) at a dilution of 1:10,000]. The membranes were visualized by using Western Blotting Chemiluminescence Luminol Reagent (BioRad).

After G418 selection of IHH-mCm and confirmation of the presence of mCm in cells by western blot, the cells were cultured on 6 well plates. The cells were incubated for 24h to reach 80 % confluence, and then Tamoxifen was added to the cells at a concentration of 800 nM to the upper row of 6 well plates. After 24 hour incubation the cells were trypsinized and collected in to an eppendorf tube. The three components of cells were separated accordingly:

- **WCL** (whole cell lysate): the pellet was lysed by RIPA buffer containing Protease inhibitors.
- **NUC** (Nuclear): The pellet was lysed by adding 1% TX100 ((100 µl TritonX+ 10 ml PBS). The lysate was centrifuged at 10,000 rpm for 10 min. The supernatant was carefully transferred to a new eppendorf and named as CYT. The pellet was lysed in RIPA again and named as NUC.
- **CYT** (Cytoplasmic fraction): as described previously.

The protein content of the three fractions was measured by Micro-BCA-Pr assay kit (Thermo Scientific, 23235). The 8% acrylamide gel was poured and 20 µg of

Pr was loaded onto the gel. After transfer to nitrocellulose membrane, and by using a rabbit anti-α-estrogen receptor antibody (Santa Cruz.sc-542) as primary antibody and HRP conjugated goat-anti-rabbit IgG we detected the merCremer presence in WCL, Cyt or Nuc. For visualization of membrane we used a SuperSignal West Pico Chemiluminescent (Thermo Scientific, 34080).

5.3.4. In situ Detection of merCremer by Indirect Immunofluorescence

For in situ detection of merCremer by Anti-Cre (Novagen,Tx), IHH-mCm were treated with tamoxifen for 48 hour to confirm the cytoplasmic location of Cre prior to tamoxifen treatment and translocation of Cre to the nucleus after tamoxifen treatment.

Cells were seeded onto glass cover slips in 12 well dishes at 10-40,000 cells per dish and incubated at 37°C, 5% CO2 and 95% air. After 24 hour the cells were washed twice with PBS, and then 3ml of 3.7% (v/v) formaldehyde in PBS was added for 15min at room temperature 25°C. After removing formaldehyde solution and washing twice with 2 ml of PBS, cells were incubated at -20 for 10 min in PBS containing 0.05 % (w/v) Triton X-100 and then washed with PBS/BSA 1%. Then cells were incubated in 2 ml PBS containing 1% BSA (PBS/BSA) for 15min at room temperature. Afterward cells were incubated for 1 hour at 37º C with primary antibody (0.5 ml fresh PBS/BSA containing 5-10 µg/ml antibody) (1:50-1:100). Rabbit anti-Cre monoclonal antibody (Dilution: 1:100) was used as primary antibody. Then we washed the cells 3 times with 2ml PBS/BSA and then incubated with secondary antibody (FITC conjugated goat anti-rabbit antibody, dilution 1:1000). Coverslips were washed twice with PBS/BSA and once with distilled water prior to mounting cover slips on cover slides with 8 µl mounting solution (add 1µl of DAPI to1ml of mounting media and for each slide load with 8µl). For control FITC-conjugated mouse IgG2a Isotype Control (1:100) as equivalent to testing Ab (Manufacturer protocol) were used. IHH-Pop10 was used as negative control.

5.3.5. GCV Killing Curve

The 10^4 IHH-mCm, IHH-PoP10 and Huh7 (as negative control) cells were cultured in 96 well plates and media was added to cell culture. The cells were incubated for 24 h and then media was exchanged with fresh media. Next GCV was added to cell cultures at doses from 0 to 2000µM, and followed for 4 days. To achieve this concentration, 25mg GCV was dissolved in 980µl sterile ddH_2O to obtain a 100mM stock solution. By adding 1 µl of the 100mM GCV stock to each ml of cell culture media, the final concentration would be 100 µM. Time and dose curves were determined by incubating IHH-mCm cells with different doses of GCV from 0 to 5000µM and culturing cells from 24 h up to 4 days.

Cell viability and survival after GCV treatment were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay kit (Biotium 30006). For this purpose cells were cultured at a 10^4 concentration in each well of a 96 well plate and 100µl of DMEM were added to each well. After 24 h GCV was added to each well at a pre-determined dose from 0 to 5000uM. Cells were cultured afterward for up to 4 days. For each time point we used a different 96 well plate. Then at each time point cells were incubated with 100 µl of MTT stock to reach a final concentration of 0.5 mg/ml of yellow tetrazolium bromide (MTT) in the well. After 2 hour incubation at 37°C, 100µl of Dimethyl sulfoxide (DMSO) was added to the wells and cells were incubated for 2 more h in dark to dissolve the formazan crystals. The plates were read by ELISA reader at 570nm. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

5.3.6. Phenotypic Characterization of IHH at mRNA level

We wanted to verify the existence of specific human hepatocyte functional capacity of IHH after long periods in culture and after de-immortalization with tamoxifen treatment and GCV sweep. RT-PCR amplifications were performed as described above. Reverse transciptase reactions were done according to

SuperScript II reverse transcriptase manufacturer protocol (Invitrogen, 18064-014) with 2µM of reverse primers of albumin, hAAT and b-actin as primers (as listed below). A 20µl reaction volume was used for 1µg of total RNA. The following components were added to a nuclease-free microcentrifuge tube: 1 µg total RNA, 1µl dNTP Mix (10mM each), 1µl of reverse primer (2 µM) with up to 12µl RNAase free water. The mixture was heated to 65°C for 5 min and quick chilled on ice. Then 4 µl of 5x First-Strand Buffer, 2µl 0.1 M DTT, 1µl of RNase out water were added to reaction mixture. The contents of the tube were mixed gently, then incubated at 42°C for 2 min. Next 1µl (200 units) of SuperScript™ II RT were added and mixed by pipetting gently up and down and incubated at 42°C for 50 min. The reactions were inactivated by heating at 70°C for 15 min.

PCR amplifications were performed in 50µl of reaction mixture containing cDNA samples (1:25) and Taq DNA polymerase (Invitrogen, 10342-020). Amplification conditions were: 94°C for 5 min; 35 cycles of 94°C for 1min, 58 °C for 1 min and 72 °C for 3 min. Primers used were: forward primer 5'-AAACCTCTTGTGGAAGAGCC-3' and reverse primer 5'-CAAAGCAGGTCTCCTTATCG-3' for albumin (596 bp product), forward primer 5'-CTGGGACAGTGAATCGACAATGC-3' and reverse primer 5'-TCTGTTTCTTGGCCTCTTCGGTG-3' for α1-anti-trypsin (560 bp product), and forward primer 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and reverse primer 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' for β-actin (610 bp product). Annealing temperature was 5-10 below the Tm of primers.

5.3.7. Albumin ELISA:

For measuring of albumin production ability of IHH in culture and after transplanting to Alb-uPA SCID mice, IHH culture supernatants and Alb-uPA SCID mouse sera were collected. By using an ELISA assay we measured albumin in the culture supernatant and serum of mice. The plates were coated with primary Antibody (Goat anti-human Alb, Bethyl A80-229A) at 1:800 dilution in Coating

Ab buffer(0.1 M Carbonate /Bicarbonate buffer(4.21g $NaHCO_3$,5.5g Na_2CO_3, dissolve in 500 ml of ddH_2O,adjust PH to9.5 with Na_2CO_3(0.1 M),up to 1 lit with ddH_2O ,store at 4 C), 100µl per well overnight at 4 C. The next day we washed the plates with 5x TBS-TWEEN and blocked for 30 min at room temperature with blocking buffer [1% gelatin/TBS-TWEEN] (200µl per well) and washed with 5x TBS-TWEEN. After that we added standard albumin protein, positive and negative control (100µl/well in duplicate diluted in block). After making standards and dilution of samples, they were added to the wells and incubated for 1 h at room temperature. Then we washed the plates and added the secondary antibody (Goat anti-human albumin HRP conjugated, Bethyl A80-229P) at 1/80,000 and incubated 60 min at room temperature in a foil paper. Next we prepared Tetramethyl benzidine (TMB) solution[0.1 M citric acid+2.6 ml 0.2 M Na_2HPO_4 + 5 ml of H_2O,PH=5 and added one tablet of TMB and shake until dissolved, filtered, and added 3µl of 30% H_2O_2]. Then we washed the plates, added 100 µl of TMB, incubated for 25 min, stopped the reaction by adding 100 µl of 1 M phosphoric acid /well and read the plates at 450 nm. Human serum was used as positive control and Balb-C mouse serum as negative control. The concentration numbers were calculated according to standards.

5.3.8. Urea Production:

Urea synthesis is a component of the ammonia detoxification pathway and is a hepatospecific function. To evaluate hepatocyte-mediated biotransformation of ammonia to urea, 6 million seeded IHH were exposed to 10 mM/L NH_4Cl in culture medium. Samples of media were collected at the beginning and after 24 h of exposure to ammonia. Urea concentration was measured colorimetrically using the urea nitrogen reagent set (BioTron Diagnostics, Hemet, CA) and an ELISA reader at 540 nm. Concentrations of urea were determined using a standard curve of urea (0–45 µg/ml), and total protein concentration of cells were measured by commercial micro-BCA-Protein-assay-kit. These results were then expressed as µg urea/day/mg protein. IHHs were treated with tamoxifen at 800 nM for 7 days

to induce de-immortalization, and were compared to IHH-mCm without treatment and IHH-PoP10 as controls.

5.3.9. Westernblot Analysis for Immortalizing Transgene Recombination:

Accuracy of the site-specific recombination and purity of the cell populations isolated were tested by western blot and indirect immunofluorescence analysis of tamoxifen treated cells and GCV-resistant cells. The cells on tamoxifen (800 nM) treatment (at least 7 days) were then treated with GCV (50 µM) for 5 days. The cells were cultured in a 6 well plate and at 75% of confluence, GCV was added to the media to hit the cells on the linear part of their growth curve to have the utmost effect in eliminating the susceptible cells which should still contain the immortalizing transgenes. Next RIPA buffer were added to the wells and cells were scraped into an eppenorf and protein content was measured by micro-BCA-Protein-assay kit. After adding 2xSDS sample buffer to the samples, 20 µg protein of final concentration of each sample were loaded on acrylamide gel, and by using a gel electrophoresis protein was separated and transferred to nitrocellulose membrane. After blocking overnight,SV40-T-Ag mouse monoclonal antibody (Santa Cruz BioTech, sc-147) (dilution 1:1000) and mouse anti-human β-actin (Sigma, mouse monoclonal, 1:10000 dilution) were added according to the protocol described before; the secondary antibody was HRP conjugated goat anti mouse antibody (dilution 1:10,000). The membranes were visualized by using Western Blotting Chemiluminescence Luminol Reagent (BioRad).

To confirm these results, the IHH treated with tamoxifen for 7 days and GCV for 5 days were subjected to western blot analysis to detect a known hepatocellular carcinoma marker called alpha-feto-protein which is a tumor marker used in detection and follow-up of liver tumors. The human fetus has the highest amount of AFP levels found in humans. These AFP levels gradually decrease after birth down to the low. AFP has no known function in healthy

adults. In adults, high levels over 500 nanograms/milliliter of AFP are seen in only three situations: Hepatocellular carcinoma, Germ cell tumors, and metastatic cancer in the liver originating from other primary tumors elsewhere (35). Antibody used was mouse anti-human-alpha-feto-protein as primary antibody and goat anti-mouse-HRP conjugated as secondary antibody. Western blot analysis was done on 8% gel according to the protocol and details explained previously.

5.3.10. Indirect Immunofluorescence for SV40 Recombination:

Complete extinction of SV40T expression in de-immortalizd cells was confirmed by examining cells with double immunofluorescence. For in situ detection of SV40Tag (Santa cruz sc-147) and merCremer (Rabbit Anti-Cre-antibody, Novagen) ; we seeded cells onto glass cover slips in 35mm dishes at 40,000 cells per dish and incubated at 37ºC, 5% CO_2 and 95% air. Cells were treated with tamoxifen for 48 h. After 48h, we washed the cover slips with PBS and then added 3% (v/v) formaldehyde in PBS for 15 min at 25º C. We removed formaldehyde solution and washed twice with 2ml of PBS. Then we incubated cells at -20º C for 10 min in 3 ml PBS containing 0.05% (w/v) Triton X-100, then in 2ml PBS containing 1% bovine serum albumin (PBS/BSA) for 15 min at 25C prior to incubation for 1 h at 37C with primary antibody(0.5 ml fresh PBS/BSA containing 10µg/ml antibody).After the incubation period , the cells were washed 3 times with 2ml of PBS/BSA (15 min each), and then incubated with 0.5 ml PBS/BSA containing 7.5 mg/ml FITC-goat-anti-rabbit-antibody and also 1:1000 Alexa-594-goat-anti-mouse antibody to bind to anti-Cre and anti-SV40, respectively. We washed the cells twice with 2 ml PBS/BSA and once with distilled water prior to mounting cover slips on cover slides with 90% (v/v) glycerol containing 50mM Tris-HCl (PH=9) and 2.5% (w/v) 1,4-diazobicyclo-[2,2,2]-octane. The cells were viewed under immunofluorescence at 100X magnification with oil immersion.

For double staining of Cre and SV40 for indirect immunocytochemistry: Primary antibody: mouse monoclonal SV40Tag-specific antibody (Santa Cruz,

Diluted 1:100), Secondary Antibody: Alexa-594-conjugated goat anti-mouse immunoglobulin (Invitrogen, Diluted 1:1000). For antibody anti-Cre rabbit monoclonal antibody (Novagen, Tx, Dilution: 1:100), and FITC-conjugated-goat-anti-rabbit (Invitrogen, Dilution 1:1000) is used in this study. The IHH cells were cultured in 12 well plates and after 70% confluency, tamoxifen were added to cell culture at 800nM for 48 hour.

5.3.11. In Vivo Tumorogenicity

To evaluate the potential tumorogenicity of immortalized and reverted cells, 5 million IHH-mCm treated or untreated with tamoxifen for 7 days and GCV (+/-) for 4 days and IHH-PoP10 cells as control were recovered from culture and re suspended in 100µl of PBS. The cells were transplanted subcutaneously in Alb-uPA SCID mice. The mice were assessed every 3 days-body weight and health status- and the injection site palpated for tumor growth and notes were collected. Mice were euthanized either when bearing tumors larger than 1cm (after 4 weeks), or at the end of the observation period (IHH, 12 weeks). All organs were examined macroscopically for the presence of tumors, while the livers and spleens were additionally subjected to microscopic analyses at the end of study. As a positive control a hepatoma cell line was transplanted, which has previously been shown to be tumorogenic in our mouse model.

5.3.12. Animal experiment and Intrasplenic IHH Transplant:

All mice were cared for by experienced animal technicians and fed a standard diet. The mice were maintained in micro-isolator cages containing autoclaved feed, bedding, and acidified water. All mice were cared for in accordance with the Canadian Council on Animal Care guidelines (1993). Experimental protocols were reviewed and approved by the University of Alberta health ethics research committee.

Once successful Cre-mediated de-immortalization had been established, cells were transplanted by intrasplenic injection to Alb-UPA/SCID mice and engraftment, expansion/proliferation and tumorigenicity were monitored by immunohistochemical analysis of liver for presence of functional IHH. As well, engraftment and expansion of transplanted cells were monitored by serum ELISA for human α-1-anti-trypsin and albumin levels and susceptibility of cells to infection with HCV or the JFH variant HCV were monitored by procedures already established in our lab.

To transplant IHH into Alb-uPA SCID mice, a small left flank incision was performed through which the spleen was delivered (Transplants performed by Lin Fu at the Department of Surgery, University of Alberta). One million viable IHH were injected into the inferior pole of the spleen. A small clip was then applied over the area through which the spleen was injected. The incision was then sutured with interrupted 6-0 vicryl sutures and the wound was covered in an antibiotic gel. After transplantation into the spleen, the IHHs travel via the splenic and portal veins into the liver sinusoids. After euthanizing mice livers from the mice were harvested for sectioning and immunohistochemical staining. Cassettes to hold the excised livers and spleens were appropriately prepared and labeled before the actual procedure.

The harvested livers were left to sit in formalin for 24 hours with gentle stirring. After that, the cassettes were serially transferred into jars containing 70%, 95%, and 100% ethanol spending 1.5, 2, and 4 hours in each solution, respectively. The cassettes were finally transferred into jars containing butanol solution before being sent for serial sectioning. Each harvested liver was cut into several serial sections using paraffin wax as the embedding medium. The cut sections were mounted onto glass slides and then heated in a 37°C oven overnight. Thereafter, the slides were subjected to either albumin or Alu immunohistochemical staining as appropriate.

5.3.13. Alu Staining of Mouse Liver:

The fixed slides were deparafinated by serial immersion into xylene, 100% ethanol, 95% ethanol, 70% ethanol, and distilled water at 5 min intervals. The slides were then washed in TBS-TWEEN solution and then incubated in 50µl Proteinase K solution in the 37°C oven for 20 minutes for antigen retrieval. The slides were then washed in TBS-TWEEN, fixed again in 1% formalin solution, and then hybridized with the human ALU probe (Alu hybridization DNA probe, InnoGenex, PR-1001-01) at 37°C overnight.

The slides were washed the next day with TBS-TWEEN and then incubated with primary antiserum to the ALU probe. This was followed by another wash and incubation with 50µl of the secondary antibody for 1 hour at room temperature. Detection was performed according to the protocol provided with the Super Sensitive™ Polymer-HRP ISH detection kit (DF300-YCX, BioGenex Inc, San Ramon, CA). Subsequently, sections were immersed in streptavidin-peroxidase substrate solution and the color was developed with DAB chromogen and the slides were counterstained with methylgreen. After washing the slides in distilled water, permount solution was used to mount coverslips on the slides before examining them with light microscopy. The human cells were counted, by counting the stained human nuclei, for each liver slide; this was done with the light microscope under 20 X magnification.

5.3.14. Human Albumin Immunohistochemistry Staining of Mouse Liver:

Paraffin fixed slides were rehydrated by serial immersion in to 2'x1 Xylene, 2' x 3, 100% EtOH, 2' x 1, 95% EtOH, 2' x 1, 70% EtOH, dH_2O, 5'. After washing slides with TBS-TWEEN, slides were blocked in normal Rabbit Serum Blocking for 1hour at room temperature (RT). Then we added primary antibody (Goat anti-human albumin) in primary antibody (Ab) buffer (Dilution: 1:100 for 1hour at RT). Next we blocked slides in avidin and then biotin block for 15min at room temperature, and then secondary Ab (biotinylated rabbit anti-goat,

5µg/ml) was added for 1h. Slides were blocked by Peroxidase block (3% H_2O_2 on PBS) 10min at RT. Then Avidin-biotin complex horseradish peroxidase reagent (ABC) reagent was added to slides for 30 min at room temperature[2.5µl A+ 2.5µl B+ 245µl PBS] and slides were developed in diaminobenzene (DAB) (20 µl/1 ml substrate for 1' 30" (300-500µl /each slide) (Wait 2-5', then check on microscope).After rinsing by dH_2O, slides were counterstained with Hematoxylin for 30sec to 1min, cleaned by graded alcohol, immerse in Xylene. The slides were covered by coverslip using tolouen paste.

5.3.15. Statistics and Data Analysis:

Quantitative in vitro results were obtained from 3 experimental repeats using IHH. Each value represented the mean ± SD (standard deviation) of triplicate determinations of three different cultures. One-way repeated measures analysis of variance (144) followed by the Tukey post hoc test multiple group comparison was used to analyze group differences of the resultant data. The threshold for statistical significance was considered $p<0.05$.

5.4. RESULTS

5.4.1. Expression of the Proviral Integrant in IHH, and Characteristics of IHH

Integration of proviral genome into genomic DNA of IHH was confirmed by PCR analysis for vTK, SV40 TAg and hTERT (Fig6). PHHs were used for controls. The primers used were: 5'primer GCGCTCTAGATGGCTTCGTACCCCTGC and 3'primer CGCGTCTAGATCAGTTAGCCTCCCCCAT for vTK (1134bp product), 5' primer CAGGCATAGAGTGTCTGC and 3' primer CAACAGCCTGTTGGCATATG for SV40 TAg (422bp product), and 5' primer CGCAGCCACTACCGC and 3' primer AAGGCCAGCACGTTC for hTERT

(250bp product). IHH showed integration of all part of transgenes, including hTERT, SV40, vTK by PCR.

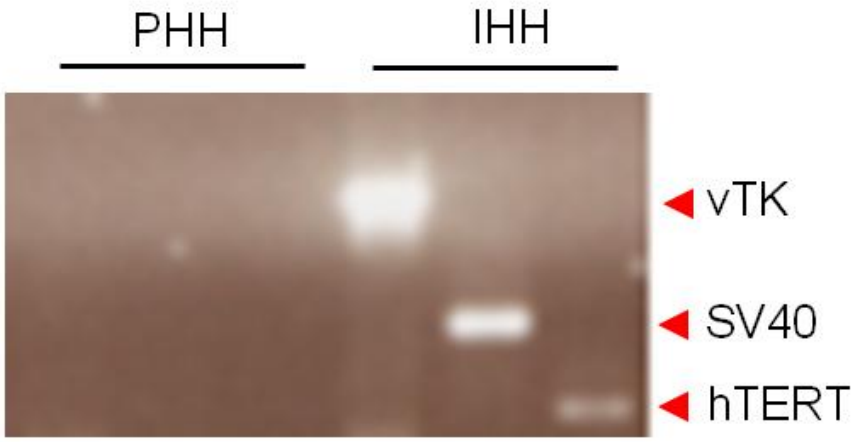


Fig 6. Expression of the proviral integrin in IHH. Integration of proviral genome into genomic DNA of IHH was confirmed by PCR analysis for vTK, SV40 Tag, and hTERT. Human primary hepatocytes were used for controls. In IHH cells hTERT, SV40 and vTK is inserted inside IHH genome (Fig from Donna Douglas).

5.4.2. Integration of Tamoxifen-dependent Cre-recombinase-expression cassette (MerCreMer) in IHH:

IHH-PoP10 clone (original IHH cells provided by Dr, Trono) were transfected with pcDNA3neo-merCremer (provided by Dr. Marek Michalak, University of Alberta) for the expression of MerCreMer using Lipofectamine™ 2000 (Invitrogen) transfection reagent, and 72 hour after transfection, selection with 5µg/ml G418 was initiated. merCremer cassette has a G418 resistant, and after high dose treatment of G418, clones 4, 9, 14, 15, 19 and 20 of IHH-merCremer were selected. Then merCremer expression was determined by immunoblot analysis using an anti-α-estrogen receptor antibody (Santa Cruz ,SC-542) that recognizes the chimeric Cre protein. IHH cells transiently transfected with pcDNA3 (empty vecor) and pcDNA3-merCremer were used as negative (-ve) and positive (+ve) controls, respectively. Clonal selection of IHH with merCremer is showed in fig 7A. The clone number 14 was selected as merCremer

expression (107kd) was detected in this clone (Fig7). This clone was repopulated for further experiments and named as IHH-mCm. In order to confirm the presence of MerCreMer protein in our engineered IHH (IHH-mCm), and to rule out the detection of endogenous estrogen, we performed immunoblot analysis of IHH-mCm and their original cell line IHH-PoP10 (Fig7). The MerCreMer were present at a 107 kd in IHH-mCm and not in IHH-PoP10. The endogenous estrogen receptor is at 66kd (Fig7B).

By indirect immunofluorescent, merCremer presence was confirmed in cytoplasm of IHH-mCm with an anti-Cre antibody. The merCremer stained as green fluorescence is present around the nucleus which is counter stained by DAPI. In IHH-PoP10, no merCremer was detected in cell cytoplasm (Fig 8).

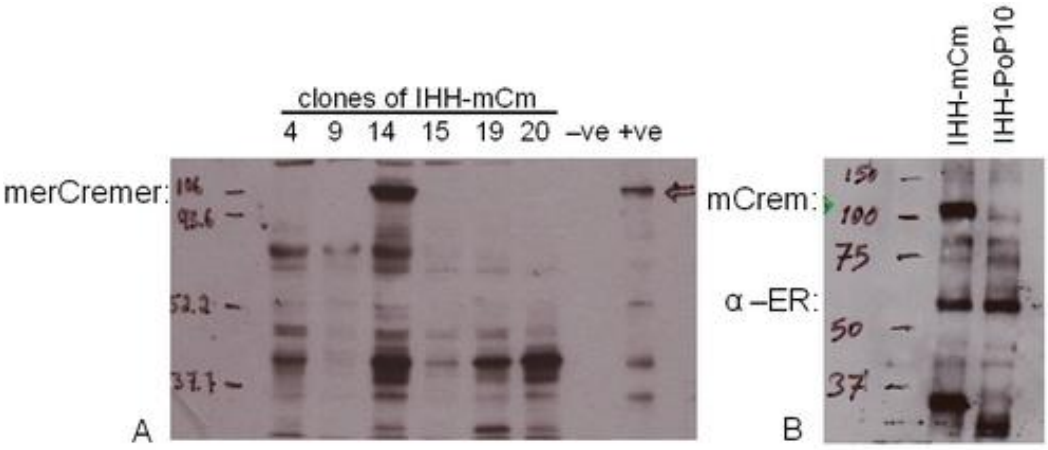


Fig 7. Expression of merCremer and clonal selection of IHHmCm. IHH were transfected with pcDNA3-MerCreMer, and MerCreMer expression of G418 resistant clones 4, 9, 14, 15, 19 and 20 was determined by immunoblot analysis using an anti-α-estrogen receptor antibody. IHH cells transiently transfected with pcDNA3 (empty vecor) and pcDNA3-MerCreMer were used as negative (-ve) and positive (+ve) controls, respectively. Westernblot analysis of merCremer expression with anti-estrogen receptor, and clone number 14 with high expression of Cre was selected and repopulated further (A). merCremer (mCrem) is expressed in IHH-merCremer but not PoP10 (original IHH) showed by westernblot analysis with anti-α-estrogen receptor at 107kd. Endogenous alpha-estrogen receptor (α-ER) is expressed at 66kd in both cell lines. (Left Fig from Dr.Donna Douglas).

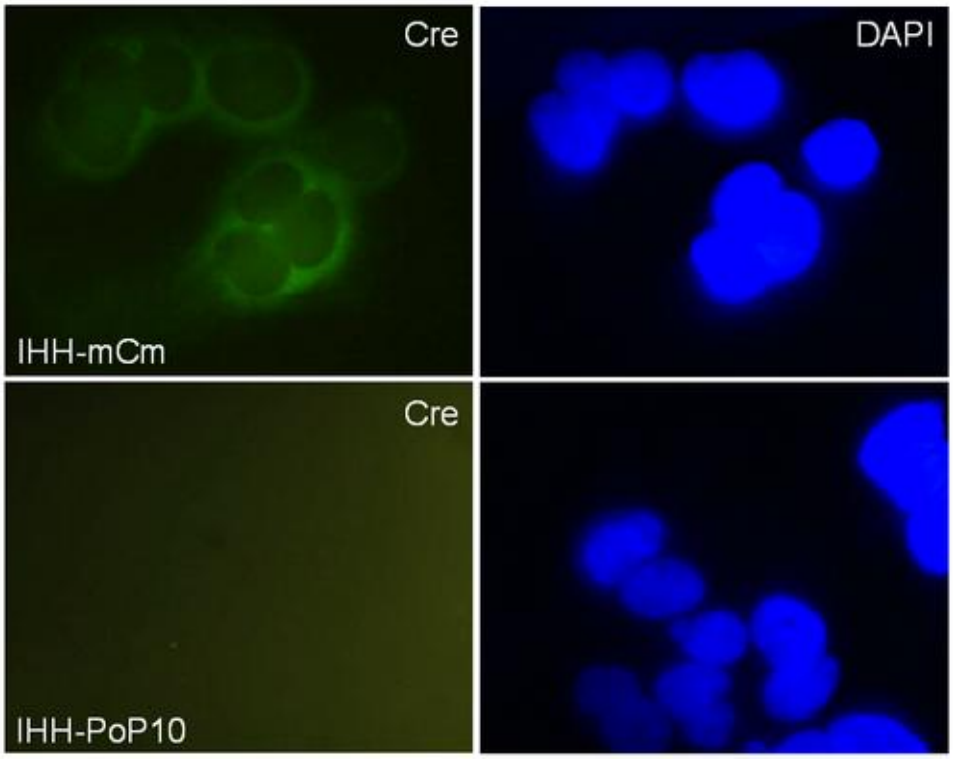


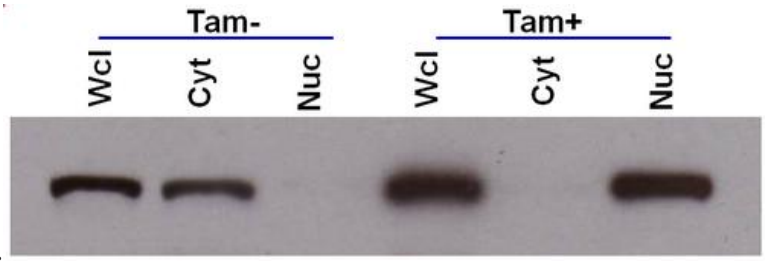
Fig 8. Indirect immunofluorescence detection of merCremer in IHH. IHH-mCm express merCremer in their cytoplasm as detected by indirect immunoflouresence with anti-Cre antibody showed by green fluorescence around nucleus. IHH PoP10 did not express any green fluorescence. Cell nuclei are stained by DAPI as blue fluorescence. Original magnification X100.

5.4.3. Nuclear Localization of MerCreMer in IHH-merCremer:

IHH-merCremer (IHH-mCm) cells were treated with 800nm OH-TAM for 24 hours (+/-TAM). Tamoxifen is estrogen receptor antagonist and preventing estrogen from binding to its receptor. Mutated estrogen receptor ligand-binding domains (MerCreMer, MCM) are insensitive to endogenous estrogen and only react to tamoxifen. MerCreMer contains two mutated estrogen receptor–binding domains, which confers tight dependency on tamoxifen binding for translocation to the nucleus. In the absence of tamoxifen, MerCreMer only resides within the cytoplasm, however, in the presence of tamoxifen, tamoxifen binds to MerCreMer and translocate into nucleus (138). Translocation of merCremer from cytoplasm

to nucleus was detected by westernblot analysis. merCremer chimeric protein was detected in whole cell lysates (WCL) and Triton X100 soluble fraction of IHH cells which contain cytoplasmic fraction (Cyt) and Triton X100 insoluble fractions containing nuclear proteins and genomic part (Nuc) by immunoblot analysis using an anti-estrogen receptor antibody. After 24hour, tamoxifen induced translocation of merCremer to the nucleus in treated cells was compared to un-treated cells (Fig9.A). To confirm the translocation of Cre to the nucleus by using an anti-Cre antibody we detected the merCremer by indirect immunefluorescent technique as green fluorescent (Fig 9B). When cells were untreated, merCremer was distributed in the cytoplasm like a hazy green fluorescence around the nucleus in all IHH-mCm but not in IHH-Pop10. It was located in the nucleus after tamoxifen treatment, like a dense ball of green fluorescence. DAPI counter-stain (which stains the nucleus of the cells as blue fluorescence) also confirmed that green fluorescence is located in the nucleus after tamoxifen treatment. Efficacy of tamoxifen effect on Cre nuclear localization is about 10-50% of cells by subjective counting through indirect immunoflouresence. These numbers are not different from what other groups have reported in this regard (64, 172, 173).

A.



B.

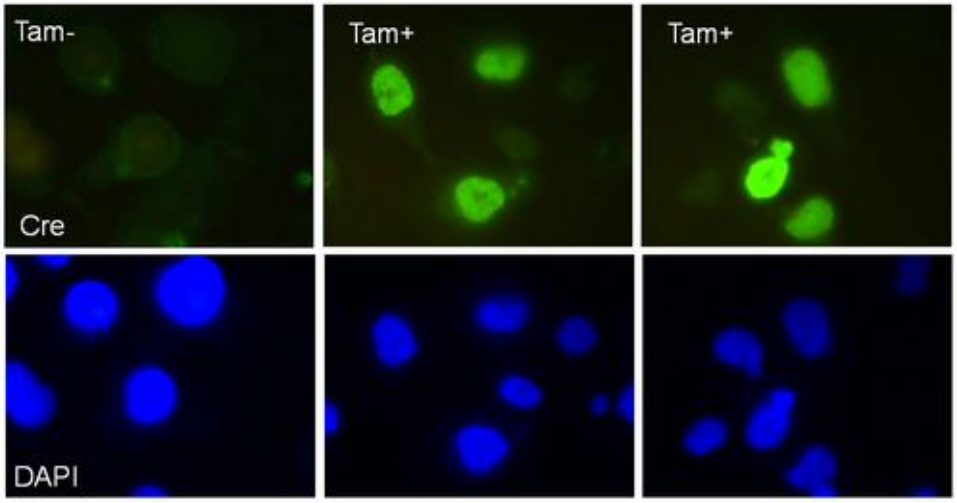


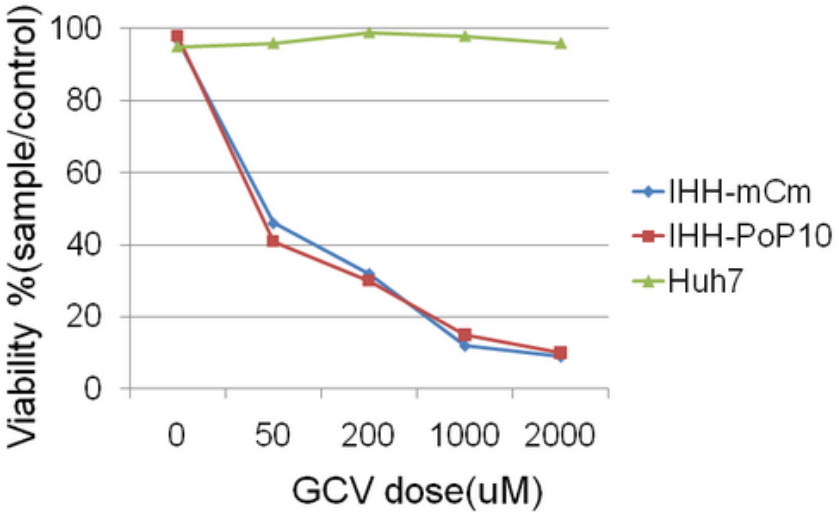
Fig 9. Translocation of merCremer to IHH-mCm nucleus after tamoxifen treatment detected by indirect immunofluorescence and westernblot analysis. merCremer is expressed in IHH-mCm in whole cell lysate (WCL) and cytoplasm (Cyt) in the absence of tamoxifen (Tam) by anti-alpha-estrogen-receptor. Cre protein translocates to the nucleus (Nuc) after tamoxifen treatment (A). Indirect immunofluorescence shows the expression of weak and dispersed fluorescence of Cre by anti-Cre antibody in cytoplasm of IHH-mCm which is slightly denser in fluorescence around the nucleus; however after tamoxifen treatment Cre accumulates in the nucleus and shows a condensed fluorescence in the nucleus as confirmed with DAPI counterstain of nucleus (B).

5.4.4. In Vitro Sensitivity of IHH-mCm Cells to Gancyclovir

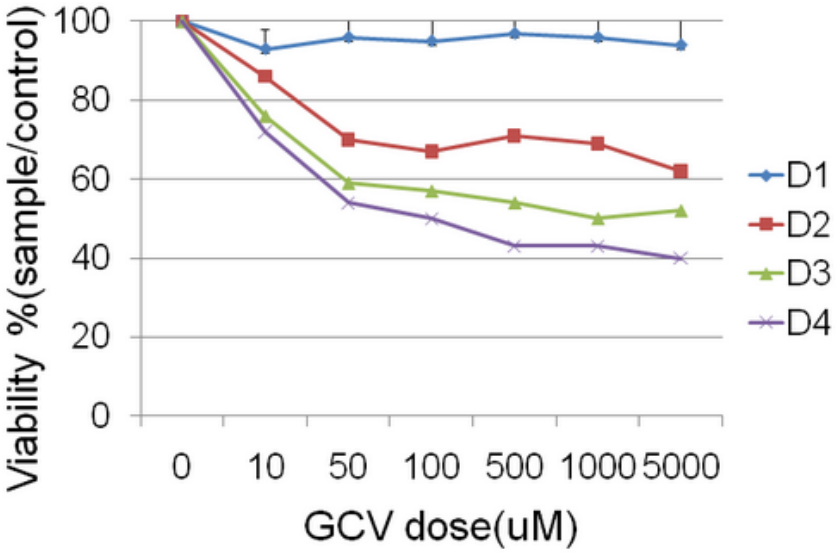
To confirm that expression of the merCremer chimeric protein does not interfere with GCV sensitivity conferred by the HSV/vTK suicide gene; IHH-PoP10 and IHH-merCremer cells were seeded in a 96 well tissue culture plate and treated with various doses of GCV ranging from 0-2000 µM for 5 days. The

cytotoxic effect of GCV on cells was determined using a colorimetric assay for the quantification of cell proliferation and viability (Cell Proliferation Kit (MTT), (Roche, Mississauga, Ont). IHH-mCm and IHH-PoP10 were sensitive to GCV killing effect but Huh7 cells were resistant to such treatment (Fig10A). IHH-mCm and PoP10 viability decreased from above 90% in 0 doses to below 50% in doses above 50 µM. In Huh7 cells as controls no killing effect of GCV was observed on these cells, and their viability stayed above 90% at all doses.

Next we wanted to check the effect of dosage and timing of GCV treatment, we dosed the IHH-mCm with different doses of GCV and at different time points. Interestingly GCV at low dose (50µM) or high dose (5000µM) do not kill a high percentage of IHH cells on day 1 which indicates the time needed for phosphorylation of GCV and incorporation of GCV into the genomic DNA of cells; however by day 2 and after GCV starts killing about 50% of IHH cells (Fig10B).



A.



B.

Fig 10. Viability of IHH cells after gancyclovir (GCV) treatment measured by MTT test. IHH-mCm, PoP10, and Huh7 were treated with different dose of GCV for 5 days. IHH-mCm and PoP10 viability decreased significantly compare to Huh7 cells with doses above 50µM GCV (A). IHH cell viability after treatment with different doses of GCV measured at day 1 up to 4 (B)(n=3,P<0.05).

5.4.5. Microscopic Appearance of reverted IHH-mCm:

The cell morphology of immortalized hepatocyte cells was assessed by light microscopy. IHH cells grew in a monolayer and displayed polygonal shape

with a round nucleus. The morphology of the cells did not exhibit any significant identifiable changes between early and late passages. The immortalized cells had the ability to grow to a high density and reached confluence within 5–7 d. They showed no sign of senescence or reduced proliferation normally shown by the non-immortalized parental hepatocytes.

Reverted IHH-mCm cells treated with tamoxifen (800 nM for 7 days) and Gancyclovir (50 µM for 5 days) in comparison to IHH do not grow with low plating efficiency and retain different broaden morphology and resistance to GCV sweep. IHH not treated with tamoxifen will die after GCV sweep as showed in Fig below (Fig11). Interestingly, according to our subjective counting of survived viable cells after tamoxifen and GCV treatment ,only 10-40% of IHH-mCm could be de-immortalized by tamoxifen inducible Cre/loxp mechanism and be resistant to GCV killing effect. This observation was not different from others (64,172,172).

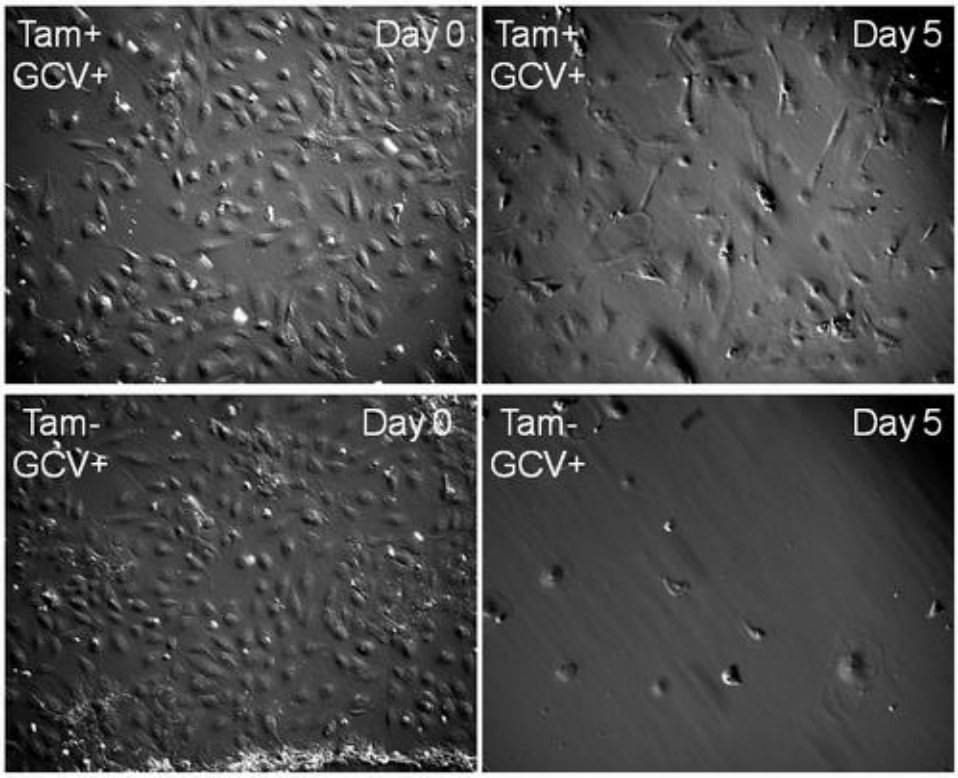


Fig 11. Light microscopy morphology of IHH. IHH show a polygonal shape in culture. After tamoxifen treatment, some IHH-mCm cells are resistant to GCV killing effect, but in cells without tamoxifen treatment GCV kill almost all of the cells on day 5. Original magnification X20.

5.4.6. SV40 Expression after Recombination:

The IHH cells were treated with tamoxifen for 2, 5 days and 7 days. Western blot analysis by SV40-T-Ag mouse monoclonal antibody (Santa Cruz BioTech, sc-147) (dilution 1:1000) was done on 8% gel; the secondary antibody was HRP conjugated goat anti mouse antibody (dilution 1:10,000). The SV40 LTag (94kd) expression was decreased in whole cell lysate after tamoxifen treatment in IHH-mCm cells compared to non-treated cells (Fig12A), especially

after 7 days in culture, which is compatible with other studies that showed decrease in SV40 after 6 to 10 days, because of the long half life of SV40 protein (53, 56). B-actin was used as control for protein loading. We also examined this decrease of SV40 expression in the nuclear fraction of IHH-mCm treated with tamoxifen. The amount of protein loaded for each group nuclear fraction is 20 µg as measured by micro-BCA-protein-assay. In the tamoxifen treated group, SV40 also decreased in the nuclear fraction (Nuc) in comparison to the non-treated group (Fig12A).

We further treated the 7 day tamoxifen treated IHH-mCm with GCV for 5 days. The cells which survive the GCV killing effect are the cells which have lost their inserted transgene part that contains vTK. By using western blot we showed that after tamoxifen treatment and GCV sweep, the remainder of surviving cells do not express SV40 at all (Fig12B). Besides, these cells lose alpha-feto-protein as a marker of hepatocellular cancer as shown by western blot (Fig12C). Alpha-feto-protein is a hepatocellular tumor marker used in clinical practice to follow-up patients with this cancer. Additionally in stem cell studies, α-feto-protein is a marker of differentiation of stem cells toward hepatocytes, and losing this marker shows differentiation to a more mature hepatocyte phenotype. In our study, after de-immortalization, IHH lose their α-feto-protein expression like human hepatocytes. The original IHH-mCm and PoP10 express this marker like hepatoma cells such as Huh7. Tamoxifen only could not de-immortalize all IHH-mCm cell population and we see expression of α-feto-protein and SV40 in IHH-mCm tamoxifen treated only. However, selected de-immortalized cells by GCV killing effect have lost expression of α-feto-protein and SV40 (Fig12 B,C).

To confirm this result we used indirect immunefluorescence double staining of IHH-mCm for Cre and SV40 with anti-Cre and anti-SV40LTAg. Our results indicated that after 48hour tamoxifen treatment IHH-mCm with nuclear localization of Cre, SV40 expression decreased accordingly (Fig13).

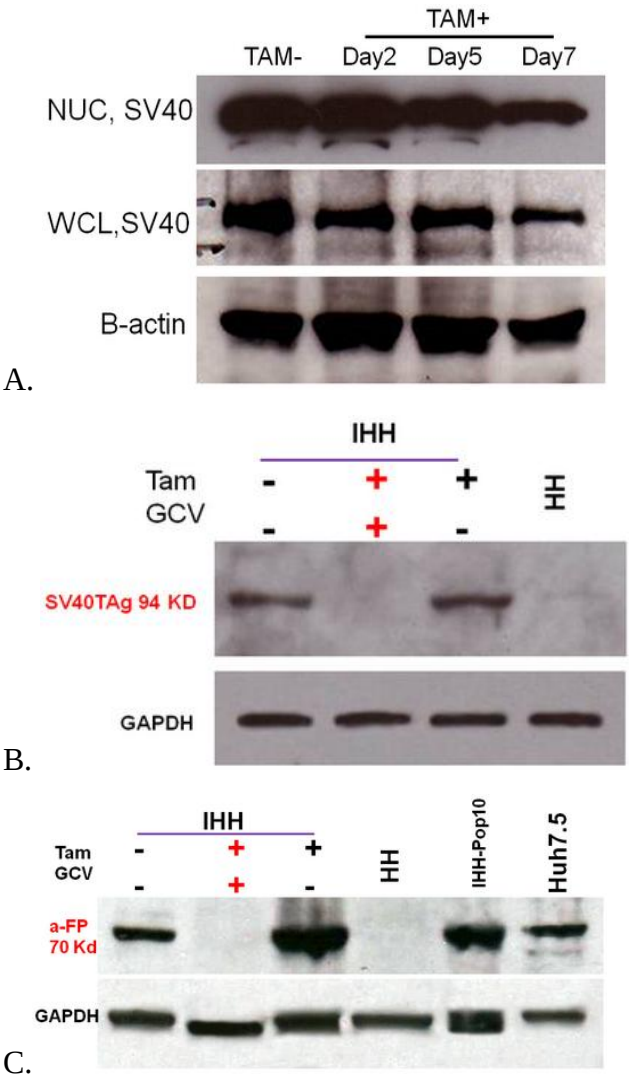


Fig 12. A. Decrease in expression of immortalizing transgenes in IHH after tamoxifen treatment. SV40 protein decreases mostly on Day 7 compared to Day 2 and 5 in both nuclear fraction and whole cell lysate detected by anti-SV40-antibody. β-actin was used as control for whole cell lysate not nuclear fraction(A). SV40 expression after 7 days treatment with tamoxifen and then 5 days with GCV was measured with western blot analysis. B. GCV kills the IHH cells that escape tamoxifen induced Cre/Loxp excision of inserted transgenes. The remaining cells from GCV killing do not express SV40 similar to human hepatocytes. C. Reverted IHH after tamoxifen treatment and GCV selection lose α-feto-protein, which is a marker for undifferentiated human hepatocytes detected by anti-alpha-feto-protein antibody. Original IHH-PoP10, non-treated IHH-mCm, and Huh7 cells show this marker of undifferentiation.

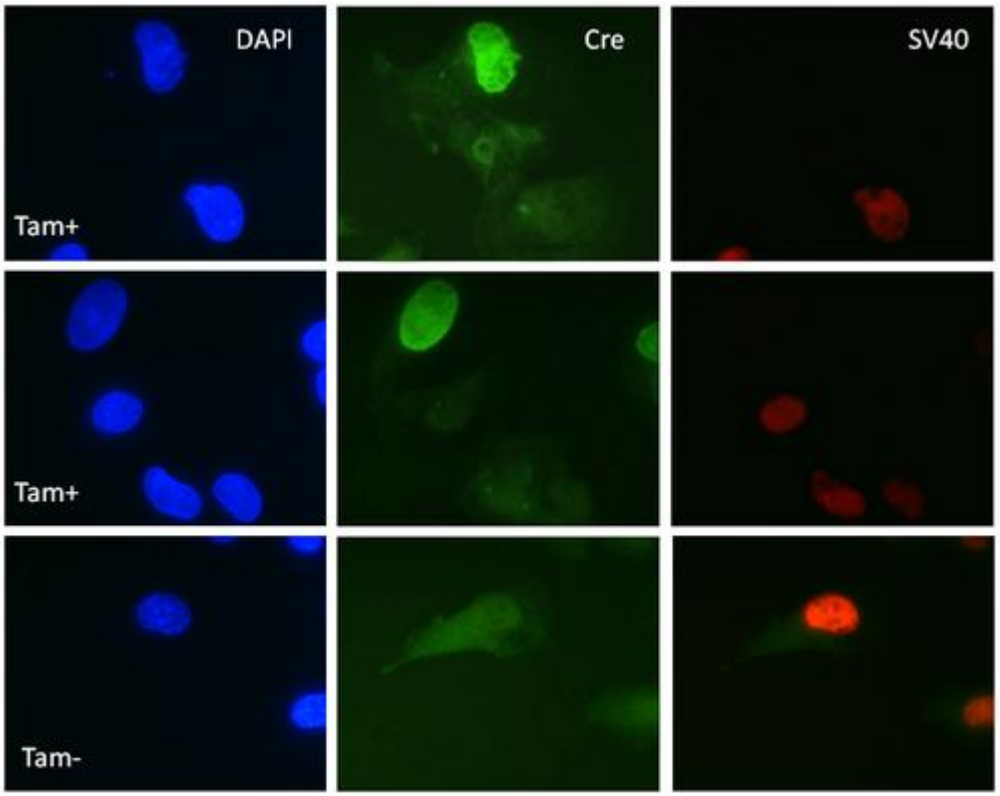
C. 

Fig 13. Decrease in SV40 after tamoxifen induced nuclear Cre. SV40 and Cre staining of IHH with indirect Immuno fluorescence after tamoxifen treatment detected by anti-Cre and anti-SV40 antibody. SV40 LTAg red fluorescence decreases in IHH-mCm that have nuclear Cre green fluorescence after treatment with tamoxifen for 7 days as shown by double staining with anti-Cre and anti-SV40 . But IHH cells without nuclear Cre green fluorescence still show SV40 (red fluorescence) in their nucleus. The nucleus is also stained by blue DAPI fluorescence. Original magnification X100.

5.4.7. Liver-specific functions of IHH:

To determine whether the IHH retained functions under the culture conditions after immortalization, we examined the expression of genes and protein products or enzymatic activity which are hepatocyte-specific and are considered hallmarks of the hepatocyte phenotype. Adult hepatocytes synthesize a

variety of serum proteins such as albumin, and alpha1-antitrypsin (hAAT). PHH were used as a positive control.

To analyze whether or not IHH cells retain such hepatocyte characteristics after de-immortalization by tamoxifen and selection of the de-immortalized cells by GCV, we first examined the mRNA expression levels of hepatocyte-enriched genes by RT-PCR analysis. We detected the production of RNA encoding albumin and alpha 1-anti-trypsin in IHH-mCm after reversion and de-immortalization. It is notable that mRNA transcription is sensitive to this de-immortalization procedure (Fig14A, B).

Albumin secretion by the de-immortalized IHH in culture was confirmed by western blot analysis; although the expression level was much lower than that of freshly isolated human hepatocytes as depicted in Fig14C. However, when using ELISA we were unable to detect albumin secretion in culture by reverted IHH-mCm, suggesting the secreted level is lower than our ELISA detection limit (4 µg/ml). These data show that in comparison to primary hepatocytes, IHH albumin secretion is at lower levels.

On the other hand, IHH under similar conditions, did not exhibit a detectable expression of hAAT at protein level in western blot (data not shown). The absence of hAAT at protein level may be due to the culture medium conditions(174, 175) or the development of a transformed phenotype, as suggested earlier with the loss of differentiated markers in SV40-transformed hepatocytes (176).

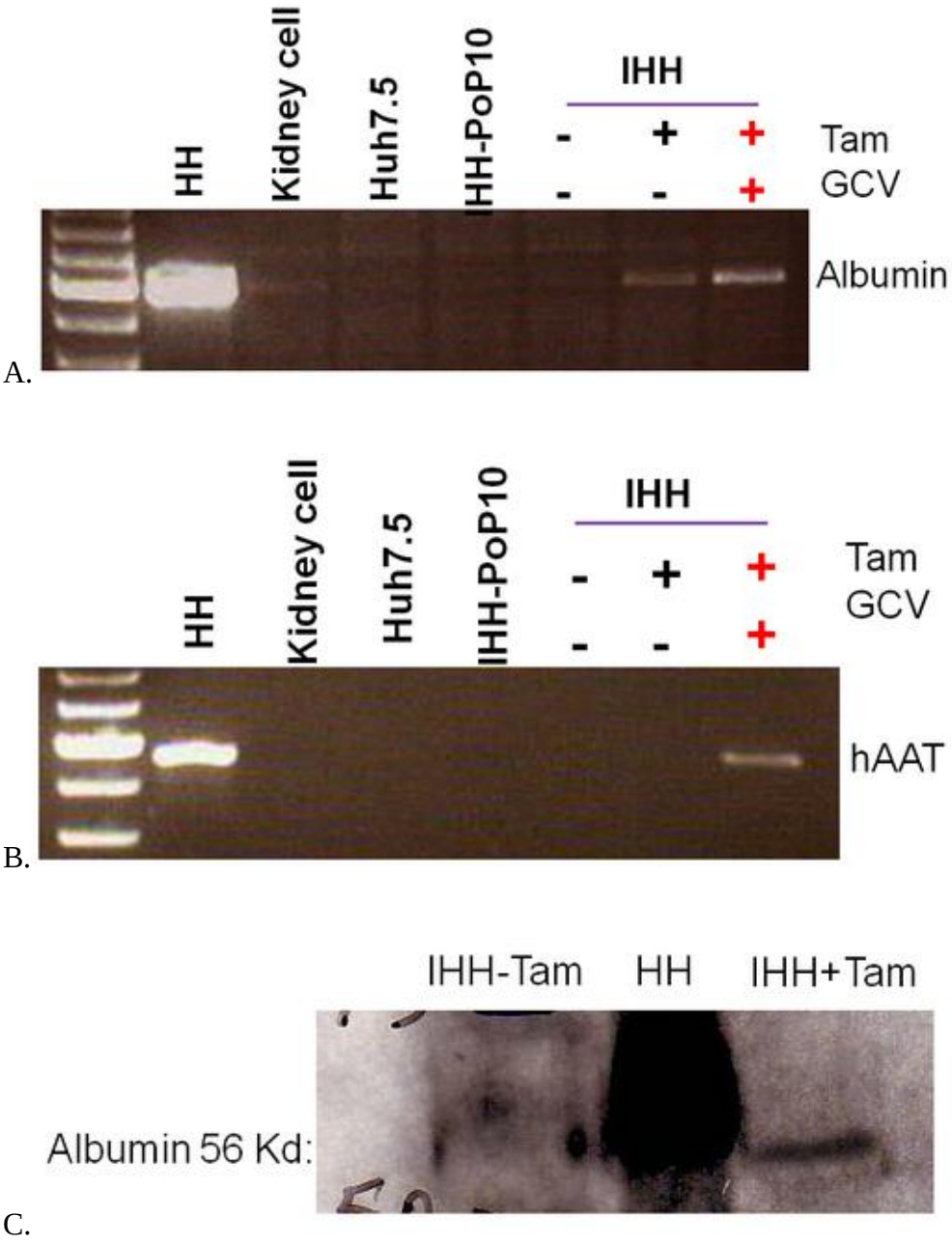


Fig 14. Hepatocyte marker expression after de-immortalization. RT-PCR analysis of IHH for hepatocyte markers albumin and hAAT at mRNA level. IHH cells show RNA expression after tamoxifen treatment and de-immortalization. Albumin RNA is expressed in IHH after tamoxifen treatment for 7 days and GCV sweeping for 5 days (A). Human alpha-1 anti-trypsin mRNA expression after tamoxifen and GCV treatment were shown by RT-PCR(B). Albumin expression (56kd) after tamoxifen treatment for 7 days was measured by western blot analysis by anti-albumin antibody. 20 µg of protein from whole cell lysate of each sample was loaded on gel(C).

Urea synthesis was measured in the different IHH culture groups to assess the effect of de-immortalization on regaining the functional ability of urea synthesis of primary hepatocytes (Fig15). Urea concentration in IHH-mCm and PoP10 groups was significantly lower than in the IHH-mCm treated with tamoxifen for 7 days ($P<0.05$). Urea production of hepatocytes in IHH-mCm and PoP10 did not have significant differences in culture. To eliminate the effect of tamoxifen itself on the values of urea concentration, we first washed the cells with PBS and then we started the experiment. Values are mean± SD and $p<0.05$.

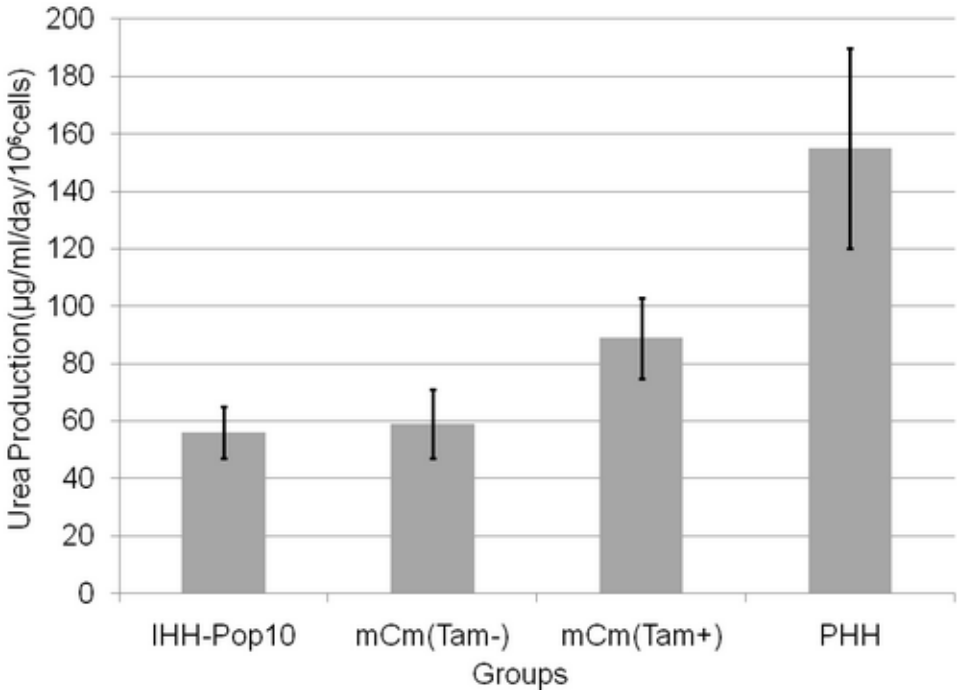


Fig 15. Urea production function of IHH after de-immortalization. Urea production to measure bio transformation of ammonia to urea as a human hepatocyte functional marker is measured in IHH-mCm treated with tamoxifen. IHH-mCm treated with tamoxifen showed significantly higher amount of urea concentration in culture than non-treated cells and IHH-PoP10 as control (,n=3, $P<0.05$). The box shows mean±SD. Primary human hepatocytes were used as positive control showed urea concentration of 155±35 µg/ml/day/10^6cells.

5.4.8. Tumorogenicity of IHH in Subcutaneus Transplant:

Subcutaneus transplantation of IHHPoP10 or mCm to Alb-uPA SCID mice was performed to evaluate tumorogenicity of IHH. None of these mice showed subcutaneous gross tumor formation within 3 month after transplantation in comparison to control tumorigenic cell line (Huh7).

5.4.9. Intrasplenic Transplant of IHH, Tumorogenicity and Histological Examination:

The results of ELISA assay for albumin on culture supernatant of IHH culture in combination with RT-PCR analysis of hepatocyte-enriched genes revealed that IHH gradually de-differentiated as passage progressed. Thus we conducted intrasplenic transplantation of $1x10^6$ million IHH into Alb-uPA SCID mice. The endogenous growth factors secreted as paracrine factors in this mouse model could be an enhancer for these IHH to retain their hepatocyte specific function. However they could also induce tumor growth in IHH. Our lab has previously observed that transplantation of Alb-uPA SCID mice with Huh7 or HepG2 cells results in aggressive tumor formation (Dr.Norman Kneteman, personal communication). However, this was not the case when mice were transplanted with IHH. Out of 40 transplanted mice followed up to 6 months post-transplant, only 1 had visible tumors, similar to the one shown in Fig 16.

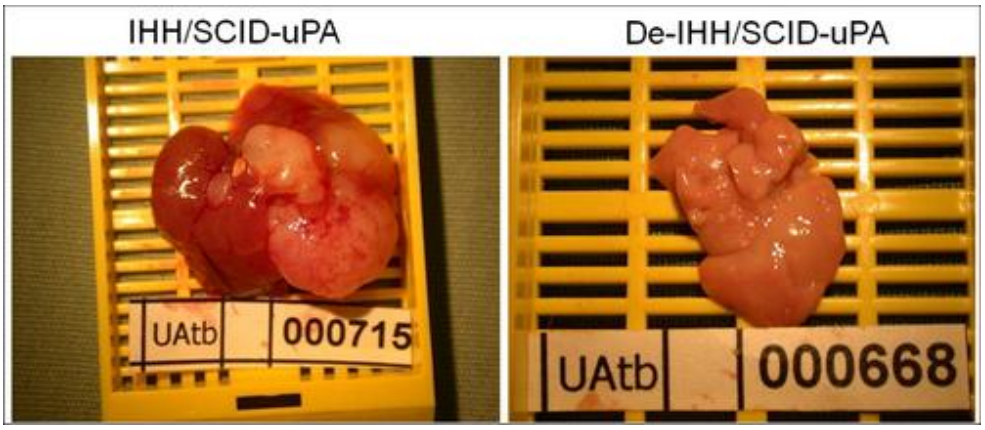


Fig 16. Tumor growth after intrasplenic transplantation of IHH. IHH cells transplanted into Alb-uPA SCID mouse model intrasplenic did not grew macroscopic tumor in mice transplanted with de-immortalized IHH (right) unlike the non tamoxifen treated IHH cells (left).

After transplantation of IHH, mouse livers were recovered to see the engraftment, presence and function of IHH after transplant. The sections were stained for albumin as a functional measure and for Alu staining as the engraftment sign. Alu staining of liver sections post-transplantation of IHH showed presence of IHH inside the liver (Fig17). The normal hepatic structure was not preserved in the engrafted parts. IHH morphology inside the liver was different from PHH transplant, although the border of the mouse part and humanized part was completely separate in both cases. Transplanted IHH showed weak staining for albumin. The dark brown staining was notable in IHH inside the liver. Staining of PHH inside the mouse liver showed stronger dark brown staining for albumin; this difference could reflect partial de-differentiation of IHH.

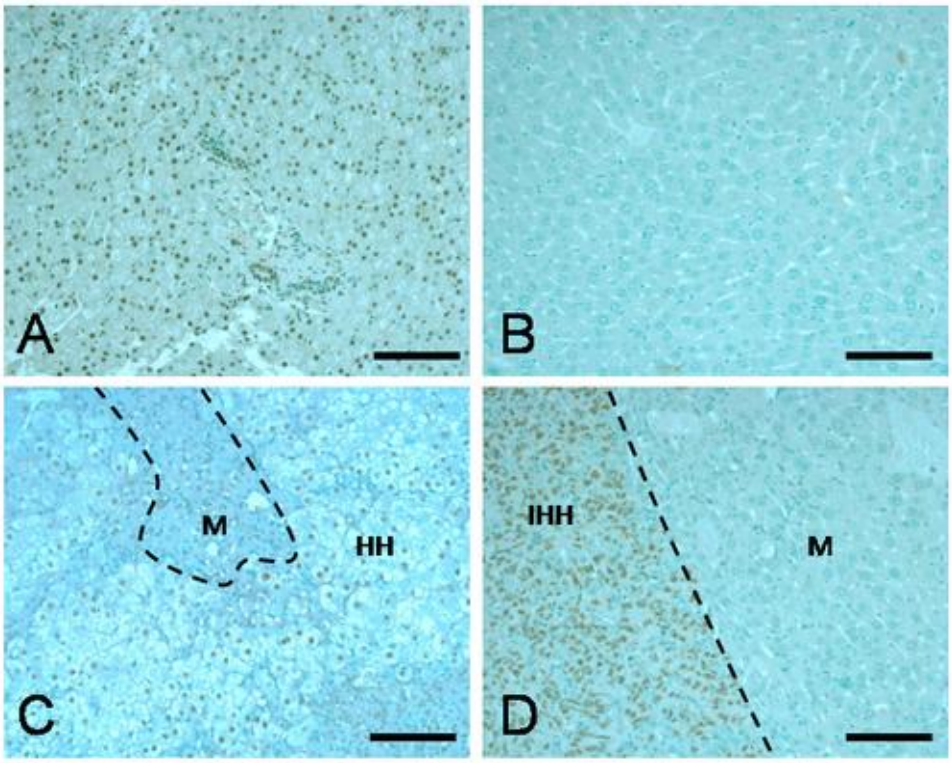


Fig 17. Engraftment of IHH transplanted into Alb-uPA SCID mouse liver. Alu staining of IHH transplant into liver of Alb-uPA SCID mouse is depicted. Human DNA probes stains nucleus of human hepatocyte (dark brown) but not mouse hepatocytes (blue only). Human liver tissue (A) is used as positive control and mouse liver (B) is negative control. Fresh human hepatocyte transplanted into Alb-uPA SCID mouse liver(C) is nested and engrafted inside the mouse liver like series of islands with dark brown nucleus. IHH integrated in mouse liver is depicted as dark brown nucleus inside the mouse liver (D). M: mouse tissue, HH: human hepatocyte, IHH: Immortalized human hepatocyte.

5.4.10. IHH in vivo function:

IHH were transplanted into SCID mouse livers and their integration was observed by Alu staining (Fig17). They were not tumorogenic in mice as established by close observation for 6 months. None of them grew tumors that could be detected by direct physical exam of mouse liver, spleen, or other organs. IHH functional status in vivo was measured by albumin immunohistochemisrty staining of mouse

livers sections transplanted with IHH and PHH. We found weak albumin staining in livers of mice transplanted with IHH and strong dark brown staining in PHH transplanted mice. No brown staining was noted in the mouse hepatocytes (Fig18). We also measured human albumin in sera recovered from mice transplanted with IHH. While no hAAT level could be detected in serum from IHH transplanted Alb-uPA SCID mice, human albumin was detectable in mice transplanted with IHH-mCm at an average of 84±45 µg/ml (Table 1). In mice transplanted with IHH-PoP10 no albumin was detected in mouse serum. Albumin level in a mouse transplanted with fresh human hepatocytes with high engraftment as confirmed by proven high hAAT (>200 µg/ml) was measured as positive control and was 1200±640µg/ml (mean±SD). The lower limit of the linear portion of our albumin ELISA assay is 4µg/ml.

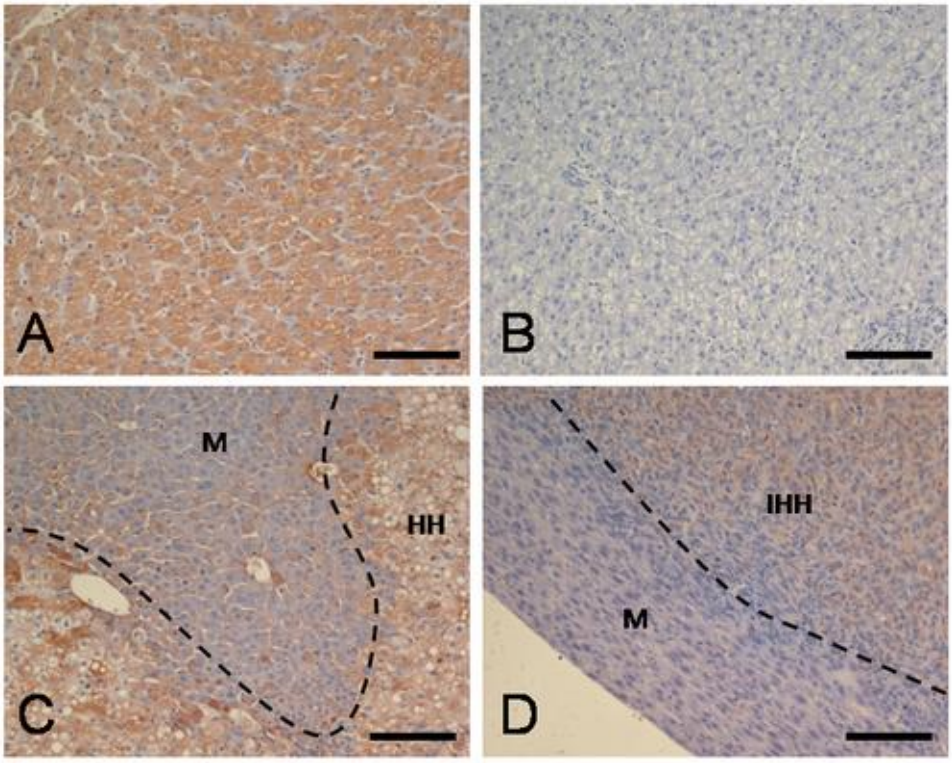


Fig 18. Retained hepatocyte function in IHH after transplant into the liver of Alb-uPA SCID mouse. Immunohistochemistry of human albumin stains as brown in cytoplasm. Human liver section (A) shows a universal staining of human albumin, and mouse liver (B) is negative for staining. Transplant of fresh human hepatocytes results in areas of human hepatocytes shown as brown cells that surround blue mouse cells (C). IHH integration into mouse liver (D) stains more weakly than fresh human hepatocytes in mouse liver. M: mouse tissue, HH: human hepatocyte, IHH: Immortalized human hepatocyte.

Table 1. Albumin secretion function of IHH post-transplant to Alb-uPA SCID mice. IHH-mCm treated with tamoxifen transplanted to Alb-uPA SCID mice showed no hAAT level, and showed albumin at a much lower level than high hAAT (high level repopulated) mice transplanted with PHH. Three mouse were transplanted in each experimental group (mean±SD, n=3).

Tx Group	hAAT(μg/ml)	Albumin(μg/ml)
IHH-PoP10	0	0
IHH-mCm	0	84±45
PHH	450±125	1200±420

5.5. DISCUSSION

HCV studies are in real need of small animal models to test new drug efficacy and side-effects for different HCV genotypes and to facilitate study of HCV biology. Freshly isolated PHHs provide a valuable tool for various research strategies and clinical applications, such as studying the regulation of certain differentiated hepatocyte functions. More importantly it is our main source for transplantation of human hepatocytes into chimeric mouse models (177, 178). However, the utility of such freshly isolated human hepatocytes in Alb-uPA SCID mouse model is hampered by difficulties in obtaining populations of high quality primary cells on a regular basis and is exacerbated by inability to expand such cells *in vitro*. Thus, other alternative sources of hepatocytes have to be explored.

Hepatocytes immortalized with SV40Tag and hTERT can make unlimited supplies of cells feasible. In our study, use of the Cre/LoxP-based reversible IHH represents an important step in the development of an appropriate strategy for resolving the current limits of primary hepatocytes for generation of chimeric mice and other uses. Different from a general gene transfer strategy, the use of this novel strategy of regulated reversible immortalization allows temporary

expansion of cell populations by transfer of an immortalizing transgene which can be subsequently excised by site-specific recombination. Several studies have confirmed the utility, safety and efficiency of the reversible immortalization procedure in transplant studies (179).

The immortalization process imparts unlimited replicative potential and allows the cells to bypass senescence. The IHH utilized in our studies were made by transduction of two immortalizing transgenes, SV40LTAg and hTERT, to PHH. Furthermore, we have engineered our IHH model to express a chimeric Cre as confirmed by our western blot and indirect immunofluorescence. We named them IHH-mCm. Detection of cytoplasmic Cre by indirect immunofluorescence microscopy was difficult due to dispersed cytoplasmic Cre and low intensity of fluorescence. However by using specific anti-Cre we managed to capture confirmatory images in every IHH-mCm cell preparation in comparison to the original IHH and isotype control images. Our results also showed that engineered IHH-mCm could be de-immortalized by tamoxifen regulated Cre/loxp mechanism. This was done using a regulated form of chimeric Cre bound to two mutated estrogen receptors, so the Cre only responds to tamoxifen and not endogenous estrogen. Our results showed nuclear translocation of Cre in IHH-mCm treated with tamoxifen compare to non-treated cells. Tamoxifen treatment resulted in Cre nuclear translocation in 10-40% of IHH-mCm by our subjective estimation. However as mentioned earlier this observation was not very different from other groups (64, 172, 173). The lack of 100% translocation could be because of resistance of cells to penetration of tamoxifen or resistance of mutated estrogen receptor to tamoxifen effect, or incomplete dissociation of merCremer from HSP90 in cytoplasm. The strategy to eliminate the cells that had escaped Cre/loxp mechanism of de-immortalization was to use GCV negative selection of cells which were resistant to GCV killing.

This cell line has an extra safety advantage of the presence of a suicide gene (vTK) which enables us to eliminate any possible post-transplant tumor

development by simple GCV treatment in our transplanted mice. Addition of ganciclovir to cells containing the HSV-TK gene will convert ganciclovir to a toxic metabolite that kills HSV-TK-containing cells. GCV treatment (50μM) of IHH cells resulted in their death within 5 days, whereas reverted cells survived. These multiple selection systems should remove any tumorigenicity from our IHH-cell line. IHH cells reverted and selected by GCV showed no SV40T expression, lost their proliferative capacity and did not cause tumors to develop in SCID mice. Introduction of suicide genes into genetically engineered cells would provide a way to eliminate the transplanted cells when their behavior becomes harmful to the host. In this work, we evaluate the in vivo cell killing effect of GCV, by transplanting IHH-mCm into mice before tamoxifen treatment and administering GCV to the mice. Interestingly, none of these transplanted mice with de-IHH grew tumor. In order to facilitate the identification of any possible tumor growth after transplantation of IHH cells into Alb-uPA SCID mice, the observation time was extended considerably up to 1 year in this study. The accumulation of data regarding these safety considerations can make the cells more reliable and increase the potential for use of such cells in humans clinical trials in future (71).

The ability to measure differentiated hepatocyte function in vitro is extremely dependent upon culture conditions for IHH cells. In this study such differentiated functions such as albumin secretion and urea production were detected in IHH cells in culture. There is evidence that an improvement in differentiated cellular responses can be accomplished simply by transplanting the cells in animals (176, 180). In this study, although IHH cells are positive for albumin at mRNA and protein levels, albumin secretion was very low compared to PHH cells that served as positive control. However, the data match with earlier studies, showing low gene expression and protein secretion of hepatic cell markers albumin and alpha-feto-protein in undifferentiated liver cells (71). It was interesting to see that although IHH showed higher functional status in albumin secretion and urea production after reversion, their functional activity was still

much lower than PHH. Another interesting finding of our study was that IHH were able to retain their albumin secretion in vitro and after transplantation in vivo, but never showed any hAAT secretion in vitro or in vivo even after de-immortalization. This could be due to the fact that hAAT secretion reflects a higher functional status and is more specific compare to albumin secretion and so de-differentiation affects hAAT secretion more profoundly than albumin secretion. It could also simply be that albumin secretion is at a log higher level and so is easier to detect.

Successful intrasplenic transplantation of hepatocytes is a procedure that has been reported by many groups to introduce freshly isolated human hepatocytes into our Alb-uPA SCID mouse model to make chimeric humanized mice which can harbor HCV infection in the humanized part of the liver. This technique involves injection of a small suspension of hepatocytes into the splenic subcapsular sheet. We demonstrated the formation of a humanized liver in Alb-uPA SCID mouse model after intrasplenic transplantation of immortalized human hepatocytes. Our study is an exception to the field where in the past every effort to replace human hepatocytes by cell lines like hepatoma cells has failed due to high potency of intrinsic tumorogenicity in the Alb-uPA SCID mouse model. We confirmed that our IHH cell line is not tumorogenic in our mouse model. This could be related somehow to their growth curve in vitro where they grow only up to confluency and stop growth in culture due to space limit and high cell contact, which could also be a limiting factor inside the mouse liver (73, 181).

As observed by our long-term follow up, IHH did not induce tumors when transplanted into Alb-uPA SCID mice, either subcutaneously or in the liver. This correlates with previous observations that expression of SV40TAg and hTERT is not sufficient to induce the oncogenic transformation of human primary cells (75, 182, 183). Instead, the addition of an oncogenic allele of ras has been shown to be necessary for the malignant transformation of SV40TAg/TERT immortalized human cells, including hepatocytes (79, 88, 184). In fact, the tumorigenicity of

SV40-IHH seems to be associated with a secondary genetic event (e.g. ras oncogene over-expression), which complements SV40 T-Ag immortalization to create the fully tumorigenic phenotype ((176, 180). The probability of the occurrence of such an event increases with the number of passages of SV40-IHH in culture before injection (185-187). However, in this study we passaged our cell lines for 3 years and yet no tumorogenic activity was observed in transplant studies. Although for confirmation kariotype analysis would be important.

We showed IHH engraftment into Alb-uPA SCID mouse liver after intrasplenic transplantation and their ability to partially regain their functional status as reflected by albumin immunohistochemistry staining and serum levels. Despite strong evidence of engraftment of IHH cells into mice liver, lack of tumorogenicity and evidence of partial reversion to a hepatocyte like phenotype, we were unable to infect mice transplanted with IHH with HCV. This could be due to many factors such as mice factors, IHH cell inhibitory factors, or even HCV virus factors in our inoculums.

Altogether, the potential advantages of using this IHH model is that, it eliminates the need for repeated human donation, the variability associated with different donors could be avoided, and the technical aspects of hepatocyte isolation would be greatly simplified. It could allow for the production of large quantities of a standardized group of cellular clones for experimental and therapeutic purpose. The cells can be infected with HCV in vitro, and so could facilitate examination of the role of HCV infection in cell cycle regulation. IHH could also be used for other areas of study, including human malaria and hepatocyte transplantation for acute liver failure and metabolic liver disease.

6. IHH FOR IN VITRO MODEL:

6.1. INTRODUCTION

Progress in understanding hepatitis C virus (HCV) biology and developing new anti-HCV therapies has remained a challenge due to the lack of an efficient cell culture system for virus growth that sustains viral replication and produces infectious particles (61, 66, 188, 189). PHH are the ideal system in which to study HCV infectivity. In vitro cultures of PHH are challenging due to their limited ability to divide and survive in culture condition (56). In order to overcome those problems researchers have tried to provide the natural liver milieu to human hepatocytes cultures. Attempts to provide space supporting conditions to the culture such as collagen or co-culture of hepatocytes with other supportive cell lines (fibroblasts or endothelial cells) have been partially successful. Hepatocytes stay differentiated and functional for at most 2-3 weeks, then start to de-differentiate and lose their capacity to grow and survive in culture (190). In addition, HCV replicates in PHH only at very low levels (29, 30, 32). Altogether, culture of PHH is not presently a suitable model to study HCV replication in vitro.

The most suitable in vitro system at present is Huh7/JFH1. Only the Huh7/Huh7.5 cell lines are permissive for replication, infection and release of the fulminant hepatitis-derived HCV-2a (JFH-1) strain and its chimeric derivatives (11, 13, 51). This virus replicates well in Huh7 cells without adaptive mutations (13, 50). Wakita et al (13) obtained virus production in cells transfected with the cloned JFH-1 genome, and Zhong et al (51) established a highly efficient system for production of infectious virus in Huh7.5.1 cells. This system has two challenges. First the virus was recovered from a rare fulminant case of HCV unlike the typical natural history of HCV which is chronic in 85%. Substantial

variations in the underlying virus are likely. In addition, Huh7 cells are a hepatoma cell line that has lost many functional and differentiated characteristics of PHH. This makes the current HCV in vitro models far from ideal reflections of the natural virology, pathology and metabolic patterns of HCV.

Expansion of in vitro models of infectious HCV to other genotypes such as 1a has not been successful to date. Nevertheless, the development of a system that can sustain the replication of HCV of various genotypes in non-transformed hepatocytes, after either transfection of non-chimeric virus or exposure to serum of patients infected with HCV virus, remains a challenging priority (191).

In this study we tested IHH as to suitability for use as a substitute model for in vitro HCV studies. Continuous proliferation of PHH could be achieved by introducing immortalizing transgenes (192, 193), such as SV40LTAg (97, 180, 194-196) and the human papilloma virus E6E7 genes (HPV/E6E7) and IHH are phenotypically and functionally similar to the parental cells (74, 197-202). These IHH will divide in culture conditions that assist their survival for potential long-term HCV replication, and besides because of their similarity to their origin, yet they may support HCVser replication in culture.

6.2. Hypothesis:

IHH are ANALOGOUS to Huh7 cells in that they support infection by transfection with JFH-1 and IHH cells are UNIQUE from Huh7 cells in that they support infection by natural HCV from patient sera.

6.3. Methods and Materials

6.3.1. Generation of JFH1 RNA

We obtained the pJFH1 plasmid in appropriate quality and quantity (400µg/µl). Plasmids were used to transform Ca^{2+}-competent DH5 cells

(Invitrogen, 18265-017) using a standard heat-shock method according to the manufacturer's protocol. To make the plates we added 15.5 g of LB(Difco Lurie Broth Base, Miller, 241420) to 1 lit and then add 15 g of Agar to that and then sterilized , let it to cool down and added 100 µM Ampicillin, then poured 30-35 ml on each 100 mm plate. Transformation reactions were plated on agar containing Ampicillin for selection and colonies were allowed to grow at 37 C for 24 h. Single colonies were selected and used to inoculate overnight cultures of LB media (5ml) containing 100 µg/mL ampicillin at 37 C while shaking (245 rpm). Plasmid DNA from expanded single bacterial colonies was extracted using Qiagen plasmid extraction kit (Qiagen, 27106). LB cultures that contained positive clones were also used to make glycerol stocks which consist of 850 µl overnight LB cultures combined with 150 µl sterile glycerol. These are kept at –80 C and are used to inoculate LB/amp solutions for overnight culture and subsequent plasmid miniprep.

We digested pJFH1 DNA with restriction enzymes by combining XbaI (100U) + NEBuffer2, and incubating for 2 hour at 37ºC. We checked complete digestion of pJFH1 DNA by separating 0.5ml of a digested sample, along with the 1Kb Plus DNA Ladder, by electrophoresis through a 1% agarose gel containing 0.1 mg/ml ethidium bromide. Next we mixed the digestion product with 50µl TE buffer, and pipeted into the tube 100µl phenol-chloroform-isoamyl alcohol 25:24:1 (vol/vol/vol). The tube was shaken vigorously, centrifuged for 15 min at 12,000g at 20-25 ºC (room temperature), and the aqueous phase was transferred to a new tube. After that we pipetted 100ml chloroform into the tube, shook vigorously and centrifuged for 5 min at 12,000g at room temperature (chloroform extraction).Then we transferred the aqueous phase to a new tube and pipette 1/10 volume 3M sodium acetate, 2.5 volume 99.5% ethanol and 1/100 volume glycogen into the tube (ethanol precipitation). We stored the sample for 20 min at –80 ºC, and centrifuged for 20 min at 12,000g and 4ºC. The supernatant was discarded, the pellet washed with 500ml 70% ethanol, and then centrifuged for 15 min at 12,000g and 4 ºC. The supernatant was discarded, and the pellet dried at

room temperature, and re-suspended in nuclease-free water and stored at -80ºC. Next we treated the plasmid with Mung bean nuclease and incubated for 30 min at 30ºC. The digested plasmid was treated with proteinase K and extracted with phenol-chloroform-isoamyl alcohol (25:24:1), then with chloroform as described, and then DNA was precipitated by ethanol as described previously. We estimated DNA concentration by micro-spectrophotometery and marked its size by electrophoresis through a 1% agarose gel containing ethidium bromide.

For H77 RNA we used a PCV-H77 plasmid obtained from Buck et al. We used the same method of bacterial transformation, and Ampicillin resistant clone selection. The only difference was the digestion enzyme which was XbaI and Reaction Buffer2. We examined the digested plasmid on 1% agarose gel to confirm the complete digestion

We transcribed the RNA with a MEGAscript kit. After that we purified RNA with TRIzol LS according to the instructions of the manufacturer. After denaturing for 5 min at 65 C, we confirmed the size of the synthesized RNA by separating 1ml of diluted sample, along with an RNA ladder, by electrophoresis through a 1% agarose gel containing 0.1 mg ml–1 ethidium bromide. We determined the RNA concentration with a spectrophotometer after a further 50-fold dilution (final dilution, 1:1000).

6.3.2. Transfection (electroporation) of IHH with JFH1 and H77c:

IHH were transfected with in vitro transcribed RNA of JFH1 or H77 (10µg RNA). For this purpose, we cultured IHH in a flask and after reaching confluency, we trypsinized the cells. The cell pellet was re suspended and washed with OptiMEM I reduced-serum medium and centrifuged. We resuspended 7×10^6 IHH cells from the pellet with 400µl Cytomix buffer. Then we mixed 10 µg RNA with 400µl cell suspension and transferred to an electroporation cuvette, and electroporated the cells with Gene Pulser II (BioRad) in conditions of 260V and 950µF. We transferred the transfected cells into two 10-cm culture dishes, each

containing 8 ml complete medium and incubated the dishes for 24 hour at 37 °C and 5% CO_2. We removed culture medium and washed the transfected cells 10 times with 10 ml PBS, the last wash was retained for quantification. Then, 8 ml of fresh complete medium was added. Every 72 hours at 60-80% confluency of the cells, we gathered the media for quantification and we trypsinized cells and collected them in a tube. After washing 3 times with PBS, we retained the last wash. We re-cultured the cells at 1/3 to $1/5^{th}$ on new plates and added 8 ml fresh media. The rest of the cell pellet was added to 375ml of Trizol-LS for intracellular RNA extraction and quantification of intracellular RNA.

We next examined the presence of HCV in IHH cell culture medium. On different days after transfection, culture medium was filtered through a 0.45µm cellulose acetate membrane (Millipore, Bedford, Mass.), concentrated to x10- to 20-fold by Millipore ultra-filtration (100-kDa cut off), and examined for detection of the HCV genomic sequence by RT-PCR.

6.3.3. Detection of HCV RNA in IHH by RT-PCR

We investigated whether IHH support HCV genome replication. The clones JFH1 and H77 contain a 5' untranslated region (5'UTR), a coding sequence which is suggested to be necessary for replication (203, 204). Total cellular RNA was extracted 5 days post-transfection. To detect the HCV genome, total cellular RNA was used for cDNA synthesis with a SuperScriptII first-strand-synthesis system (Invitrogen), following the supplier's protocol and using a 2 µM antisense primer. PCR amplification was performed with cDNA as a template, using sense (5'-CACTCCCCTGTGAGGAACTACTGTCT-3') and antisense (5'-TGGTGCACGGTCTACGAGACCTCCC-3') primers from 5'UTR at 94°C for 30 sec, annealing at 55°C for 60 sec, and extension at 72°C for 90 sec for 40 cycles. b-actin was used as an internal control, using specific primers described in method section 5.3.6 (205). Reverse transcription-PCR (RT-PCR) analyses suggested amplification of sequence from the 5'UTR.

6.3.4. Quantification of HCV RNA in infected IHH by Realtime RT-PCR

Real Time RT-PCR was performed by KMT Hepatech Inc (Edmonton,Alberta,Canada); their protocol is briefly described. The samples were saved from supernatant and intracellular content of IHH infected or transfected with HCV. Intracellular RNA was extracted by TRIZOL LS according to the manufacturer's protocol. After extraction, intracellular RNA was dissolved in RNAse DNAse free water and the samples were sent to KMT.

Real-time RT-PCR protocol was provided to us by KMT hepatech. Briefly, HCV RNA was extracted from supernatant by using GTC (GuSCN-Silico, "Boom" method) method , using extraction by 560 µl AVL lysis buffer containing 5.6 µl carrier RNA+ 20 µl size fractional silica, and vortexed 10 second, and incubated for 15 min at room temperature and then centrifuged for 10 second to result in a silica pellet. The silica pellet was washed with buffer (Guanidine thiocyanate 60g, 0.1 M Tris-HCl (PH 6.35-6.45) , and then washed again by 70% Ethanol and acetone. Then pellet was dried for 10 min at 56° C in the heating block. After centrifugation for 5 min at max speed, 6 µl of the supernatant containing RNA was aliquoted to RNase free tubes.

The reverse transcription of HCV RNA into cDNA was performed by primers homologous to the desired genetic sequence. The HCV NCR T-342-R sequence: 5'-AGGTTTAGGATTCGTGCTCAT is computer generated to provide maximum specificity. Appropriate controls are run with each reaction.

Five µl RNA was added to the PCR tube, and 2x RT buffer +20x enzyme mix+10 µM T-342-R primer were added. RT protocol was at 37ºC for 60min and 95 ºC for 5min. PCR primers were Forward T-149-F sequence: 5'-TGCGGAACCGGTGAGTACA. HCV probe was labeled with dye, sequence: 6-FAM-CACCCTATCAGGCAGTACCACAAGGCC-TAMRA. It is crucial to run a blank (no cDNA) each time to ensure the primer or contamination is not a problem. A curve is created for each set of PCR reactions which can be used for

all of the samples in the same RT-PCR. The following reagents were added to reaction: Taqman® Gene Expression Master Mix+ Primer T-149-F+ primer T 342- F+ probe –FAM. PCR thermal cycling conditions were set as 50º C for 2 min, 95º C for 10 min,95 ^{o}C for 45 x19 second, and 60 ^{o}C for 1 min.

6.3.5. In Vitro Infection of IHH with serum-derived HCV

HCV infection experiments were carried out using sera from patients infected with HCV. Infections were performed 2 days after plating by overnight incubation of cell monolayers with 100 µl inoculum derived from HCV positive patient sera, which contains 1x10^{5} HCV RNA/ml virus determined by real-time-RT-PCR, in 3 ml culture serum free medium. The cells were incubated with the HCV positive patient's sera for 8 h. Following exposure, cells were washed ten times with PBS and incubated in fresh serum-free medium. The medium was changed every 2±3 days until harvest. Supernatant of infected IHH cells were collected at various times during the culture period and stored at -80 °C. The cells were also extracted and following extraction from culture; cells were centrifuged and washed three times thoroughly with PBS. RNA was then extracted from the cells as described above by TRIZOL LS according to manufacturer protocols.

6.3.6. In Situ HCV Protein Detection by Indirect immunofluorescence

To further examine intracellular expression of HCV protein, we grew un-transfected and transfected IHH cells (1-2.5×10^{5}) on a sterile coverslip. Then we washed the cells and fixed and premeabilized them in acetone-methanol (1:1 vol/vol) for 10 minutes at −20°C. Cells were then incubated for 1 h in immunofluorescence (IF) buffer (PBS, 1% bovine serum albumin, 2.5 mmol/L EDTA) at room temperature. Then cells were incubated for 1 h with anti-NS3a mouse antibody (Chemicon) diluted (1:100) with IF buffer at room temperature. We aspirated the antibody and washed the cells 3 times with PBS. The cells were incubated for 1 h with fluorescent alexa-488-conjugated anti mouse IgG (Alexa Fluor 488; Invitorgen, Cat. No. A11029) (1:1000) at room temperature in the

dark. Then we aspirated the antibody and washed the cells 2 times with PBS and 1 time with ddH_2O. Coverslips were mounted on glass slides with Perma-Fluor™ mounting solution and DAPI was added for nuclear staining.

6.3.7. Detection of HCV Protein by Immunoblot

IHH transfected or infected with HCV were collected at passage on day 7. Plates were washed once with Phosphate buffered saline (PBS) and lysed with RIPA buffer. Protein content of each sample was measured by micro-BCA-Protein-Standard kit. After that 2XSDS sample buffer were added to the samples, and then 1.5 µl of B-Mercapto-ethanol were added to 100 µl 1xSDS sample. Afterward, 20 µg total protein of each sample was loaded on acrylamide gel. Proteins were separated on 8% acrylamide gel by gel electrophoresis, and then were electrophoretically transferred to a nitrocellulose (BioRad) membrane. The membranes were blocked in 5% milk-PBS-TWEEN overnight and the next day after wash with PBS-TWEEN, incubated with the primary antibodies [mouse anti-NS3a antibody, Chemicon, 1:1000 dilution), then after 3 times wash with PBS-TWEEN, they were incubated with secondary antibodies [Hourse-raddish-peroxidase-conjugated anti-mouse IgG antibodies (Invitrogen), were used at a dilution of 1:10,000]. The membranes were visualized by using Western Blotting Chemiluminescence Luminol Reagent (BioRad).

6.3.8. Infection of Naïve IHH with HCVcc:

We collected culture medium 72 hour after transfection, cleared it using low-speed centrifugation and passed it through a 0.45 µm filter. Part of the filtrate was concentrated 1/30 using an Amicon Ultra- 15 (cut off: 1×10^5 Da; Millipore). We seeded cells 24 hour before infection at a density of 5×10^4 cells/well in a 12-well plate, or at 1×10^5 cells/well in a 6-well plate. We infected cells with 100 µl of inoculum for 3hour, washed them, added complete medium and cultured cells for 12, 24, 48, 72 and 96hour. We performed RT-PCR analysis of intracellular HCV RNA for detection of infection in the cells post inoculation.

6.3.9. Inoculation of Alb-uPA SCID mouse with HCVcc

We collected culture medium 72 hour after transfection and added fresh medium to the cells, then repeated culture medium collection every 2–3 d after cell passage. Day 7 culture medium was used to inoculate mice. We removed cell debris by low-speed centrifugation (20min at 1,000g) and passed the culture medium through a 0.45µm syringe-top filter unit. We concentrated medium using an Amicon Ultra-15 device. First, we pipeted filtered culture medium into the Amicon Ultra-15 device (maximum volume is 15ml) and centrifuged for 30min at 3,000g and 4ºC. Culture medium with virus was concentrated until the medium reaches 50X concentrations.

We next inoculated Alb-uPA SCID chimeric mice with supernatant from infected or transfected IHH to see if this supernatant is infectious to the mouse. Eight weeks after transplantation of mice with freshly isolated human hepatocytes, 2 mice were inoculated intraperitoneally with 100µl inoculums from IHH infected with serum derived HCV (HCVser) from HCV positive donors (viral genotypes 1a) or transfected with JFH1. One and 2 weeks after inoculation with the supernatant, mice serum samples were analysed for quantification of positive-strand HCV RNA by real time reverse transcriptase PCR.

6.4. RESULTS

6.4.1. Detection of HCV in IHH transfected with JFH1 (2a) and H77 (1a) RNA:

We investigated whether IHH supports HCV genome replication. We transfected IHH and Huh7 with JFH1; transfected IHH were passaged up to 18 days in culture (Fig19). Transfected IHH showed intracellular HCV RNA by RT-PCR up to 18 days. Control negative was non-infected IHH. Quantitative RT-PCR analysis of HCV RNA in supernatant of transfected cells showed a pattern of

rapid decrease from 10^6 to 10^3, but then was stable up to 18 days (Fig20). Unlike IHH, Huh7.5 cells transfected with JFH1 showed a pattern of stable production of JFH in supernatant and intracellularly. This suggested a higher permissiveness of Huh7.5 cell lines compare to IHH. Non-infected cells were negative at all time points in real time PCR analysis. The drop in intracellular RNA in IHH is much slower than the drop in supernatant. This could be due to faster replication of the virus intracellular than egress of virus to supernatant.

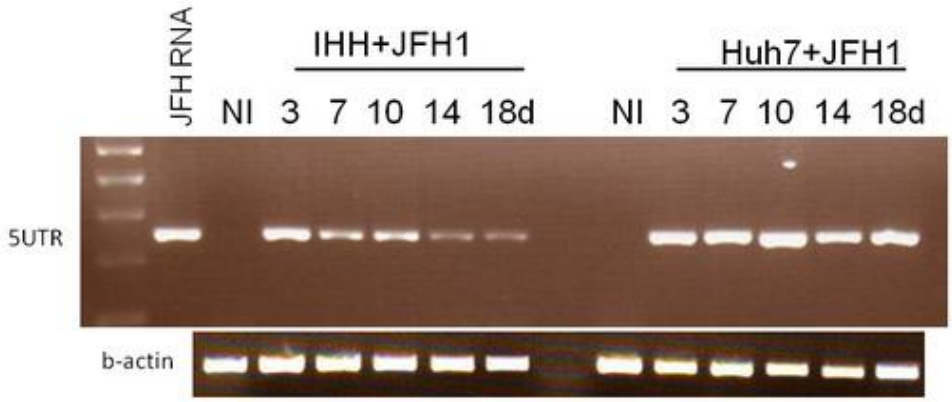


Fig 19. HCV RNA replication after transfection of IHH. RT-PCR analysis of Intracellular HCV RNA of IHH and Huh7 cells transfected with JFH1 and cultured up to 18 days in culture. Intracellular HCV RNA presented inside the IHH transfected with JFH1 up to 18 days in culture. The primer was 5'UTR from untranscribed region of HCV RNA detected at 244bp. The non-infected cells (NI) as control negative were negative for HCV RNA.

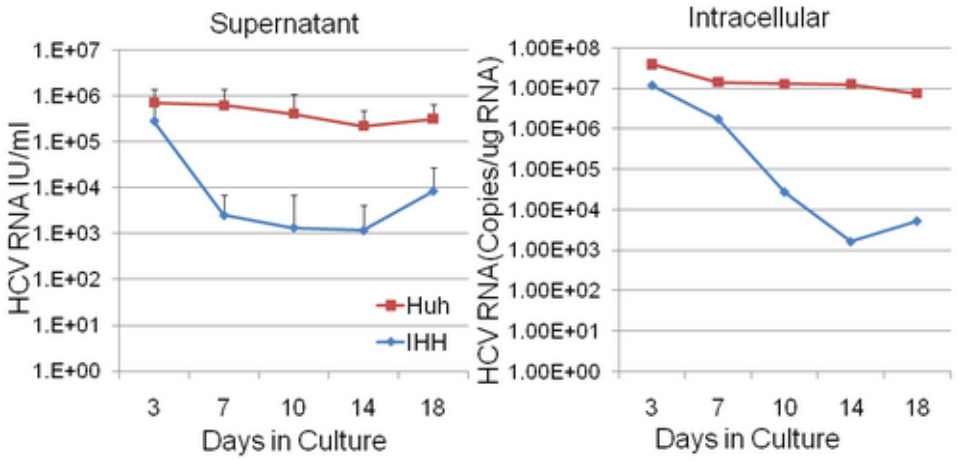


Fig 20. Time course of HCV RNA secretion by JFH1 (2a) transfected IHH. Levels of HCV RNA in transfected cells (right) and corresponding culture supernatants (left). Cells were transfected with given HCV RNAs and passaged up to 18 d. Viral RNA was determined 3,7,10,14 and 18days after transfection. HCV RNA level in supernatant of Huh trasfected with JFH is maintained between 10^5 to 10^6, however in IHH transfected with JFH, the level of RNA declined rapidly by Day 7 to between 10^3 to 10^4 IU/ml. Intracellular RNA in this cell line follows a similar pattern and in IHH it declined to 10^3 to 10^4IU/ml by day 14. Numbers present mean±SD (n=3, P<0.03).

Transfection of IHH with H77 also showed a pattern of decrease from 10^6 to 10^3 IU/ml in supernatant of culture. However this decrease was at a slower rate in compare to JFH1. Quantification of intracellular HCV RNA by real time PCR showed a decrease from 10^7 to 10^4IU/ml. In IHH transfected with JFH1, intracellular RNA decreases more. Real-time PCR suggested that maximal HCV RNA accumulation from transfection of H77 occurred at the intracellular level on Day 3 and declined by Day 7 (Fig21). Similarly, JFH1 RNA-transfected IHH supernatant displayed a peak genome copy number per ml of 10^6 on Day 3. Supernatant from Day 7 of transfected cell cultures from both JFH1 and H77c transfection (100µL of 10^4 IU/ml) was added to 3mL culture media and used to

inoculate naive cell cultures which themselves became infected as demonstrated by RT-PCR analysis of intracellular HCV RNA (Fig25A).

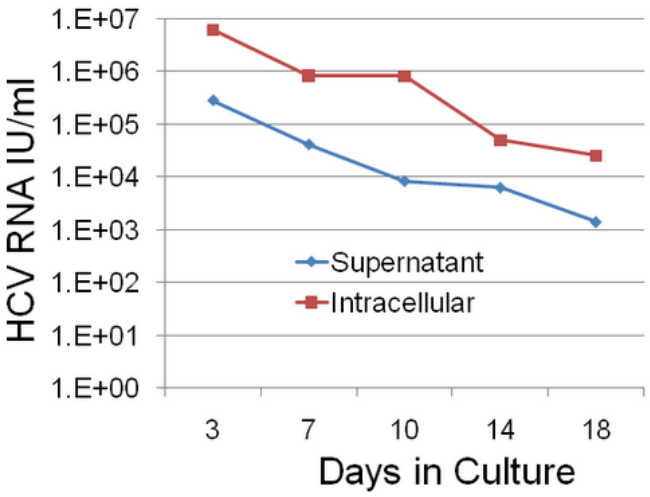


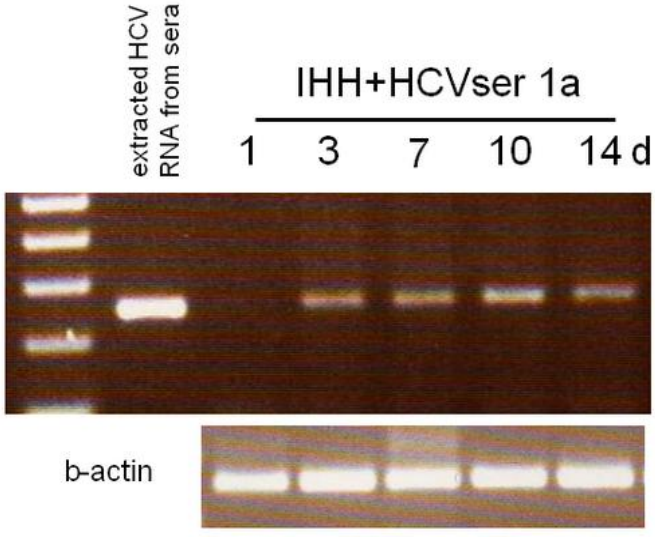
Fig 21. Time course of HCV RNA secretion by H77 (1a) transfected IHH. Levels of HCV RNA in transfected cells and corresponding culture supernatants are depicted in this Fig. Cells were transfected with given HCV RNAs and passaged up to 18days. Viral RNA was determined 3,7,10,14 and 18days after transfection. Transfected IHH maintains HCV RNA levels between 10^3 to 10^4 in supernatant and between 10^4 to 10^5 IU/ml intracellularly. Numbers present mean±SD (n=3, $P<0.05$).

6.4.2. Infection of IHH with different HCV genotypes:

We further assessed the HCV infectivity of IHH by infection with serum derived HCV. IHH and Huh7.5 cells were separately infected with serums derived from 2 different HCV patients (genotypes 1a and 3a) or by JFH-1 concentrated medium (HCV-2a). Inoculated virus titer was adjusted to be the same in all cases. IHH cells showed reproducible infectability for the different HCVser genotypes

type 1 and 3. RT-PCR analysis of intracellular HCV RNA in IHH showed positive at all time-points up to 18 days in culture.

After inoculation with wild type RNA, IHH shed HCV into the culture medium for 18 days in a cyclical pattern, with peaks at 3 and 14 days after inoculation. HCV levels reached a peak ranging from 10^3 to 10^5copies/ml during the peaks at 3 and 14 days culture period (Fig22). The HCV titers on days 7,10, and 18 were less than 100 IU/ml. Fluctuation of HCV levels has been observed both in infected chimpanzees and in Huh-7.5 lines infected with a chimeric JFH1 genome (11, 36) and may reflect the effect of host responses to the virus, as discussed below. In marked contrast, in IHH cultures transfected with HCV RNAs (both JFH1, H77c) HCV RNA levels progressively declined. The progressive decline of virus levels after transfection of HCV RNA viruses reported here is almost identical to the pattern described by Wakita et al for Huh-7 cells transfected with JFH1 mutants. Although the rate of decline in Huh7.5/JFH1 is slower than IHH/JFH1 or IHH/H77, which could be due to lack of internal viral response mediators which is described in details below. Further measurements of viral levels in IHH cells and the culture medium revealed that HCV viruses are slowly released from the cells into the medium for up to 30 days.



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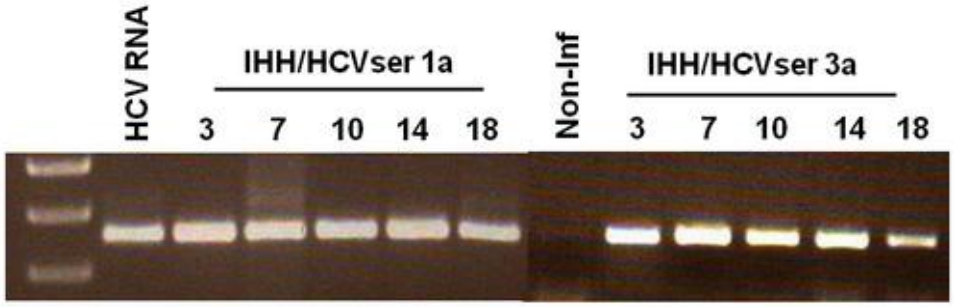

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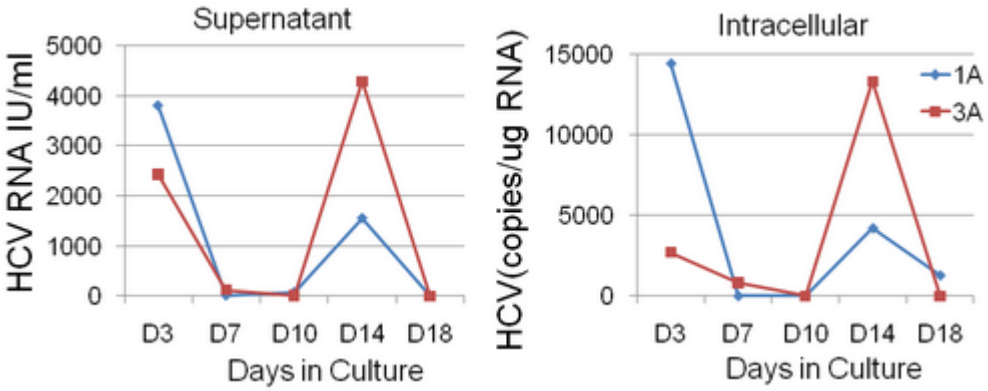

Fig 22. HCV RNA replication and titer in culture of inoculated IHH with HCVser. RT-PCR analysis of IHH inoculated with serum derived HCV (HCVser) from positive patient with 1a HCV showed presence of HCV RNA intracellular after Day 3 up to 14 days in culture (A). IHH inoculated with HCVser from different patients with genotype 1a and 3a were followed up to 18 days in culture by RT-PCR analysis for intracellular HCV RNA (B).Time course of HCV RNA replication in IHH inoculated with HCVser genotype 1a and 3a (C). Levels of HCV RNA in transfected cells (right) and corresponding culture supernatants (left) have been depicted. Cells were infected with given inoculuum from HCV positive patient's sera containing HCV viral particle and passaged up to 18 days. Viral RNA was determined 3, 7, 10, 14 and 18 days after infection. Infected IHH maintains HCV RNA levels between 10^3 to 10^4 in supernatant and between 10^4 to 10^5 intracellular. The primer was 5'UTR from untranscribed region of HCV RNA detected at 244bp. The control negative values of non-infected (NI) IHH cells were negative. The lower limit of HCV titer in real-time-RT-PCR is 296 IU since dilution factor of serum is 296 during the experiment procedure. Numbers present mean±SD (n=3, $P<0.05$).

6.4.3. Detection of HCV Proteins in IHH:

Cytoplasmic NS3 was detectable by immunofluorescence in clusters of cells infected with HCVser and in cells tranfected with HCV RNA (JFH1) (Fig23). We observed cytoplasmic expression of NS3a in about 20% IHH after 5 days of transfection counted subjectively. HCV genotype 2a (clone JFH1) has been shown to grow in Huh7 cells or its derivatives (13, 51, 68, 206). We have also used Huh7.5 cells transfected with JFH1 RNA as positive controls and observed NS3 expression by indirect immunofluorescence. Core staining in cells was not detectable after transfection with HCV RNA or after infection with HCVser in both immunocytochemistry staining and westernblot techniques.

Western blot analysis was used for NS3a protein of HCV inside the IHH cells infected with HCVser or transfected with JFH1 or H77 (Fig24). We used mouse anti-NS3 Antibody (Chemicon, MAB8691) and 8% acrylamide gel. The NS3 was detected in infected IHH with HCVser from genotype 1a, and transfected with JFH1 and H77c. Besides, we were not able to detect any core protein by western blot. This could be due to variation in idiotype of antibody that we used in this study which is regularly used to detect core protein in Huh7/JFH1 in our lab. It could also be result of mutations in amino acid tandem of core protein after transcription in IHH. Another reason could be difference in intracellular site of expression of core protein in IHH and Huh.

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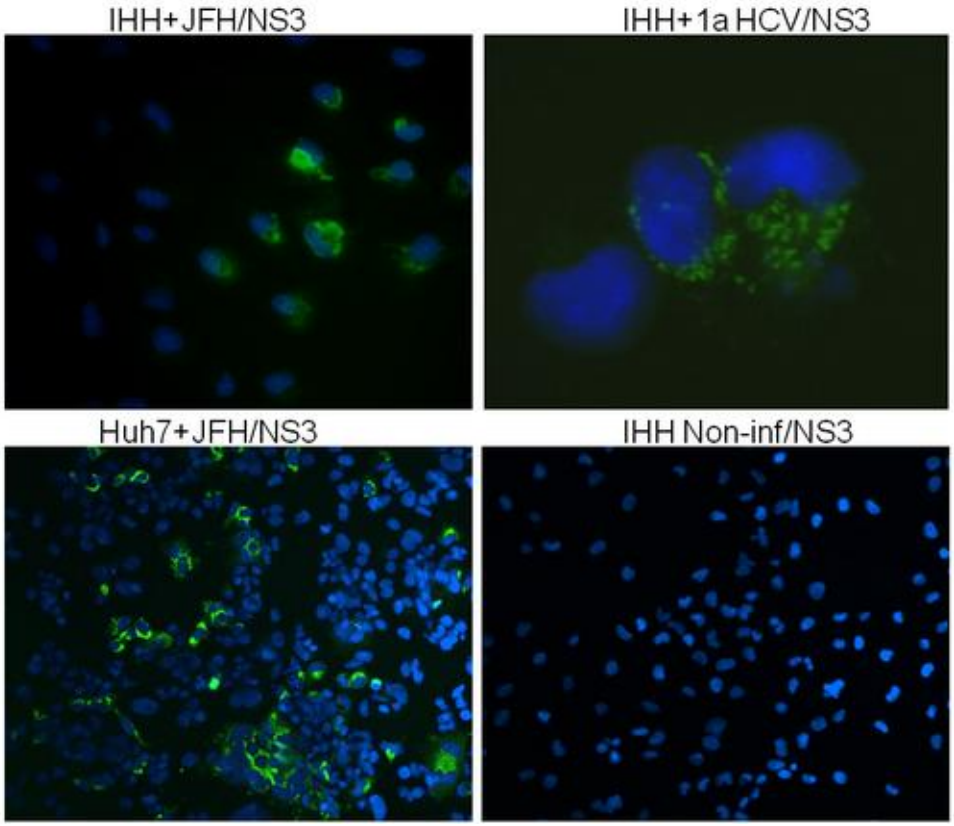


Fig 23. HCV protein expression in infected or transfected IHH. Immunofluorescence microscopy of HCV NS3 proteins in IHH cells transfected with JFH-1 RNA or infected with wild type HCV genotype 1a detected by anti-NS3 antibody. Transfected cells were seeded on coverslips 5 days after transfection or infection and HCV proteins were detected with antibody to NS3a protein.Huh7/JFH1 infected were used as positive control. Original magnification, x200 for left upper Fig, X1000 for right upper Fig, X100 for lower figures. The non-infected cells did not show any green fluorescent.

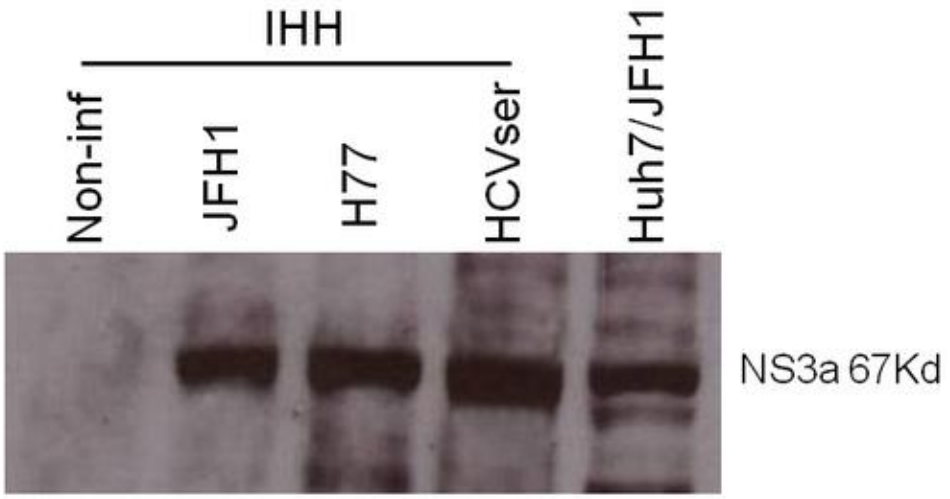


Fig 24. Detection of HCV non-structural protein (NS3a) in IHH transfected with HCV cloned RNA (JFH1, H77) and HCVser (genotype 1a) by western blot analysis detected by anti-NS3 antibody. Huh7 transfected with JFH1 were used as control positive and non-infected IHH were used as negative control.

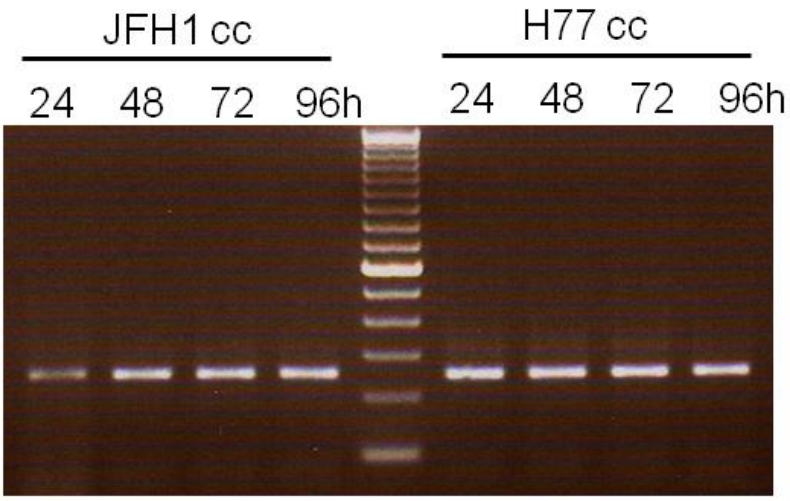
6.4.4. Infectivity of HCV Recovered from Cell Culture Supernatants

To determine whether transfected or infected IHH cells produce infectious HCV virions that can propagate HCV infection into non-infected cells, we cultured naive IHH in 6 well plates and incubated for 1 day. We inoculated naive IHH cells with supernatant harvested from JFH1 RNA of transfected cells, and 48 to 96 hour later we did RT-PCR analysis for the presence of intracellular HCV RNA (Fig25a). Only cells inoculated with JFH1 medium were positive. To exclude the possibility that residual *in vitro* transcripts were captured by inoculated cells, we prepared supernatant from cells treated with the same amount of JFH1 RNA but without the electroporation. Upon inoculation of naïve IHH cells, we observed no Intracellular RNA by RT-PCR, as was the case with supernatant from non-infected cells. Before inoculation supernatant containing HCVcc titer was determined and set at 10^4 IU/ml HCV RNA. Cells were incubated for 4 h, infected media was removed and fresh medium was added to the wells, and the newly infected cells were cultured independently. Medium was collected from

culture. In both cultures HCV was detected with cyclic fluctuations, reaching from 10^3 on day 3 to 0 on day 7-10 and then start rising to a concentration of 10^3 to 10^4 copies/ml at peak expression on day 14. Thus, virus released by IHH cultures transfected with JFH1 RNA infected naı¨ve IHH cultures. In similar experiments using IHH cultures transfected with the H77, we had the same pattern.

More interestingly, our supernatant from infected IHH was infectious in vivo. IHH transfected with JFH1 and infected with HCVser 1a supernatant was collected 7-14 days after infection. The supernatant were concentrated as described in methods and materials section 6.3.7. The concentrated virus was inoculated by intra-peritoneal injection to Alb-uPA SCID mice. The Alb-uPA SCID mice had low hAAT levels as shown in Table 2 below. Unfortunately these were not optimal mice for experimentation due to low engraftment of PHH. Ideal high hAAT mice were not available for inoculation at the time the tissue culture supernatant viral samples were ready for evaluation. After injection, we followed the mice for HCV infection with collection of serum samples 1 and 2 weeks after inoculation. Real-time-RT-PCR for HCV RNA quantification in mice sera showed presence of low-level HCV titer in the mice. This result supports production of infectious HCVcc in IHH after transfection with JFH1 and infection with HCVser genotype 1a. Although, all the HCV titers in this experiment are below the cutoff for the quantitative RT-PCR. These results could be the effect of low hAAT mice or the low infectivity rate of expelled virus from IHH.

A.



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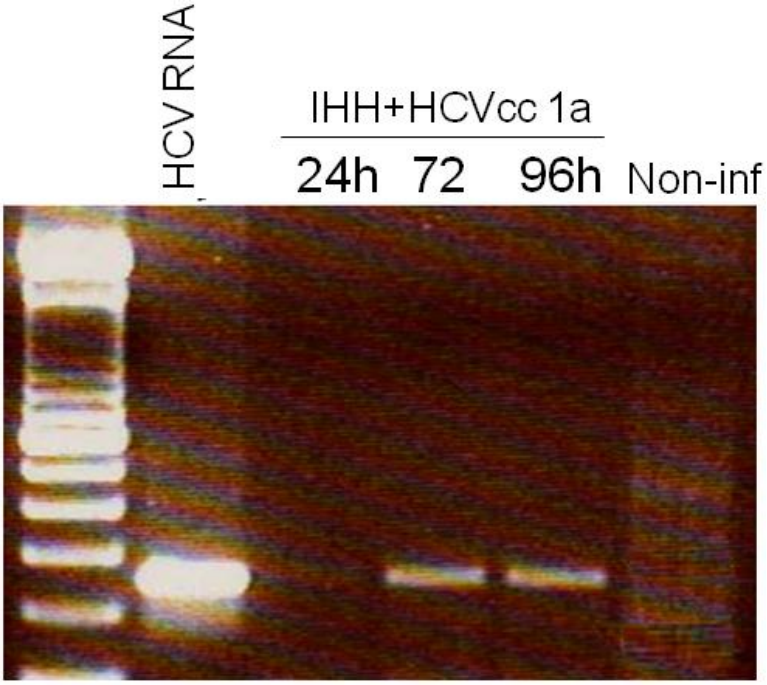
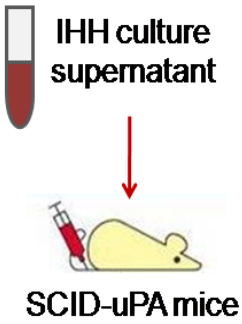


Fig 25. Infectivity of supernatant of HCV infected or transfected IHH. Naïve IHH/mCm cells were inoculated with cell culture derived HCV (HCVcc) from IHH transfected with JFH1 or H77 and cultured up to 4 days (A). Panel B shows intracellular HCV RNA was positive after we inoculated IHH with HCVcc retained from genotype 1a infected cells culture supernatant (B).

Table 2. HCVcc (cell culture derived HCV) from IHH transfected with JFH1 RNA or infected with serum derived HCV (HCVser) from positive patient were collected from supernatant, and then inoculated into Alb-uPA SCID mouse. HCV titers were positive in all mice indicating production of infectious HCVcc in IHH. This showed that IHH cells secrete viral particles that are infectious in animal model. The hAAT values of the mice were below optimal for human hepatocyte engraftment. The supernatant of non-infected mice was not infectious and HCV titers were 0 in mice 1 or 2 weeks after inoculation.



Source of HCVcc	Mice hAAT	1w HCV titer	2w HCV titer
HCVser 1a	48	0	1.91E+02
HCVser 1a	91	2.55E+02	5.39E+02
JFH RNA (2A)	45	2.11E+02	1.09E+02
JFH RNA (2A)	79	1.75E+02	2.91E+02

6.5. DISCUSSION

PHHs are the natural targets for HCV infection, yet it has been difficult to establish culture systems of PHH that sustain HCV replication. HCV is a heterogeneous virus composed of different genotype and subtypes. Each genotype has different epidemiologic distribution and may require different therapies and management strategies (207). Scientists are not able to investigate different genotypes of HCV due to lack of an established in vitro model for full natural virus. Together, this is an important obstacle to further invention of new treatments for various genotypes.

Rapid and impressive progress has been obtained in the recent studies of the replication of the JFH-1 virus in Huh-7 cells; however this established model is a very rare type of virus isolated from a Japanese patient with fulminant hepatitis (JFH1) that is genotype 2a. Moreover this rare virus only replicates in a hepatoma cell line (Huh7/Huh 7.5) which has a phenotype quite distinct from PHH. The course of infection of the JFH1 strain differs from most infectious clones, usually causing higher viremia and occasionally HCV-specific sero-conversion or hepatocellular injury. Alternatively, the JFH1 strain, although replicating efficiently in vitro, may be less infectious in vivo.

In this study we embarked on developing a novel cell culture system for HCV by using IHH that can sustain the replication of HCV of various genotypes by either transfection with in vitro transcribed HCV RNA or infection by HCVser. We demonstrated that IHH culture could support the infection and replication of natural HCV derived from patient sera (HCVser). Unlike recombinant HCV, which have been required to adapt to sub lines of Huh7 cells, the population of the HCVser is fairly polymorphic, demonstrating different responses to a variety of anti-viral agents (77). Thus IHH offers an important advantage in the study of natural HCV infection and replication, and the response of natural HCV to anti-HCV drugs.

In this study we tried to transfect IHH with two HCV RNA clones 1a (H77), 2a (JFH1). The route of delivery of HCV genome into cells (proper infection versus RNA transfection) might have a strong influence on the replication capacity of a given strain. However, HCV RNA clones are not able to replicate in any other systems than JFH1/Huh7. In fact, even the H77 clone is not able to replicate to the same titer as JFH1 in Huh7 cells. In this study we demonstrated that an H77 clone could replicate in IHH unlike Huh7 cells.

In the second part of this study we tried to infect IHH with different genotypes of HCVser. Earlier attempts to infect Huh7 cells with sera from infected individuals were not successful. Recombinant viral particles have a homogeneous density, whereas HCV in human sera shows much lower and heterogeneous densities, suggesting an association with cellular components, especially lipoproteins, which may interfere with or facilitate infection in varying conditions – eg in vitro vs. in vivio (208). Another major problem in study of serum-derived HCV infection is the low level of replication of HCVser in current in vitro models. The efficiency of replication of a virus in cell culture depends on both viral and host factors. In this study many HCV strains have been used to either infect or transfect (i.e. after in vitro transcription of cloned genomes) cells. One possibility of low infectious rate of HCVser is that HCV infection and viral spread occurred only in a small fraction of cells.

We also did not observe any increase in the number of infected cells over time, arguing for limited spread of HCV in the cultures. Several factors could contribute to this phenomenon, including limited numbers of infectious particles, heterogeneous polarity (spatial hydrophobic/hydrophilic properties of the bilayer surface), or an inherent or acquired refractory nature of a proportion of cells. Furthermore, certain critical host factors may be heterogeneously expressed and therefore limiting in some cells, rendering them resistant to infection or unable to sustain HCV RNA replication. Although our data demonstrate that IHH can produce infectious virus, the titers are low and few infectious virions are available

for spread. Besides, the genetic and viral structure variability observed in the serum of HCV infected patients is important parameters to define the infectivity potential of serum-derived HCV. It is important to show that the IHH cell culture system could allow the replication of all HCV strains, irrespective of these parameters, as no changes can be made on serum-derived viral innocula.

In this study, experiments using two different genotypes from HCV positive sera coming from different patients have been performed to generalize the results and further extend our cell culture model to various HCV genotypes. Our quantitative RT-PCR results from intracellular and supernatant HCV RNA determined whether persistent HCV infection can be obtained in IHH cells. In spite of the latter point, our work opens some interesting ways to carry on the improvement of cell culture models to study serum-derived HCV.

At present, we cannot clarify the discrepancy between the drops in extracellular RNA at passage 3 at day 7 when the IHH cells are infected with HCVser. However, the extracellular RNA level was recovered in the subsequent passage. We cannot rule out several possibilities, including the restriction of virus growth from autophagy (Autophagy is a homeostatic mechanism of lysosomal degradation. Defective autophagy has been linked to various disorders such as impaired control of pathogens) or the generation of defective interfering particles or antiviral cytokines. Further work is needed to determine if additional passages of the virus increase the infectious HCV titer. Nevertheless, our study showed the production of HCVcc from serum derived HCV (HCVser) in cell culture, which can infect both cells and Alb-uPA SCID mouse.

It is unclear why the spread of infection in IHH cells is limited. Activation of innate immunity in transfected or infected cells is one possibility. Alternatively, JFH1 RNA containing cells may have a growth disadvantage and therefore be displaced during passage. Moreover, formation of assembly-competent envelope protein complexes and virus release may be a rate-limiting

step, reducing virus production to a level lower that replication of HCV observed in Huh7 cells.

One important component defining host permissiveness is the cellular innate immune response that leads to an antiviral status in infected cells as well as in the surrounding uninfected cells. For HCV, it was recently shown that intracellular double stranded RNA (dsRNA), that are produced upon genome replication, can induce a type I interferon response and therefore restrict HCV replication (209, 210). This interferon response is established after cascade events including the (i) detection of viral replication (i.e. dsRNA) by ‘‘sensor molecules’’, i.e. the retinoic acid inducible gene I (RIG-I), toll-like receptor 3. Despite the ability of the virus to counteract cellular responses, the development of models enabling the replication of HCV in vitro is challenging (58, 59). After years of research, it was shown that HCV replication is stronger in cells deficient for innate antiviral responses. Thus, Huh7.5 cells that are derived from cured-Huh7 cell lines that harbored subgenomic HCV replicons and are mutated in RIG protein are more prone to HCV transfection and infection (49). In vivo, a productive HCV infection at the cellular level is likely to occur after a ‘‘battle’’ between host responses and virus-encoded resistance. But, unlike in vitro, infection in vivo is persistent, as contamination with HCV leads to chronic infection in about 70% to 80% of clinical cases (211). It seems that replication of HCV in hepatocytes, or hepatoma cells, in vitro is more difficult to achieve for unclear reasons. It might be that cells grown outside of their natural context may produce and diffuse more antiviral cytokines or chemokines, thus inhibiting replication and spreading of infection. It is conceivable that a better inactivation of the IFN pathway may lead to an even better permissiveness of IHH.

We showed that HCV replication is sustained for week’s cultures transfected with HCV genotype 1a, the most common genotype in United States, or infected with patient sera containing genotype 1a, and 3. Virus produced by transfected cells was also capable of infecting naive cultures, and chimeric Alb-

uPA SCID mice. In an addition to check that IHH are able to produce infectious particles, we injected Alb-uPA SCID with supernatant from infected IHH. The supernatant was infectious to the mice.

After transfection or serum infection, HCV levels in the medium fluctuated with a cyclical pattern that persisted through culture periods of 18 days. Clusters of cells expressed HCV NS3 protein. The overall proportion of cells expressing viral proteins in the cultures was approximately 10 to 20%. Unfortunately, we were not able to detect HCV core protein in IHH by current antibody used in detecting JFH1 core in Huh7 cells. This might be due to restriction of core protein in IHH to other part of cell or with different motifs not detectable for that specific antibody.

We showed that IHH cells maintain their hepatocyte phenotype for several months or even years. However, we have not examined which HCV receptors are required for HCV infection in these cells. Besides in contrast to Huh-7 cells, IHHs can be maintained in primary culture without passaging for more than 2 weeks, with complete replacement of culture medium at each medium change. This has the advantage of observing replication of virus only, not virus using of replicative machinery of replicating cells such as Huh7 cells.

One other advantage of our IHH is that we could study HCV interactions and effects on cell cycle mediators after de-immortalization. Long lasting chronic HCV infection predisposes patients to hepatocellular carcinoma (212). To date, the underlying molecular mechanisms linking HCV infection to HCC are not yet fully understood. The ability to infect non-transformed hepatocytes with HCV in vitro and maintain a persistent infection for a long period of time could be an interesting but difficult approach to further understand viral induced carcinogenesis. The tremendous progresses obtained in this study in cultivating recombinant or serum-derived HCV open realistic perspectives of reaching such a goal as our IHH are able to de-immortalize and be studied for any HCV induced HCC tumor markers and proto-oncogens.

In summary, Immortalization of PHH is an interesting approach, as it allowed replication with serum-derived HCV. Improvements are yet possible to further widen the utilization of such a system to study patient derived HCV strains. It would be of interest to grow IHH in a context closer to physiological condition, for instance in the presence of other cell types, that are naturally present in the liver (e.g. cholangiocytes or endothelial cells), and/or in the presence of proteins of the matrix, to determine whether infection can be enhanced (213). Indeed it was shown, for instance, that liver sinusoidal endothelial cells (LSEC) in co-culture system induce an increase of the level of expression on hepatocytes of the human low density lipoprotein receptor (LDLr) (214, 215), which is a potential receptor for HCV entry (216, 217). Alternatively, it would be interesting to determine whether other cells than hepatocytes, including adult liver stem cells (218) or hepatic oval progenitor cells (30, 219) could support HCV infection and what could be the physiological role of such infection.

7. Future Direction

The IHH cell culture system should be validated with current HCV antiviral therapies based on HCV genotype. Our cell culture system is designed to be de-immortalized by a Cre/loxp mechanisem, which will enable us to study de-immortalized IHH like PHH without having difficulty of culturing PHHs. The future direction in this sense could be to determine if growth arrested cells maintain HCV infection; and how HCV infection has an impact on the regulation of cell cycle proto-oncogenes and oncogens and tumour suppressors like Rb or P53 in growth arrested cells (virus-induced cell-cycle regulation) and how this relates to viral-induced carcinogenesis

The identity of the infectious form of HCV in “real life” remains open to debate because HCV in sera is strongly associated with lipoproteins; certain features (such as the infectious form's density) appear to differ from those seen in

the HCV particles produced in cell culture systems (HCVcc). New information concerning the receptors involved and other important determinants of permissiveness will be valuable for improving the culture system. These new insights could enable researchers to grow clinical HCV isolates and study their particular features, and evaluate novel antiviral therapies.

For future research IHH enable usage of them as an appropriate model for permissiveness of HCVser by new drugs or blocking antibodies.

8. Summary and Concluding Remarks

In summary our IHH model has the potential to eliminate the need for repeated human donation, and to avoid the variability associated with different donors and the technical hurdles of hepatocyte isolation. It allows for the production of large quantities of a standardized group of cellular clones for experimental and therapeutic purposes that can be infected with HCV in vitro to determine the role of HCV infection in cell cycle regulation. It also could support the use of IHH for other areas of study, including human malaria and hepatocyte transplantation for acute liver failure and metabolic liver disease.

We also showed that IHH are permissive for HCV viral entry from a natural HCV derived from patient sera and IHH are permissive for HCV viral replication and are able to secret infectious HCV viral particles. Our study showed that IHH could support HCV entry, replication, assembly and release. This system could provide a powerful ground breaking tool for various aspects of HCV biology.

In conclusion, the novel in vitro culture system of immortalized hepatocytes described in this study demonstrated support of natural HCV infection and replication. This system may be used in future virological studies to define new anti-HCV strategies. It may also prove useful for the specific design of effective individual therapy according to patient-specific strains.

In vitro and vivo outcomes summary:

1. IHH Showed qualitative evidence for HCV RNA REPLICATION after transfection or infection
2. Transfection or infection with HCV leads to Expression of HCV NS3 protein, Intracellular HCV RNA are TRANSLATION COMPETENT in IHH
3. Quantification of supernatant of IHH cells Transfection/infection showed detectable titre of HCV RNA
4. JFH1 or H77 transfected IHH secrete infectious HCV viral particles (*in vivo & vitro*)
5. HCVser infected IHH secrete infectious HCVcc (*in vivo & vitro*)
6. Higher titre of HCV in Huh/JFH but limited to only JFH1
7. merCremer localized to nucleus by Tamoxifen in IHH-mCm
8. Cre could decrease expression of tumorogene transgenes and Loss of SV40 expression *in vitro*
9. De-immortalized IHH are not tumorogenic in SCID/uPA mice
10. The de-immortalized IHH could show some aspects of PHH phenotype

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