

**Approaches to the development of *in vivo* propagation systems for  
Hepatitis C Virus (HCV)**

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**In  
The Faculty of Science  
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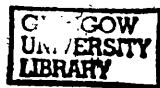
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## Abbreviations

ABC	avidin biotin complex
Alb-Upa	albumin-urokinase
ALT	amino liver transferases
BCIP	bromo-Chloro-Indoly-phosphate
BNX	beige/nude /X-linked immunodeficient
CMV	cytomegalovirus
DAB	3,3 diaminobenzidine tetrahydrochloride
DEPC	diethylpyrocarbonate
DMEM	dulbeco's modified essential medium
DMDP	dichloromethylene diphosphate
DMSO	dimethyl sulfoxide
EPG	epidermal growth factor
FCS	foetal calf serum
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis delta virus
H&E	Harris's Haematoxylin and Putt's Eosin
HGF	hepatocyte growth factor
HIV	immunodeficient virus
HPV	human papilloma virus
HVR1	hypervariable region 1
ICC	immunocytochemical staining
ISH	<i>In situ</i> hybridisation
IL6	interleukin-6
IP	intraperitoneally
IV	intravenously
NBT	nitro blue tetrazolium

NK	natural killer
PAP	peroxidase anti peroxidase
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PEG	polyethelene glycol
PHHC	primary human hepatocyte
PH <sub>x</sub>	partial hepatectomy
PTFE	polytetrafluoroethylene
PVP	polyvinylpyrrolidone
RT PCR	reverse transcription polymerase chain reaction
SCID	severely combined immunodeficient
STW	scotts tap water
SV40	simian virus 40
T <sub>3</sub>	thyroxin
TBS	tris buffered saline
TGF- $\alpha$	tumour growth factor- $\alpha$
TNF- $\alpha$	tumour necrosis factor- $\alpha$
UTR	untranslated region
UW	University of Wisconsin
VLDL or LDL	very low density or low density lipoproteins

I dedicate this thesis to my father and my dear friend Patrick  
Conway

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## **Declaration**

The studies described in this thesis were carried out in the Institute of Virology at the University of Glasgow between March 1997 and May 1999 and were part of two concomitant projects. These were funded by Roche Discovery, Welwyn Garden City, UK and the Wellcome Trust. All the surgical and liver perfusion and RT PCR procedures were performed by a team including the author. The author was responsible for writing this manuscript and all the histopathology results except where it is stated otherwise.

No part of this thesis has been presented to any other university.

Selma Rebus, May 2000



## Summary

Hepatitis C virus (HCV) is a recently discovered major human pathogen infecting 170 million individuals worldwide. It was the first hepatotropic virus to be isolated by molecular biology techniques. Since its discovery in 1989 considerable progress has been made in the identification of the viral functional regions, the interactions between viral products, and viral pathogenesis. However, little is known about its replication cycle and the only experimental *in vivo* HCV infection model is limited to the chimpanzee. Thus, the development of a cheap and accessible *in vitro* or *in vivo* culture system has become a priority.

The work in this thesis explores a variety of different approaches to an *in vivo* culture system using the severely combined immunodeficient (SCID) mouse. These animals lack functional T and B cells and will accept xenografts from a range of species including humans.

Hepatocyte derived cell lines and primary human hepatocytes (PHHC) were transplanted intrasplenically and under the renal capsules after performing a partial hepatectomy. The rationale rests on the hypothesis that these cells would attain a biologically functional state of differentiation *in vivo*, increasing their sensitivity to HCV infection and acting as targets for HCV replication in an *in vivo* culture system. HCV positive serum was used to infect the cells prior to transplantation or 3 days post transplantation. PHHCs were also mixed with anti-*met* antibody and interleukin 6 (IL6) prior to transplantation to increase their survivability and proliferation *in vivo*; silica was administered intraperitoneally to deplete host macrophage cells.

All the animals transplanted with hepatocyte derived cell lines developed extremely large metastatic tumours which were hemorrhagic, additionally these cells were not susceptible to HCV infection, irrespective of the route of infection and source of the HCV positive serum. PHHCs failed to engraft irrespective of

treatment and were HCV reverse transcription polymerase chain reaction (RT-PCR) negative.

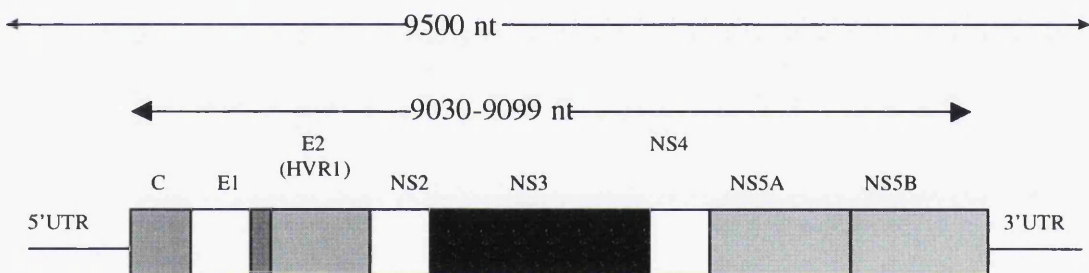
## CHAPTER 1

### Introduction

Hepatitis C virus (HCV) has been identified as the main cause of more than 90% of posttransfusion and sporadic non-A, non-B hepatitis (Alter *et al* 1992). It chronically infects about 170 million individuals worldwide (World Health Organization 1997), the global prevalence is estimated to average 3%. In industrialized countries, HCV accounts for 20% of acute hepatitis cases, 70% of chronic hepatitis cases, 40% of end-stage cirrhosis, 60% of hepatocellular carcinomas and 30% of liver transplants (EASL International consensus conference on hepatitis C, 1999). Disease development is associated with low grade, persistent viraemia, with various rates of progression. About 15% of HCV infected individuals recover spontaneously and 25% have an asymptomatic illness with normal aminotransferases (ALT) and benign histological lesions (EASL International Consensus conference on hepatitis C, 1999). But, chronicity is the most usual outcome of infection, where patients present with a relapsing, remitting disease with recurrent bouts of hepatitis marked by fluctuating ALT levels, but normal levels have been observed in one third of cases that are HCV RNA positive. 20% of patients will develop liver cirrhosis after 20 years of infection (Kiyosawa, 1994). Infection is also linked with most cases of type II and type III cryoglobulinemia, which are B lymphocyte proliferative disorders characterized by polyclonal B cell activation and autoantibody production (Silvesteri *et al*, 1996).

## 1.1 Genetic organization of Hepatitis C Virus (HCV)

The complete sequence of HCV was published in 1989 (Choo *et al* 1989). HCV was identified as a positive strand enveloped RNA virus with a length averaging 9.5 kb. Its genome, summarised in figure (1.1), includes two untranslated regions at the 3' and 5' ends, and a large open reading frame that encodes for a 3010-3030 amino acid polyprotein. This polyprotein is translated and cleaved into structural and non-structural proteins. The structural proteins, at the N-terminal region, are the core protein followed by two glycosylated putative envelope proteins: E1 and E2 (Brechot 1996). The hypervariable region 1 (HVR1) is located on the 5' end of the second envelope gene E2 and appears to be the one of the main targets of anti-HCV neutralising responses (Farci *et al* 1994). The non-structural domain encodes six proteins: NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Brechot 1996). The 5' untranslated region (UTR) is highly conserved among HCV isolates and shows secondary structures with several stems and loops (Smith *et al* 1995).



**Figure (1.1) Genetic organisation of HCV (Brechot, 1996 and Pawlotsky, 1998)**

The precise structure and function of the 3' UTR region is unclear, though it consists of a short stretch of poly (U) or poly (A) nucleotides and is heterogeneous among different isolates (Brechot 1996). Because HCV shares structural and functional homologies with flaviviruses and pestiviruses, by

analogy with the replicative strategies of these viruses (Cleaves *et al* 1981, Chambers *et al* 1990), it is possible that this region might influence viral replication and pathogenicity.

The NS3 domain codes for three enzymes: serine proteinase, helicase and ATP dependent nucleotide tri-phosphatase. NS2 and the N-terminal sequence of the NS3 region also code for a second protease; and a heterodimeric complex is formed with NS4A and the NS3, which acts as a cofactor for NS3 proteinase activity (Hirowatari, *et al* 1995, Failla *et al* 1994). A GDD motif conserved among RNA dependent RNA polymerases is contained in the NS5B domain, which probably acts as the RNA polymerase (Brechot, 1996). For HCV replication to occur, the viral polyprotein will probably be translated from the virions's single stranded RNA by the host ribosomes and the protein required for HCV replication cleaved. The resulting RNA dependent RNA polymerase may then recognise the 3' end of the viral genome and allow transcription to proceed from the 3' to the 5' end. Using this negative strand as a template, positive sense genomic RNA can then be transcribed. Negative strand intermediates have been detected in HCV infected patients (Saleh *et al*, 1994, Lohr 1995)

As viral RNA polymerase lacks "proof-reading" activity, replication errors made are not corrected. Consequently accumulation of mutations that might confer different biological properties (Martell *et al*, 1992), for example the emergence of HVR1 variants could partly explain viral persistence (Pawlotsky, 1998).

As there is no reliable and sensitive tissue culture system, progress in understanding the pathogenicity and life cycle of HCV has been limited. There is no consensus on HCV cell tropism; the mechanism of viral entry and the cellular receptors have not been identified. However, Pileri *et al* demonstrated binding of HCV envelope protein E2 to human CD81, a tetraspanin expressed on various cell types including hepatocytes (Pileri *et al*, 1998) suggesting a possible receptor site. Further the close association of HCV with VLDL or LDL (very low density or low density lipoproteins) could explain its hepatotropism, as the liver is the main site of lipoprotein metabolism (Gurr and Harwood, 1991).

The development of an efficient *in vitro* culture system and readily accessible *in vivo* infection model has thus become a priority for the assessment of antiviral strategies, the development of vaccines and analysis of viral replication. Currently, the only successful *in vivo* model is the chimpanzee (Shimizu *et al*, 1990).

## **1.2 *In vitro* culture of HCV**

### **1.2.1 HCV culture in permissive cell-lines**

A number of permissive cell-lines have been used in attempts to produce HCV culture systems. Two approaches have been used to introduce HCV genome to the cells: infection and transfection.

#### **1.2.1 a) Infection of permissive cell-lines**

HCV positive serum or plasma was used to infect either lymphocytic cell-lines: MOLT4 (Shimizu *et al*, 1993), MT2C (Kato *et al*, 1995; Sugiyama *et al* 1997), B cells (Bertolini *et al*, 1993); or liver derived cell-lines: WRL68 and HepG2 (Tagawa *et al*, 1995), HepG2 and Huh7 (Seipp *et al*, 1997).

Viral replication in lymphocytic cell-lines was found to be transient. In the MT2C cell-line, HCV was detected by reverse transcription polymerase chain reaction (RT PCR) up to 30 days post inoculation, which in turn was increased to 80 days after a reduction in the incubation temperature from 37°C to 32°C (Mizutani *et al* 1995). Conversely, Nakajima *et al* demonstrated the production of infectious HCV for more than one year after infecting the T cell line HPBMa 10-2 and the B cell line Daudi. This was achieved using an inoculum with HCV virions that were determined to be free of antibodies by immuno-precipitation with anti-human immunoglobulin (Nakajima *et al* 1996).

Several groups have attempted to infect a number of liver derived cell-lines with limited and variable success rates. Seipp *et al* found that HCV RNA was detected in Huh7 cells for up to 10 days with a low HCV RNA copy number (around 10<sup>4</sup> copies). The efficiency of detection was increased slightly after the

addition of polyethelene glycol (PEG) and/ or dimethyl sulfoxide (DMSO). However, there was no increase in viral titre in long-term cultures, suggesting enhanced HCV attachment to the cells but not infection. Further modification to the culture conditions and stimulation of the low-density lipoprotein receptor with Lovastatin caused persistence of HCV in cells and supernatant up to 130 days, but negative strand viral RNA was absent (Seipp *et al*, 1997). Conversely, negative strand RNA was detected in infected WRL68 cells for more than two months and in the supernatant for up to 62 days (Tagawa *et al*, 1995).

The detection of HCV negative strand RNA is required to prove replication but potential artefacts such as self-priming (Brown *et al* 1992) and mispriming (Willems *et al* 1993) that were not addressed in earlier publications have led to misrepresentation of results. Laskus *et al* recently demonstrated the absence of HCV negative strand RNA in circulating peripheral blood mononuclear cells (PBMC); also the sequences identified in serum were identical to those found in the PBMCs (Laskus *et al* 1997). New procedures developed by Lanford *et al*, which rely on the use of either tagged HCV primers or thermostable reverse transcriptase to optimise negative-strand detection (Lanford *et al* 1995), should clarify the issue of HCV T cell tropism.

### **1.2.1 b) Transfection of permissive cell-lines**

HCV transfection is an alternative approach for the production of a continuously infected cell line. Several investigators have transfected subgenomic HCV cDNA either into HepG2 (Harada *et al* 1995) or HeLa cells (Mizuno *et al* 1995). Structural and non-structural HCV proteins were produced in different expression systems, but the constructs did not permit viral replication.

The transfection of full length HCV RNA has been more rewarding. Yoo *et al* transfected HCV RNA produced from a recombinant cDNA clone into Huh7 cells. HCV negative-strand RNA was detected in both supernatants and cells, and 50% of cells were found to be HCV positive by *in situ* nucleic acid hybridisation. The viral yield was low and intermittent in the supernatants, and appeared to follow cell division (Yoo *et al* 1995). Recently, Dash *et al* developed a more

successful culture system by transfecting HepG2 cells with 6.7, 9.6 or 9.4 kb RNA. The viral yield in culture was found to be high, at  $10^8$ - $10^{10}$  copies, and constant over a period of 60 days. Further, virus-like particles were detected by electron microscopy (Dash *et al* 1997). Considerable scepticism, however, has been expressed about various technical aspects of this work including the infectivity of HCV clones lacking a 3' end, the large amount of RNA needed for RT-PCR, the number of virus-like particles viewed by electron microscopy and the high specificity of their RNA negative strand assay (Fausto, 1997). One of the main disadvantages of using liver derived cell-lines is that some of them originate from hepatocellular tumours, and thus will vary in their states of differentiation (Sigal *et al* 1992). This in turn may lead to the loss of important hepatocyte characteristics that could be crucial for virus attachment, infection and replication. For example, the cell-line Hep G2 does not express of the CD 81 receptor (personal communication from Dr Arvind Patel at the Institute of Virology, MRC Virology Unit, Glasgow 1999), which is known to bind E2. This in turn could explain the varying culture success rates of HCV in this cell line.

### **1.2.2 HCV culture in primary hepatocytes**

The use of primary chimpanzee or human hepatocyte culture systems for the production HCV could be more rewarding. Lanford *et al* infected chimpanzee primary hepatocytes, isolated from liver wedge biopsies, with an inoculum from an immunosuppressed HCV-positive heart transplant recipient. Using either tagged cDNA primers or thermostable reverse transcriptase, they first demonstrated HCV negative-strand RNA 4 days post-infection. It persisted for the duration of the experiment (25 days) but was cell associated with little RNA detected in the medium (Lanford *et al*, 1994). This is not unusual for viruses cultivated *in vitro*, but it is plausible that this culture system might not support a complete replication cycle. Moreover, the authors reported significant batch variation between hepatocytes taken from different animals with regards to their infectability.



Fournier *et al* infected primary normal adult differentiated hepatocytes with 33 different HCV positive sera with various virus loads from three different genotypes. Intracellular negative strand RNA was detected in only 10 of the 33 cultures, usually at day one post infection, rising at day five, then followed by a decrease at two weeks post infection. Conversely, positive strand RNA was detected in all cultures, but levels decreased after a few days, though remained detectable for up to eight days. There was no significant batch variation between hepatocytes taken from different individuals, they maintained their differentiated state and were metabolically active for up to 35 days; (demonstrated by active cytochrome P450). However, this culture system did not support the production of progeny virions in the culture supernatant (Fournier *et al*, 1998).

The use of HCV infected primary hepatocytes to generate a culture system was explored by Ito *et al*. Primary hepatocytes were isolated from liver biopsies taken from five patients with either liver cirrhosis or chronic active hepatitis. Using a competitive RT-PCR and negative-strand assay, HCV RNA was detected and quantified in cells and supernatant through out the culture period (14-56 days). The culture supernatant was then used to infect HCV-negative primary hepatocytes. Double staining experiments for anti-HCV core and anti-human albumin antibodies, suggested that HCV replication was restricted to hepatocytes (Ito *et al* 1996). One of the disadvantages of using cirrhotic livers to generate this culture system is that the cell yield and viability is reduced (Kusano *et al* 1997), thus experiments are difficult to repeat.

Primary adult hepatocytes have a long life span and rarely divide. When kept in serum free medium supplemented with growth factors, they will undergo one or two rounds of replication without losing their differentiated state. They then tend to degenerate and die (Fausto *et al*, 1995). However, after priming with DMSO and epidermal growth factor (EGF) stimulates them to enter DNA synthesis after DMSO is removed. This process induces enhanced hepatocyte proliferation, which lasts for 2 days. Re-exposure to DMSO and EGF will induce the same response (Michalopoulos, 1990). This was exploited to induce infection of hepatitis B virus (HBV) in HepG2 (Gripin *et al* 1988), then applied to HCV

culture in the infection of Huh7 cells (Seipp *et al*, 1997). Its use in promoting HCV infection in primary adult hepatocyte cultures has yet to be investigated.

Maintenance of differentiated primary adult hepatocytes has been explored in different culture systems in addition to growth in serum free chemically defined medium (Isom and Georgoff, 1984). The use of a three-dimensional collagen gel sandwich, for example, will allow the hepatocytes to remain differentiated for up to one year in culture (Blaheta, *et al* 1998), and thus could be used to investigate the life cycle of HCV.

Human foetal hepatocytes can remain differentiated. Iacovacci *et al*. detected HCV RNA negative-strand in cells and culture supernatant with an increase in HCV genomic templates for three months post-infection (Iacovacci *et al*, 1993). Free virus particles in the supernatant were later demonstrated (Iacovacci *et al*, 1998). But access to a reliable source of foetal cells can be difficult for ethical reasons, limiting their potential for generating continuous culture systems.

To overcome the cultivation restraints of primary hepatocytes, the development of conditionally immortalised human hepatocytes using simian virus 40 (SV40) that can sustain HCV replication *in vitro* should be explored. Rodent hepatocytes can be successfully immortalised by SV40 (Nakamura *et al* 1997). But difficulties associated with special growth requirements of primary hepatocyte *in vitro* cultures can be eliminated in *in vivo* systems by the initiation of liver regeneration (Michalopoulos, 1997).

One of the main draw-backs of *in vitro* culture systems is that they are not useful for studying immuno-pathogenicity of disease.

### **1.3 Hepatocyte transplantation**

#### **1.3.1 Sugical transplantation**

Surgical transplantation into a permissive site of an animal could be a more reliable method for cultivating primary hepatocytes. The intact liver can provide important physiological conditions, as its extracellular matrix will allow

polarity to develop in the hepatocytes thus retaining their differentiated state (Sawa *et al* 1997).

Allogeneic and syngeneic ectopic hepatocyte transplantation (in to the portal vascular bed, through the spleen) has been successfully demonstrated in rodents (Sawa *et al* 1997). Recently, allogeneic human hepatocyte transplantation was also demonstrated by Fox and colleagues in a patient with Crigler-Najjar syndrome type I. The patient was treated with an infusion of primary hepatocytes (5% of the normal hepatocyte mass) through the portal vein (Fox *et al* 1998). The number of hepatocytes that can be transplanted safely is limited due to the high risk of a portal embolism and subsequent hepatic failure (Sawa *et al* 1997). Therefore, a suitable stimulus to elicit hepatocyte proliferation is a prerequisite for successful engraftment.

### **1.3.2 Liver regeneration**

Hepatocyte proliferation can be attained by a partial surgical hepatectomy (Michalopoulos, 1997) or a chemical hepatectomy with either CCl<sub>4</sub> (Sawa *et al* 1997) or D-galactosamine (Dabeva *et al* 1993). These compounds will cause gradual liver injury and necrosis, which, in turn, will initiate liver regeneration. Further, DNA synthesis following the use of these compounds occurs 1-3 days after partial hepatectomy (Michalopoulos, 1997).

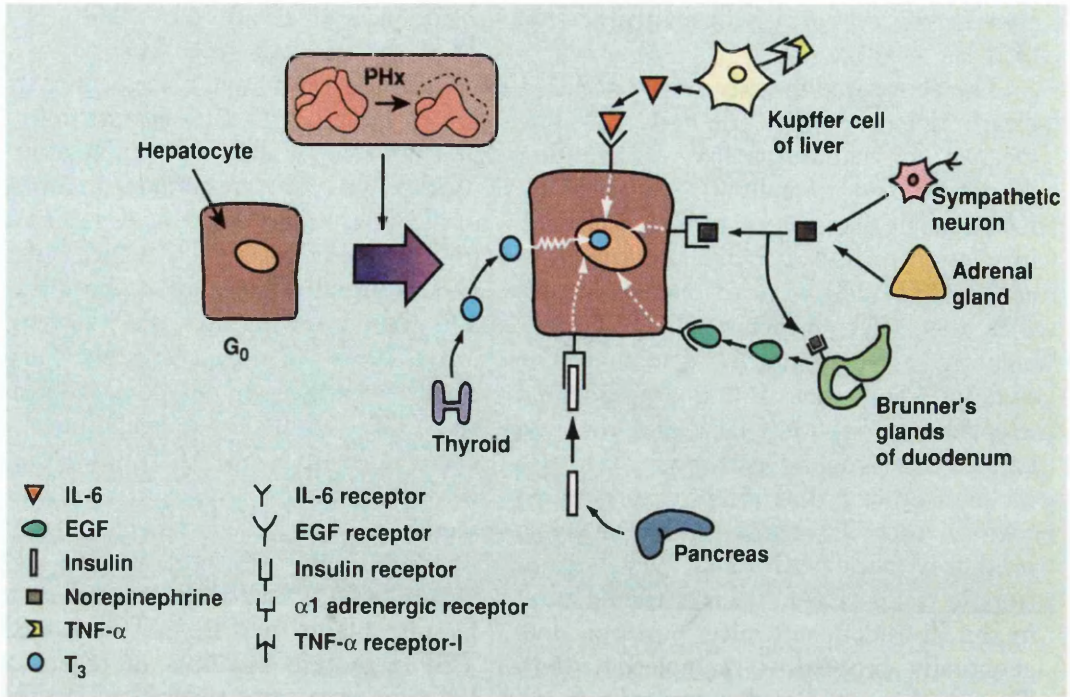
As a result of liver injury, there is increased secretion of mitogens and growth stimulants. The initial mitogenic stimulant after a partial hepatectomy is hepatocyte growth factor (HGF), which with its receptor *c-met* is a key factor in liver regeneration. HGF is detected in the plasma within one hour after a partial hepatectomy and in a rat its levels rise 20 fold then return to normal levels after 72 hours (Michalopoulos, 1997). It is a potent mitogen for primary hepatocytes in culture (Fausto *et al* 1995). Other mitogens include EGF, tumour growth factor- $\alpha$  (TGF- $\alpha$ ) and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), the latter stimulating the secretion of interleukin-6 (IL6) by Kupffer cells, that are also involved in liver regeneration. Insulin and noradrenaline are also important as they amplify the mitogenic responses to EGF and HGF (Michalopoulos, 1997).

Generation of the mitogenic stimuli for hepatocytes depends on the integrity of many signalling pathways. These pathways and the tissue sources of origin are presented in Figure (2).

A proliferative stimulus can also be provided by means of gene transfer using a recombinant adenovirus vector that expresses a modified urokinase gene into hepatocytes of the recipient animal. This results in asynchronous hepatocyte degeneration, which is likely to be caused by intracellular conversion of hepatocyte derived plasminogen to plasmin. Plasmin is a protease that results in the activation of a number of proteins and is thought to be toxic within the cells. This will cause the proliferation of the donor cells, allowing the reconstitution of 8.6% of the recipient's liver (Peeters *et al* 1997). Alternatively, complete replacement of the recipient's liver with the donor's hepatocytes was demonstrated in the albumin-urokinase (Alb-Upa) transgenic mouse model, in which hepatocyte targeted expression of a hepatotoxic transgene creates a functional liver deficit, resulting in a chronic stimulus for liver growth. The model elegantly demonstrated successful transplantation of either normal mouse primary hepatocytes (Rhim *et al*, 1994) or rat primary hepatocytes (Rhim *et al*, 1995). These models have potential for developing a HCV animal model.

### **1.3.3 Artificial support for hepatocyte transplantation**

The use of solid supports, consisting of either non-biodegradable synthetic fibres or biodegradable polymers, for *in vivo* culture of primary hepatocytes is another approach to transplantation. These provide a three-dimensional framework, allowing sufficient space for infiltrating hepatocytes to be exposed to the host's environment. The cells can adhere to the framework, restoring the cell to cell and cell matrix interactions. When transplanted, they become vascularised and the cells regain their polarity (Arkadopoulos *et al*, 1997). Several examples of solid support include liver beads, which are living hepatocytes entrapped within an alginate matrix (Fremond *et al*, 1993); polytetrafluoroethylene (PTFE) fibres, which do not produce an intense inflammatory response and Cytodex 3 collagen coated micro-carriers. These can



**Figure (1.2)**

**Signalling pathways of the mitogenic stimuli for hepatocytes taken from Michalopoulos, 1997. (Norepinephrine American nomenclature for Noradrenaline)**

be transplanted into the peritoneal cavity or under the renal capsule of the animal (Arkadopoulos *et al*, 1997). But the number of hepatocytes attached to these solid support units is limited by the size of the animal. For example, liver beads only contain 400 cells/ bead (BIOPREDIC International, France), therefore to transplant  $10^6$  cells per site, will require around 2500 beads, making this an unpractical and expensive model. Further, to retain their differentiated state *in vivo* the hepatocytes would also require proliferative stimuli, to ensure their prolonged survival.

## **1.4 Animal models of infection**

### **1.4.1 HCV infection in chimpanzees**

The chimpanzee is the only successful animal model for HCV and was first used in infection and characterisation experiments for non-A, non-B hepatitis (Feinstone *et al*, 1981). To investigate early events in HCV infection, Shimizu *et al* successfully infected two chimpanzees intravenously with 0.5ml of undiluted plasma obtained from a patient with post-transfusion acute non-A, non-B hepatitis (Shimizu *et al*, 1990). RNA transcripts from full-length HCV functional clones were also found to be infectious and to cause disease when injected intra-hepatically into two HCV naïve chimpanzees (Kolykhalov *et al* 1997).

The successful infection of chimpanzees with a single clone of HCV can provide a model where viral evolution and persistence can be studied (Yanagi *et al* 1997). Yanagi *et al* injected RNA transcripts from full-length cDNA clones of the H77 strain intrahepatically into 2 chimpanzees. Similar to earlier reports of HCV challenge in chimpanzees, HCV RNA was detected 8 weeks postinfection (Yanagi *et al* 1997). The disadvantages of these chimpanzee animal models are cost and ethics. Consequently, only a very limited number of animals can be used experimentally as a results accessibility of such a model for widespread experimental studies is restricted.

### **1.4.2 Rodent infection models**

Rodents have been used as an alternative model of HCV infection. Yamamoto *et al* transfected HCV cDNA into rodents *via* either the tail vessel or the portal vein using asialoglycoprotein receptor mediated gene delivery. This receptor is expressed on hepatocytes. The cDNA delivered codes for the core and part of the envelope protein of HCV; the core protein was detected in the liver by immunohistochemical techniques (Yamamoto *et al* 1995). The practicalities and applications of delivering full length infectious HCV RNA by this method should be investigated.

Transgenic mouse lines that express HCV proteins in the liver are alternative possible animal models. A cDNA fragment containing the entire HCV core gene was placed down stream of a transcriptional regulatory region from HBV. Two distinct lines were created. Southern blotting showed a distinct integration site. Expression of the core protein in the liver started at birth and continued for at least 19 months. These mice developed hepatic steatosis as early as 3 months and the long term consequences of this model was that some of the mice from both lines (25.9% and 30.8%) developed hepatocellular carcinomas (Moriya *et al* 1998).

### **1.4.3 Role of xenotransplantation in animal infection models**

Xenotransplantation of permissive cells or tissue fragments has become one of the most frequently used methods for the construction of animal infection models. The severely combined immunodeficient (SCID) mouse accepts xenografts from a range of species including humans, because it lacks functional B and T cells resulting from defects in the T-cell receptor and an immunoglobulin variable region (Bosma, *et al* 1983).

#### **1.4.3 a) Transplantation of transfected cells or infected tissue**

Transplantation of permissive cells into a SCID mouse transfected with an infectious clone is one example of xenotransplantation, which can be applied to construct an HCV animal model. In nude mice, transplantation of HepG2 cells

transfected with the hepatitis B genome led to Dane particles and HBV antigen production *in vivo* (Zhai *et al* 1990). Alternatively, human/SCID chimeras can be infected with agents that can only replicate in human cells. This technique has been successful for other virus animal models, for example the human papilloma virus (HPV). An animal model was generated from grafting anogenital and laryngeal papillomas harbouring either HPV-6 or HPV11 (Sexton *et al*, 1995) and a cytomegalovirus (CMV) mouse model was generated from the transplantation of hemopoietic tissue inoculated with CMV (Mocarski *et al* 1993). Recently, Bronowicki *et al* tested HCV persistence in transplanted haemopoietic cells in a SCID animal model where PBMC from 14 HCV infected individuals with histologically documented chronic hepatitis, were injected intraperitoneally. After an eight-week follow up, 7 of 26 serum samples and 8 of 27 PBMCs were positive for HCV RNA positive strand. However, negative strand HCV RNA was only detected in 2 of the 10 mice tested, which were inoculated with PBMCs from patients with either non-Hodgkins's lymphoma or acute lymphoblastic leukemia. (Bronowicki *et al*.1998).

#### **1.4.3 b) *In vivo* infection of transplanted cells**

*In vivo* infection of transplanted permissive human cells was demonstrated in the malaria mouse model and could have potential applications in an HCV model. Here primary human hepatocytes were transplanted under the renal capsule and subsequent intravenous infection with *Plasmodium falciparum* sporozoites took place one week post- transplantation. The grafts produced a well-defined cell mass that was histologically distinct from the kidney and were also permissive to infection with *P falciparum* for up to 4 months post-transplantation (Sacci *et al* 1992).

#### **1.4.4 Shortcomings of SCID/human chimera**

One disadvantage of the SCID/human chimera is that mouse mononuclear phagocytic cells are known to attack transplanted cells, thus preventing successful engraftment. This phenomenon was observed when HCV infected primary foetal



hepatocytes were transplanted in SCID nude mice and were subsequently attacked two weeks post transplantation (personal communication, Dr Frederick Prince from the New York Blood Centre). However, to promote engraftment of donor human hepatocytes, mononuclear phagocytic cells can be removed by either the administration of silica to the mice after transplantation (Adno *et al*, 1993), or alternatively, liposome-mediated macrophage "suicide" technique can be applied, where the dichloromethylene diphosphonate is encapsulated. On ingestion of the liposomes, the macrophages will be destroyed (Van Roojen, 1989). This approach was applied to facilitate the successful engraftment of human hematopoietic cells in SCIDS. Histological examination of the spleen showed transient elimination of all macrophage subsets and unexpected graft failures were not observed (Terpstra *et al*, 1997).

Another disadvantage of the SCID mouse model is that its phenotype is known to develop populations of peripheral mature lymphocytes in a time and strain dependent manner; that is, they become "leaky". This may be due to somatic reversion events or to rescue of the liberated coding regions in SCID pre-lymphocytes by alternative recombination mechanisms (McBride *et al* 1995). Consequently, some grafts may fail (Krensky, 1997).

#### **1.4.5 Infection models in SCID Beige human/chimeras**

SCID Beige mice are a potential substitute for SCID mice, for they lack natural killer (NK) cells and the *beige* defect results in defective lysosomal granule formation. This could allow successful engraftment and the establishment of viral infections (McBride *et al* 1995). Further, these mice are less prone to develop "leaky" individuals; in fact, less than 2% produce T cells or immunoglobulin (Krensky, 1997). Adult human PBMC can also be delivered into SCID/beige mice by intraperitoneal injection. This was demonstrated in the human immunodeficient virus (HIV) mouse model (McBride *et al* 1995). Secondary type immune responses, have also been exploited in protection studies involving adoptive transfer of PBMCs taken from volunteers immunised with vaccinia expressing HIV-1 LAV/Bru 160 Dalton envelope glycoprotein and

subsequently given booster injections of recombinant gp 160 protein (Mosier *et al* 1992). The passive transfer of human HIV specific antibodies into mice bearing human effector cells may lead to a better understanding of protective mechanisms involved (McBride *et al* 1995).

Gallun *et al* used lethally irradiated Beige/nude /X-linked immunodeficient (BNX) mice that were intravenously injected with SCID mouse bone marrow. After transplanting human tissue, a mouse model that comprises three genetically disparate sources of tissue, a “trimera” *in vivo* model, will result (Gallun *et al*, 1995). The transplanted tissue used was human liver fragments from chronically infected HCV patients and uninfected persons, after *ex vivo* incubation with HCV serum. This was grafted under the kidney capsule.

In spite of the morphological changes reported in the grafts, HCV RNA was detected by RT-PCR in the serum from mice with liver fragments taken from 3 patients with chronic HCV infection. This was first observed 2 weeks after infection and was detected intermittently for 2 months post-transplantation. A similar pattern was noted with the fragments infected pre-transplantation with HCV serum, with HCV RNA detected in 20-30% of the transplanted mice. Since the RNA negative-stand assay performed in the animals that received pre-infected liver fragments was positive, it is plausible that HCV replication had taken place in the transplanted grafts *in vivo* (Gallun *et al* 1995). A similar HBV “trimera” animal model was developed by Ilan *et al* where BNX mice or (CB6F1) strain of mice that were thymectomised at 6-8 weeks were used. Transplantation of the grafts was either under the renal capsule or in the ear pinna and the animals remained viraemic for up to one month post-transplantation (Ilan *et al*, 1999).

“SCID-hu” chimera/trimera systems are limited by the absence of human macro- and microenvironments, the lack of soluble factors (including nutrients, cytokines, and hormones) and differences in matrix and cell surface interaction molecules. For these models are not physiological, and are of limited use for drug testing (Krensky 1997). To resolve some of the above deficiencies Sultan and his colleagues developed a variation of the “hu/ PBL/SCID” system to study transplantation rejection. This was achieved by grafting human skin on to SCID

beige mice and then followed by an intraperitoneal injection of adult lymphocytes. This model did not represent true allogenic rejection, but was useful to study molecular and cellular interactions (Sultan *et al* 1997). It is plausible therefore, that the “hu/PBL/SCID” rationale can be applied to an HCV infection animal model. One approach is where primary human hepatocytes are transplanted intrasplenically, the animal is then injected with syngeneic PBMCs and subsequently infected *in vivo* with HCV positive serum.

To date all rodent HCV infection models have failed to replicate natural HCV infection, though intermittent negative strand RNA was demonstrated in transplanted *ex vivo* infected liver fragments by Gallun *et al* indicating a small window of viral replication. Therefore, long term testing of drug strategies in this model will be limited.

Apart from the technical difficulties in establishing this model, cryopreservation of whole liver fragments is still experimental. Additionally, there will be considerable batch variation, as the material used originates from different donors.

The absence of HCV negative strand RNA in circulating PBMCs demonstrated by Laskus *et al* questions HCV lymphotropism and the use of T cells as potential permissive sites for viral replication. It is possible that HCV has lymphotropic and hepatotropic tendencies, since CD 81 receptor that binds the HCV envelope protein is expressed on PBMCs as well as hepatocytes. Or that PBMCs are important in that they act as chaperones for HCV infection.

Transplantation and *in vivo* infection of hepatotropic cell lines that are permissive to HCV infection *in vitro* and primary human hepatocytes has not been documented to date. This approach if successful can demonstrate a repeatable and easily accessible model for primary and acute HCV infection and thus can be used to test drug strategies.

## 1.5 Aims and experimental approach

The aim of this project is to establish a reproducible *in vivo* cell culture system that would support the complete replication cycle of HCV.

Huh7, HepG2 and WRL68 cell-lines, cryo-preserved primary human hepatocytes from liver that cannot be placed for transplantation and cryo-preserved primary human hepatocytes obtained from HCV positive explant livers will be transplanted into SCID mice after performing a partial hepatectomy. These cells will be infected with HCV positive serum from the same HCV infected individual by either intra-venous or intra-peritoneal routes. *Ex vivo* infection will also be attempted prior to transplantation.

To test successful engraftment of the cell-lines, paraffin fixed sections will be examined using hematoxylin and eosin staining. However, detection of human primary hepatocytes will require cytokeratin 18 staining and albumin detection using immunocytochemistry and *in situ* hybridisation techniques.

The animal tissues and whole blood will also be tested for the presence of HCV using positive strand RT-PCR at time intervals.

## CHAPTER 2

### Materials

All chemicals and reagents were purchased from BDH Chemicals UK or from Sigma-Aldrich Company Ltd UK, unless otherwise stated.

#### 2.1 Animals

Mice: Sex: male; Strain:CB-17 SCID ( on ICR Background)  
(Harlan UK Ltd)

#### 2.2 Surgical instruments and reagents

*Instruments* Microfine Scissors, HB-040-01-J Scissors, Microfine Forceps (B.Brone, Downs Surgical, UK); Insulin syringes: 1ml 2.7mm Microfine U-100 (B D Microlance, Becton Dickinson, UK); Scalpel blades (Swann Morton, UK); Sutures: Vicryl 3/0 code W94444, Mersilk 5/0 code W580 (Ethicon, UK).

*Reagents* Halothane (Zeneca Ltd, UK).

#### 2.3 Explant Livers

Professor James Neuberger from the Liver and Hepatobiliary Unit, Queen Elizabeth Hospital Birmingham, supplied five HCV positive explant liver wedges from the left lobe of HCV positive transplant recipients.

(a) Donor 1, age 46 years, patient also had alcohol related liver disease. Date received was 20/8/97.

(b) Donor 2 age 40 years, date received was 14/1/98.

(c) Donor 3 age 57 years, date received was 21/4/98.

(d) Donor 4 age 46 years, date received was 7/7/98.

(e) Donor 5 age 67 years, date received was 14/1/99.

There was no HCV subtype data available on the above donors.

Mr Bryon Jaques at the Transplant Unit, Addenbrookes Hospital, Cambridge supplied wedges from three livers that could not be placed for transplantation:

(f) Donor 6 age 7 years, date received was 17/5/98.

(g) Donor 7 age 56 years, date received was 3/6/98.

(h) Donor 8 age 8 years, date received was 14/6/98.

A ninth liver obtained from a 42 year old woman was received on 29/5/98 and supplied by Mrs Walsh, the transplant coordinator at the Renal Transplant Unit, at the Western Infirmary Glasgow.

Donors 6 and 8 tested negative serologically for HBV, HCV, HIV and CMV, but donor 7 was positive for CMV. No serological tests were available from the ninth donor.

## **2.4 Human sera**

Various staff members of the lab 109 donated negative (control) sera during the project.

Dr Douglas Thorburn supplied HCV antibody positive successive sera from an asymptomatic patient, 001, (without severe liver disease) attending the Liver Clinic at Gartnavel Hospital, Glasgow.

HCV antibody positive serum from a patient with cryoglobulinemia/macronodular liver disease was titrated to  $10^7$  copies/ ml HCV by Adna Ochert, Roche Discovery, Welwyn Garden City, UK. This was obtained from Sacramento Blood Center and supplied by Roche Discovery, Welwyn Garden City, UK.

Pelispay HCV-RNA run control, is a weak positive plasma standard for the validation of HCV-RNA amplification test runs. It contains 3800 genome

equivalents /ml and was obtained from Central Laboratories of the Netherlands Red Cross Blood Transfusion service.

## 2.5 Cell lines

HepG2 (Aden *et al.*, 1979; Knowles *et al.*, 1980) and WRL68 (Apostolov, 1976; Gutierrez *et al.*, 1994) cell-lines were obtained from the European Collection of Animal Cell Cultures, PHLS, Center for Applied Microbiology and Research Department, Porton Down, UK. The Huh7 (Nakabayashi *et al.*, 1982) cell-line was available for use at the Institute of Virology, University of Glasgow.

## 2.6 Buffers, Solutions and Materials for the following procedures:

### 2.6.1 Liver Perfusion

#### *Krebs-Henseleit (KH) solution*

117mM NaCl, 2.4mM NaHCO<sub>3</sub>, 11mM Glucose, 10mM Hepes, 4.7mM KCl, 1.2mM MgSO<sub>4</sub> .H<sub>2</sub>O, and 1.2mM KH<sub>2</sub>PO<sub>4</sub>.

*Buffer A* A 10 mM solution of EGTA was prepared with the KH stock solution; pH was adjusted to 7.4 with 0.1 M NaOH.

*Buffer B* This was prepared by diluting the KH solution with H<sub>2</sub>O (1/4 v/v); pH was adjusted to 7.4 with 0.1M NaOH.

*Buffer C* A 4.2 mM solution of CaCl<sub>2</sub> was prepared with the KH stock solution, pH was adjusted to 7.6. This was then used to produce a 0.5mg/ml Collagenase D solution (Roche Diagnostics, UK).

*Buffer D* A 0.75mM solution of CaCl<sub>2</sub> was prepared with the KH stock solution.

All buffers were supplemented with 40mg/ml Gentamicin (Life Technologies Ltd UK).

*Marseilles's Freezing Medium*

L15 Medium (Life Technologies Ltd, UK) containing 2% (v/v) Polyvinylpyrrolidone (PVP) 2.5% (w/v) Bovine Serum Albumin fraction V, 20% Foetal Calf Serum (FCS) (Advance Proteins Products, UK) and 10% Dimethyl Sulphoxide.

*Isotonic Solution* 0.15M NaCl, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.3 mM KH<sub>2</sub>PO<sub>4</sub>, 1.0mM MgSO<sub>4</sub>, 2.0 mM CaCl<sub>2</sub>, 20mM Hepes-NaOH (pH 7.5).

*Nycodenz Gradient* 40%(w/v) Nycodenz containing 6.5 mM KCl, 1.0mM CaCl<sub>2</sub>, 10mM Hepes-NaOH (pH 7.5).

**2.6.2 *In vivo* and *in vitro* culture medium***Cell lines*

Dulbecco's modified essential medium (DMEM) 100IU/μl Penicillin, 100IU/μl Streptomycin, and 2mM L-Glutamine (Life Technologies Ltd, UK), 10% Foetal Calf Serum (Advance Proteins Products, UK) trypan blue, Trypsin, Versene, (supplied by the media service at the Institute of Virology, Church Street, Glasgow).

*Primary Hepatocyte Transplantation*

DMEM, Bovine serum albumin (1%), 0.02μg/ml EPG, 10U/ml Insulin, 1000U/ml Interleukin 6 (Roche Diagnostics, UK), 60μg /Animal Anti-*met* antibody supplied by Ken Hillan (Genentech, USA), 38.5mg/ml, Silica and Silicon discs, known as Immobasil D (Integra Biosciences, UK).



**2.6.3 General Pathology use**

Harris Haematoxylin, Putt's Eosin, Paraffin wax, and Trigene were purchased from R and J Lamb, UK; Goat serum was obtained from Scottish Antibody Production Unit, Law Hospital, Glasgow; Formol saline, Hydrogen Peroxide/ Methanol (1/60), Tris buffered saline, 0.01M Citrate buffer, Tween 20, Xylene.

*Scotts tap water (STW)*            3.5g sodium hydrogen carbonate, 35.7g magnesium sulphate dissolved in one litre of water.

*Apes coated slides*                Slides immersed in a 2% solution of 3'-aminopropyltriethoxysilane/ acetone and dried at 60°C.

**2.6.4 *In situ* Hybridisation and Southern blot Hybridisation**

All reagents were prepared with Diethylpyrocarbonate (DEPC) treated ultra pure H<sub>2</sub>O (Elga UHP).

*Denhardt's Reagent, X50*

5g Ficoll (Type 40), 5g PVP, 5g Bovine serum albumin dissolved in DEPC treated H<sub>2</sub>O to a final volume of 500mls.

*20 X SSC*                                3M NaCl, 0.3MNa<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> (pH7.2).

*Denaturation Solution*

1.5MNaCl, 0.5MNaOH.

*Neutralisation Reagent*

1.5MNaCl, 0.5MTris-HCl pH7.

*Pre Hybridisation solution*

5XSSC, 50% deionised Formamide

*Hybridisation solution*

5M Tris pH7.5, 10X Denhardt's, 2X SSC, 0.5% SDS, 50% deionised Formamide, 10% Dextran Sulphate 20µg/ml sonicated Salmon Sperm DNA.

Hybond N(+) Membrane (Amersham, Pharmacia Biotech, UK).

X-OMAT S film (Kodak Ltd, UK)

The following reagents were used in *in situ* Hybridisation only:

*End-labelling of probe reagents*

Biotin-16-dUTP, (Roche Diagnostics,UK), dCTP (Amersham Pharmacia Biotech, UK), 5X tailing buffer (Life Technologies Ltd, UK).

*Digestion and fixing reagents*

HCL, TritonX and Paraformaldehyde.

*Dig(1) buffer* 100mM Tris base, 100mM Tris HCl, 150 mM NaCl pH 7.6.

*Dig(3) buffer* 100mM Tris base, 100mM Tris HCl, 150 mM NaCl pH 9.5.

*Visulisation Reagents Stock solutions*

Nitro Blue Tetrazolium (NBT): 7.5mg/ 100µl 70% Dimethyl Formamide; Bromo-Chloro-Indoly-Phosphate (BCIP): 5mg/100µl 100% Dimethyl Formamide; Levamisole : 2.3mg dissolved in 10µl Dig (3) buffer.

*NBT/BCIP Levamisole working solution*

10ml Dig(3), 10µl Levamisole stock solution, 34µl BCIP stock solution and 44µl NBT stock solution, the solution was mixed filtered and stored at 4°C.

## 2.6.5 Immunocytochemical staining (ICC)

*Antibodies*

Anti-mouse monoclonal against cytokeratin 8 and18 antigens (Novocastra Laboratories Ltd, UK).

Anti-rat peroxidase monoclonal and Anti-digoxigenin, Fab fragment from sheep conjugated with alkaline phosphatase (Roche Diagnostics,UK).

Alfa fetoprotein rabbit anti-human polyclonal; swine antigen anti-rabbit biotinylated polyclonal and rabbit antigen anti-mouse polyclonal, mouse peroxidase anti peroxidase (PAP) antibody (Dako Ltd, UK).

Anti-rat monoclonal against murine macrophage lineage (F4/80) was a generous gift from Dr Jim Brewer at the Department of Immunology, Glasgow University, but also can be obtained from Sero Tec, UK.

### *General visulisation Reagent*

*3,3 Diaminobenzidine Tetrahydrochloride (DAB)*

### **2.6.6 RNA extraction**

All reagents were prepared with DEPC treated molecular biology grade H<sub>2</sub>O.

*Stock solution D* (450g in 293ml H<sub>2</sub>O) Guanadinium isothiocyanate, (26.4ml) of 10% N-lauryl sarcosine, 17.5ml of (0.75M) Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> 2. H<sub>2</sub>O filtered with a 2µm filter.

*Lysis Buffer* 180µl β- Mercapthoethanol in 25ml stock solution D.

*Other reagents* RNA carrier (yeast tRNA), 2M CH<sub>3</sub>COONa, Phenol, Chloroform, Isoamyl alcohol and Ethanol.

### **2.6.7 RT-PCR analysis**

50mM MgCl<sub>2</sub>, 10X PCR buffer, 5XRT buffer, DTT and 100bp Ladder were all purchased from Roche Diagnostics,UK Taq Start <sup>TM</sup> Antibody (Clontech Laboratories Inc., Palo Alto CA) mineral oil.

*10 X dNTPs* 100mM each of dATP, dTTP, dCTP, dGTP (Roche Diagnostics,UK).

*10 X TBE* 89mM Tris base, 89mM Boric acid, 10mM EDTA.

*Gel loading buffer*     50% Glycerol, 0.025% Bromophenol blue (1%) 1X TBE.

*Agarose Gel*             1.5% Agarose in 1X TBE buffer, 50µl (1mg/ml) Ethidium Bromide per 300mls of Agarose.

## 2.7 Plasmids

pGEM3Zf(-) containing a 2Kb cDNA sequence of Human Albumin obtained from ATCC Rockvile, Maryland, USA.

pBSK9 containing 5'UTR, Core, E1 and E2 regions of HCV (Nucleotides 1-2545) synthesized in Roche Discovery, Welwyn Garden city, UK.

## 2.8 *In situ* plasmid Linearisation Buffers

*10X NTPs*             10mM each of ATP,CTP,GTP and UTP ( 6.5nM) and Dig labeled UTP (3.5 nM) (Roche Diagnostics,UK)

5X Transcription buffer (Roche Diagnostics,UK).

## 2.9 Kits

Vector VIP Peroxidase Substrate Kit (Vector Laboratories, UK).

Universal Vectastain ABC Kit (Vector Laboratories, UK).

ECL Random prime labeling kit (Amersham Pharmacia Biotech, UK).

## 2.10 Enzymes

Proteinase K, DNase I, RNase free, SP6 RNA polymerase, EcoRI restriction endonuclease were purchased from Roche Diagnostics,UK.

Taq<sup>TM</sup> DNA Polymerase ,Reverse Transcriptase M-MuLV, Rnasin RNase inhibitor were either purchased from Roche Diagnostics,UK or Life Technologies Ltd, UK Terminal deoxynucleotidyl transferase was purchased from Life Technologies Ltd, UK.

## 2.11 Oligonucleotides

Oligonucleotides are summarised in tables 2.1 and 2.2:

Oligo	Size	Sequence	Reference
VICTAG-RT Anti-sense *	40	<5'CGTATGCTACATTCG AGGGTTTTTCTTTGAGGTTTA GGA3'>	M <sup>o</sup> Kechnie 2000
VICTAG-1 Anti-sense **	20	<5'CGTATGCTACATTCG AGGGT3'>	M <sup>o</sup> Kechnie 2000
VICTAG-940 Sense *	40	<5'TGAGCGAGACCTAT AGGGCCTTCACGCAGAAAGC GTCTAG3'>	M <sup>o</sup> Kechnie 2000
VICTAG-2 Sense **	20	<5'TGAGCGAGACCTAT AGGGCC3'>	M <sup>o</sup> Kechnie 2000
NCR4 Antisense **	26	<5'CACTCTCGAGCACC CTATCAG3'>	M <sup>o</sup> Kechnie 2000

\*Synthesised in-house, \*\* Synthesised by Cruachem, West of Scotland Science Park and MWG-Biotech UK Ltd.

**Table 2.1 HCV oligonucleotide sequence used in nested RT-PCR for the 5' UTR region.** HCV sequence adapted from Kato *et al.*, 1990 and used by Dr McCrudden's laboratory at the Institute of Virology University of Glasgow.

Oligo	Size	Sequence	Reference
# 5** Anti-sense	22	<5'TCGCAAGCACCCCTATCAGGG CAG 3'>	Hsu <i>et al</i> 1991
#6 ** Sense	23	<5'GGAAGTACTGTCTTCACGCAG A3'>	Hsu <i>et al</i> 1991

\*\* Synthesised by (Cruachem, West of Scotland Science Park and MWG-Biotech UK Ltd).

**Table 2.2 HCV oligonucleotide sequence used in single round RT-PCR for the 5' UTR region. These oligonucleotides were used by Roche Discovery .**

## CHAPTER 3

### Methods

#### 3.1 Perfusion of normal and HCV infected human explant livers

Livers and lobes were obtained from HCV positive transplant recipients or perfused normal donors, whose livers could not be used for organ transplantation. All HCV positive liver explants were perfused with University of Wisconsin (UW) preservation solution after hepatectomy. Normal livers were perfused *in situ* prior to hepatectomy. A portion of the organ, usually the left lobe (300g-500g), was resected, then transported in UW preservation solution on wet ice and processed usually within 24 hours.

The tissue was processed aseptically in a class II hood under category III containment level in accordance with the health and safety regulations.

To isolate primary hepatocytes, digestion of the liver was performed using four perfusion buffers A, B, C and D in succession: (chapter 2, section 2.6.1), prepared shortly before processing and stored at 4°C. Just prior to processing the tissue, the buffers were allowed to equilibrate to 37°C. Starting with buffer A and using a cannula, 500 mls of each buffer were injected through a suitable vein in the tissue with a 50ml syringe (flow rate was approximately 20-30ml/minutes). The objective of perfusing with a pre-perfusate (buffer A) was, to wash out the remaining blood from the liver and to remove Ca<sup>2+</sup>, which is a cellular adhesive ion. The EGTA in this solution is a chelator of Ca<sup>2+</sup> removing extracellular Ca<sup>2+</sup>. Buffer B washes out any residual buffer A. Buffer C contains collagenase, which is a crude extract from *Clostridium histolyticum* and includes various proteolytic enzymes which act effectively to disperse cells. The enzymatic activity of

collagenase is  $\text{Ca}^{2+}$  ion dependent and maximum activity is obtained with a perfusate containing 4 mM  $\text{Ca}^{2+}$ . To disperse the cells the lobe was then washed in buffer D (Kasai and Mito, 1997) and the cells released by mechanical scraping. The tissue was then cut into fragments of 2 cm and a spatula used to release cells mechanically into a suspension of buffer D. The original cell suspension was filtered through gauze and washed twice by slow centrifugation (50 rpm). Cell count and viability was determined by trypan blue exclusion.

### **3.1.1 Cryo-preservation and storage of cells**

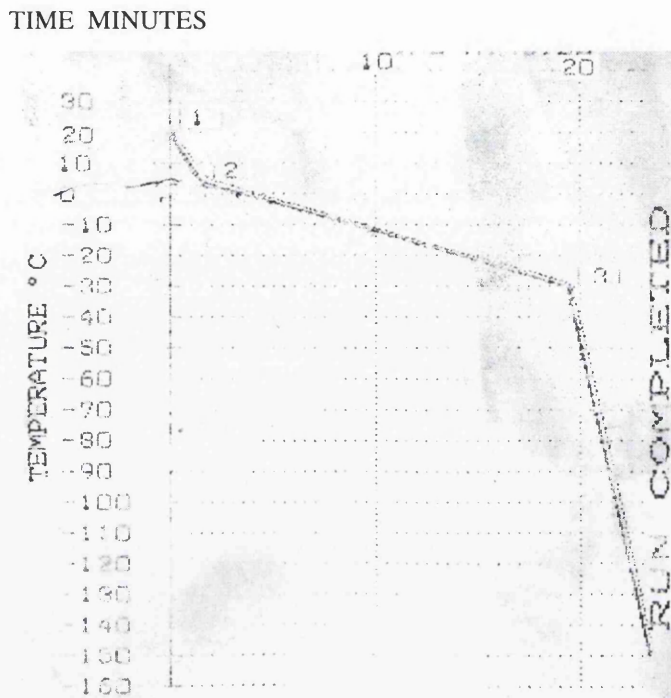
Using Marseilles's medium the hepatocytes were suspended at a cell density of  $10^7$  cell/ml, aliquoted in sterile polypropylene vials and placed in either a Nalgen cell freezing storage container in the presence of propan-2-ol. Where the vials were placed initially at  $-70^\circ\text{C}$  for 24 hours, after which they were transferred to liquid nitrogen for long time storage. Alternatively, the vials were transferred to a cell-freezing machine (model Planer, KRYO10 III Series). The freezing cycle was monitored, the optimum gradient temperature was  $-1.9^\circ\text{C}/\text{minute}$  from  $4^\circ\text{C}$  to  $-30^\circ\text{C}$  (2 in figure 3.1) and  $-30^\circ\text{C}/\text{minute}$  from  $-30^\circ\text{C}$  to  $-150^\circ\text{C}$  (3 in figure 3.1). Cryopreserved hepatocytes were stored at  $-196^\circ\text{C}$  in liquid Nitrogen.

### **3.1.2 Preparation of primary hepatocytes for transplantation**

Frozen ampoules were thawed quickly in a  $37^\circ\text{C}$  water bath; cells were suspended in isotonic buffer and centrifuged at 1000 rpm for 10 minutes. In some experiments, the cells were layered on a Nycodenz gradient and centrifuged at 1500rpm for 10 minutes at room temperature to further remove non-parenchymal and damaged cells. The cells were removed at the interface, washed in  $\text{L}_{15}$  medium and resuspended in either phosphate buffered saline (PBS), or hepatocyte growth medium (DMEM, 1% Bovine serum albumin  $0.02\mu\text{g}/\text{ml}$  EGF,  $10\text{U}/\text{ml}$  Insulin). A sample of the cells was also preserved in formal saline and cytopsin preparations were made for immunocytochemical staining and *in situ* hybridization analysis, using a Shandon Cytospin 2.



In some experiments,  $3 \times 10^6$  cells were mixed with either Interleukin-6 (IL6) (100U) or with anti-*met* antibody (14 $\mu$ l), which is equivalent to 60 $\mu$ g/ animal. In another set of experiments,  $3 \times 10^6$  cells were also mixed with both IL6 and anti-*met* antibody.



**Figure (3.1)** Freezing cycle with optimum temperature gradients ( 2 and 3)

### **3.1.3 Preparation of transformed human hepatocyte cell lines**

$10^6$  cells were seeded in a T175 flask in 20ml DMEM medium supplemented with 10% FCS, 2mM L-glutamine, 100 IU/ $\mu$ l penicillin and 100 IU/ $\mu$ l streptomycin. The cultures were incubated at 37°C in the presence of 5% CO<sub>2</sub> and allowed to reach confluence. The cells were harvested using trypsin: versene at a ratio of 1:1 and suspended in PBS for transplantation. Alternatively,  $10^5$  cells were seeded on to Immobasil D silicon disks, placed in 2cm tissue culture grade petri dishes and allowed to proliferate and infiltrate the matrix for 36 hours. The disks were then removed and suspended in PBS for transplantation. Proliferation of the cells was determined by treating the disks with trypsin: vesene at a ratio of 1:1 and washing with PBS to release the adhering cells. The disks were also preserved in formol saline and processed after embedding in paraffin for microscopic examination.

### **3.1.4 *In vitro* infection of cells for transplantation**

To infect the cells, HCV positive serum neat or 20% v/v with normal serum was mixed with  $10^7$  cell/ml, suspended in either DMEM medium or hepatocyte growth medium, incubated at 37°C and agitated intermittently for one hour. The cell suspension was then administered into the animals.

## **3.2 Transplantation: surgery and anaesthetics**

All procedures were performed in a class II hood under category III laboratory conditions in accordance with health and safety recommendations and the Animals (Scientific Procedures Act 1996).

### **3.2.1 Pre-surgical preparation and anaesthesia**

5-6 week old mice were acclimatized to the positive pressure isolator (appendix 1) for one week prior to surgery.

Surgical instruments, swabs and drapes were sterilised at 121°C and 15psi for 20 minutes. The hood was sequentially disinfected with 2% Virkon and

70% ethanol spray. A sterile drape was used to cover the operating area in the hood. The animals were shaved approximately 24 hours before surgery.

To induce anaesthesia, the animals were placed in a chamber where a mixture of oxygen and halothane vapour (2% v/v) was delivered from an anaesthetic machine (appendix 2). Maintenance of anaesthesia was achieved during surgery by inserting the animal's head in a mask attached to the anaesthetic machine, from which a mixture of oxygen and halothane vapour at a concentration of 1-1.5% was delivered. Sufficient depth of anaesthesia was sustained; vital signs for example breathing and mucous membrane colouration and the pedal withdraw reflex were frequently monitored. The animals were placed with limbs restrained on a heated pad to maintain body temperature during surgery, in a supine position, swabbing with 70% ethanol to disinfect the skin.

### 3.2.2 Surgery

To expose the peritoneum and the abdominal cavity, a midline laparotomy incision was made (figure 3.2; solid line) using a size 15 scalpel blade. A further incision extending from the right border of the sternum, along the xiphoid process (figure 3.2; interrupted line) was made using scissors, taking care to avoid accidental penetration of the chest cavity (curved lines represent costal margins) (Doerr *et al.*, 1990).

Exposure of the liver was achieved by manual compression of the thorax. The four major lobes were identified (right, median, left and caudate); the two segments of the median lobe are partially divided by the gallbladder (figure 3.3) (Doerr *et al.*, 1990).

A 4/0 silk suture was used for mass ligation of the lateral portion of the median and right lobes (figure 3.3) avoiding the gallbladder, extrahepatic biliary tree, abdominal aorta and portal vein (figure 3.4a). The right lobe and the lateral segment of the median lobe were resected with scissors (figure 3.4b). After haemostasis was obtained, the liver was gently eased back into the abdominal cavity.

Both kidneys and spleen were individually exposed through the incision and supported in position. Cells were then injected under both renal capsules ( $10^6$  cells/ 50 $\mu$ l volume) and into the spleen using either a 25-gauge (G) needle or 27G insulin syringe (figures 3.5a and 3.5b).

In some experiments transplantation of cells was performed by inserting inert silicon disks containing cells into the abdominal cavity close to the liver.

In macrophage depletion experiments 200 $\mu$ l of silica (38.5 mg/ml) was injected into the peritoneal cavity. To restore the anatomical integrity of the muscle and skin layers a continuous suture pattern with either catgut or vicryl sutures was used (Waynforth and Flecknell, 1992).

### 3.2.3 **Post-operative care**

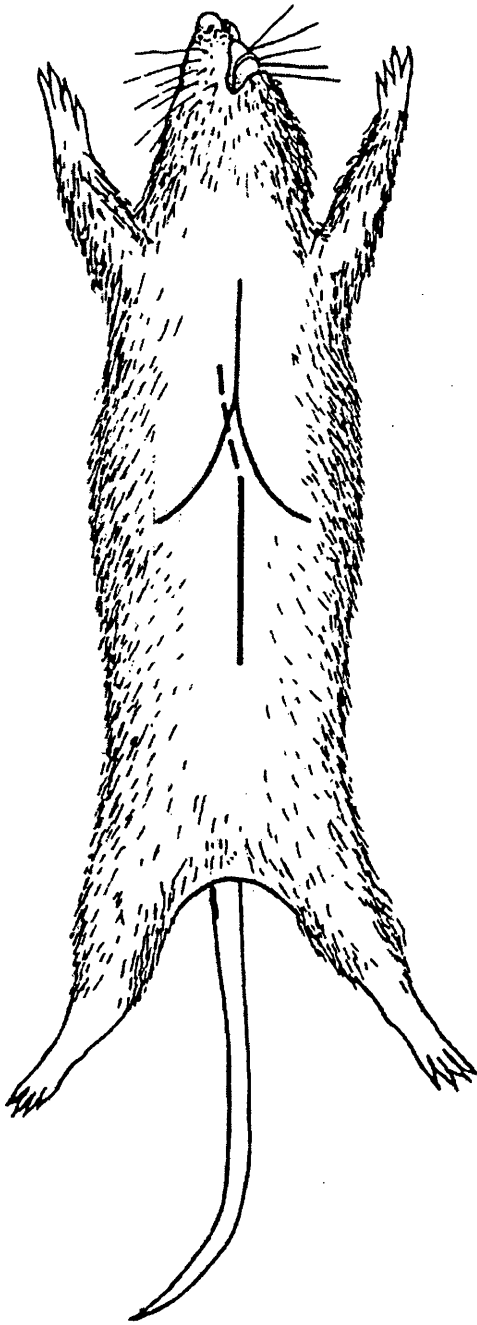
The animals were allowed to recover in a sterile cage placed on a heated mat. Vital signs such as breathing and colour of mucous membranes were monitored until the animal recovered consciousness. It was then returned to the negative pressure isolator (appendix 1). If, however, there was excess bleeding during the surgery, 100 $\mu$ ls of 0.18% isotonic saline was injected subcutaneously.

### 3.2.4 ***In vivo* infection of transplanted cells**

After 24 hours post-operative recovery, the animals were again anaesthetized; 50 $\mu$ l of HCV positive serum was injected either intraperitoneally (IP) or intravenously (IV) using the tail vein with a 27G needle.

### 3.2.5 **Phlebotomy**

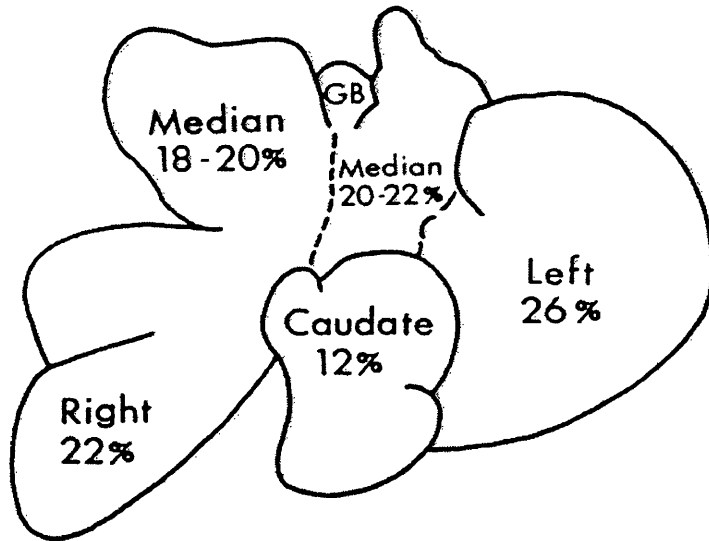
After anaesthetizing the animals, a small incision was made in the base of the tail using a 15-scalpel blade. The blood was encouraged to flow by applying pressure from the base to the tip of the tail. 50 $\mu$ l of blood was aspirated using a micropipette and mixed with sodium citrate (1/10 dilution).



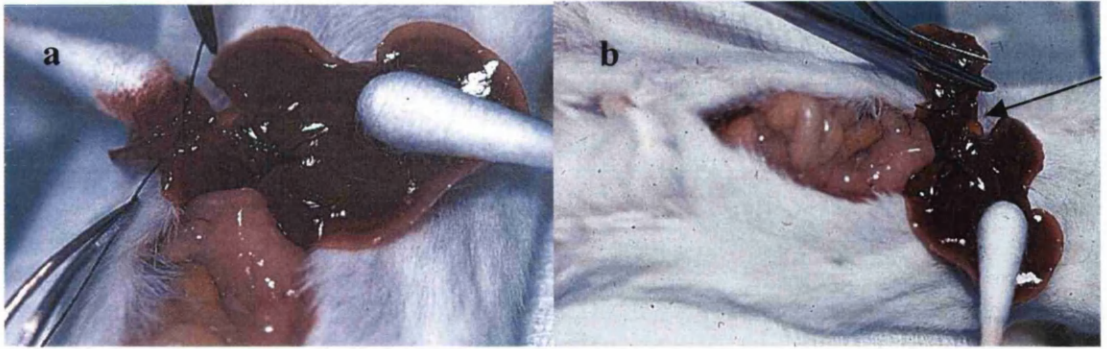
**Figure 3.2** A drawing of a mouse midline laparotomy incision.

Solid line represents the scalpel incision, interrupted line represents the second incision made with scissors.

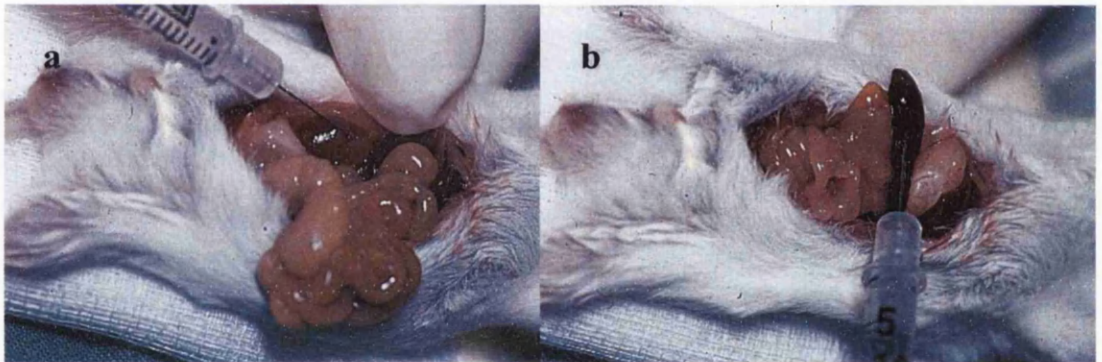
Taken from **Doerr *et al.*, 1990.**

**MOUSE LIVER**

**Figure 3.3 Drawing of the inferior surface of the liver** The drawing shows the gallbladder (GB) and four lobes with relative percentages of total by weight. Taken from Doerr *et al.*, 1990.



**Figure 3.4** (a) Silk ligature *in situ* for resection of the right lobe and lateral segment of median lobe.  
(b) Resection of right lobe and lateral segment of median lobes with scissors, avoiding the gallbladder (arrow).



**Figures 3.5** (a) Transplantation of cells under the renal capsule  
(b) and into the spleen.

### 3.2.6 Euthanasia and post-mortem examination

The animals were sacrificed by dislocation of the cervical vertebrae using standard protocols (the Animals Scientific Procedures Act 1996).

To perform the post-mortem examination, a mid-line incision was made with a 15-scalpel blade and scissors. The abdominal organs were exposed; small samples of fresh tissue for RNA analysis (approx.20-40mg) were taken from the spleen, kidney and liver. The samples were placed in sterile eppendorf tubes, with 400µl of RNA lysis buffer (chapter 2, section 2.6.5) where they were macerated with a plastic pestle to ease tissue lysis, and stored at -70°C. Blood was collected from the thoracic cavity and heart using a 21G needle (200-300µl) and stored in a sterile eppendorf tube with 400µl RNA lysis buffer at -70°C.

The abdominal and thoracic organs, myocardium and lungs were eviscerated and placed in a jar containing 30ml of formal saline to fix for 24 hours for histological processing.

### 3.3 Pathological processing of fixed tissue

Specimen pots were transferred from the category III laboratory to the pathology laboratory using all appropriate safety protocols, and immersed in Trigene disinfectant for 24 hours.

The abdominal and thoracic organs were examined grossly and any abnormality, in particular, the presence of tumor was noted. The liver, spleen, kidneys, heart and lungs dissected out; suitable blocks were selected for histological examination and processed to paraffin wax (appendix 3).

Using a Leitz microtome (model 1212), 3 µm sections were cut, dewaxed and stained with Harris's Haematoxylin and Putt's Eosin (H&E) for routine microscopic examination. APES coated slides were used to aid tissue section adhesion for immunocytochemical staining and *in situ* hybridization.



### 3.4 Immunocytochemical staining (ICC)

The following method was adapted from the standard operating procedures of the Department of Pathology, Western Infirmary, Glasgow and was used for the detection of cytokeratin 18 and 8, F4/80 and alpha fetoprotein antigens.

The sections were dewaxed and hydrated and endogenous peroxidases were blocked with 3% aqueous H<sub>2</sub>O<sub>2</sub> solution. Antigen retrieval was performed using the microwave method with 0.01M citrate buffer (pH 6.0) and the power set at 750 W for three 5 minute cycles (Pileri *et al*, 1997). After which the sections were blocked with 20% normal goat serum for 10 minutes. No antigen retrieval was required for sections stained with Alpha fetoprotein polyclonal antibody. Primary antibodies were applied to the sections at the following dilution and allowed to incubate overnight at 4°C:

- (1/100) Cytokeratin 18 and 8 mouse monoclonal.
- (1/200) F4/80 Macrophage mouse monoclonal.
- (1/500) Alpha fetoprotein polyclonal

The sections were then washed with TBS/Tween buffer 3 times at 5 minute intervals. A secondary antibody at a 1/50 dilution (rabbit anti-mouse polyclonal, for Cytokeratin antigen detection), (anti-rat peroxidase monoclonal, for F4/80 detection) and (biotinylated swine anti rabbit polyclonal, for Alpha fetoprotein antigen detection) was applied and incubated for 30 minutes at room temperature. Sections were washed again with TBS/Tween buffer 3 times at 5-minute intervals. Cytokeratin stained sections were also incubated with mouse peroxidase anti peroxidase (PAP) antibody for 30 minutes at room temperature. The Avidin- biotin complex (ABC) was applied to the sections stained with biotinylated antibodies (following manufacturers instructions of the vector ABC kit). Finally, all sections were washed as before and visualization of the antigen was performed using either the vector VIP kit for the peroxidase-conjugated antibodies or DAB for the biotin-conjugated antibodies. The sections were then examined microscopically, counter stained with Harris's Haematoxylin, dehydrated and mounted with Histomount for analysis.

### 3.5 *In situ* Hybridisation

The following method was adapted from the standard operating procedures of the Department of Pathology, Western Infirmary, Glasgow, and was used for the detection of human albumin (hAlbumin) (Hillan, 1990).

A 2 Kb cDNA fragment (5µg) corresponding to part of the coding sequence of human albumin was ligated into a pGEM3Zf(-) plasmid. 1µg was then linearised with 10 units of EcoRI restriction endonuclease for 3 hours at 37°C. After purification with phenol/chloroform, the digest was then visualised on a 1% agarose gel against uncut plasmid. 1µg of linearized plasmid was used to produce a Digoxigenin 11UTP labeled anti-sense riboprobe: the reaction was set up with 4µl of 5x transcription buffer, 2µl DTT, 0.5µl RNASin, 2µl 10 xNTP and 2µl SP6 in a 20µl reaction volume.

The sections were dewaxed and hydrated, immersed in 0.02N HCl for 15 minutes at room temperature, washed in DEPC PBS, 3% TritonX 100 solution for 15 minutes at room temperature; followed by 100µg/ml Proteinase K digestion at 37°C for 25 minutes. The hAlbumin probe was applied to the sections and hybridized overnight at 42 °C using the Hybaid Omnislid chamber. Cover slips were used to prevent dehydration of the sections during the process.

Stringency washes were performed at room temperature using 2X SSC buffer for 30 minutes, followed by 0.1X SSC at 50 °C for 30 minutes. Alkaline Phosphatase conjugated anti-Digoxigenin antibody (1:200) was applied to the sections for 1 hour and washed off with Tris buffered saline. The probe was visualized using Nitro-Blue Tetrazolium/ Bromo-Chloro-Indolyl-Phosphate (NBT/BCIP) substrate and Levamisole. The slides were washed counterstained with Haematoxylin and mounted in Glycergel.

### 3.6 RNA extraction

200µl of serum/ plasma mixed with 600 µl of RNA lysis buffer, or 400µl of lysed tissue mixed with 200µl of RNA lysis buffer were combined with 1µl of tRNA

(10µg/µl), 600µl phenol, 100µl chloroform and 50µl of 2M CH<sub>3</sub> COONa and vortexed vigorously for 10minutes. The tubes (1.5ml) were then spun at 8000rpm for 15 minutes, after which the aqueous phase was transferred to a fresh tube with 600µl of chloroform and spun for 15 minutes at 8000rpm. The aqueous phase was then transferred to 600µl of propan-2-ol and the RNA was allowed to precipitate at -20°C overnight, after which the mixture was spun for 30 minutes at 14000rpm. The pellet was washed with 70% ethanol, dried, suspended in 20µl of DEPC treated water and stored at -70°C (Chomczynski and Sacchi, 1987).

### **3.7 Oligonucleotide synthesis and purification**

Oligonucleotides were made “in-house” using a Cruachem PS250 synthesizer, by Mr David Macnab and Mr Alex Orr at the Institute of Virology, University of Glasgow. The DNA was covalently linked on a column to an inert support. To remove the oligonucleotides from the resin, they were cleaved with molecular biology grade ammonia solution (1.5M) through a 5ml syringe, subsequently deprotected at 55°C for 5 hours and dried overnight in a centrifuge evaporator (Sarant “speedivac” concentrator). The pellet was resuspended in 100µl DEPC treated water.

### **3.8 Quantitation of DNA/RNA by spectrophotometry**

RNA and DNA were quantified by reading the optical density (O.D) at 260nm using a Beckman DU-62 spectrophotometer. The following formula was used to determine nucleic acid concentration:

Concentration (µg/ml) = O.D<sub>260nm</sub> x reading x dilution factor.

One O.D<sub>260</sub> = 50µg/ml Double stranded DNA, or 40µg/ml single stranded DNA or RNA or 20µg/ml for oligonucleotides(<30 bases in length)

### **3.9 Reverse Transcription (RT)**

100 units of M-MLV Reverse Transcriptase were used to synthesise first strand complementary DNA (cDNA) from either 3 µl of extracted RNA or 1µg extracted

RNA/ reaction mixture. The reaction mixture contained 4µl of 5x first strand buffer; 3mM MgCl<sub>2</sub>; 10mM DTT; 1mM each of dGTP, dATP, dTTP, dCTP; 40pmol of HCV specific antisense primer and 1 unit of RNAsin in a final volume of 20µl. Incubation was for 60 minutes at 37°C and 95°C for 10 minutes and subsequently stored at 4°C.

### **3.10 Nested polymerase chain reaction (PCR)**

#### **3.10.1 First round PCR**

The reaction mixture for the first round contained: 0.5 units of Taq DNA polymerase mixed with Taq start antibody (according to manufacturers instructions) in 2µl of 10x (Tris/KCl) buffer supplied by the manufacturer; 1.5mM MgCl<sub>2</sub> 0.2mM each dGTP, dATP, dTTP, dCTP; and 16pmol each of the outer pair of primers, VICTAG-1/ VICTAG-940 (Table 3.1) in a final volume of 20µl. The reaction was overlaid with 10µl of mineral oil to prevent evaporation and 4µl of cDNA product was then added. Amplification conditions are summarised in Table 3.1.

#### **3.10.2 Second round PCR**

1µl of first round product was amplified in a second round PCR using the same reaction mixture described above but using 16pmol of each inner primer VICTAG-3/ NCR4 (Table 3.1) in a final volume of 20µl. Taq start antibody was used to provide an automatic “hot start”. The PCR was performed on a Biometra TRIO Thermoblock. Amplification conditions are summarised in Table 3.1.

### **3.11 Agarose gel electrophoresis**

Analysis of PCR results was by electrophoresis of 10µl of PCR product with 2µl of loading buffer on a 1.5% agarose gel containing 50µl (1mg/ml) ethidium bromide. The gel was visualised under short wave UV light (254nm). The following methods were used by Roche Discovery laboratory as part of a collaboration with our laboratory.

<b>Primercombination</b>	<b>Number of amplification cycles</b>	<b>PCR Temperature /Time</b>
VICTAG-1/ VICTAG-940 First round PCR	1 30 30 30 1 pause	95 °C /4 minutes 94 °C / 30 seconds 55°C /40 seconds 70 °C/2 minutes 30 seconds 70°C/ 7 minutes 10°C/
VICTAG-3/ NCR4 Second round PCR	1 35 35 35 1 pause	94 °C /5 minutes 94 °C / 1 minute 55°C /1 minute 70 °C/1 minutes 70°C/ 5 minutes 10°C

**Table 3.1 Summary of nested HCV PCR conditions used in first round and second round amplification.**

### 3.12 Single round PCR

The reaction mixture contained 0.5units of Taq DNA polymerase in 5µl of 10x (Tris/KCl) buffer supplied by the manufacturer; 2.5mM MgCl<sub>2</sub>; 0.2mM each dGTP, dATP, dTTP, dCTP and 5 pmol of each pair of primers, #5 / #6, (table 3.2) in a final volume of 50µl. The reaction was overlaid with 10µl of mineral oil to prevent evaporation and 2µl of cDNA product was then added. Amplification conditions are summarised in Table 3.2. Analysis of the PCR product was performed as section 3.11.

The specificity of the amplification product was confirmed by Southern blotting analysis with a non-radioactive HCV probe.

<b>Primer combination</b>	<b>Number of amplification cycles</b>	<b>PCR Temperature /Time</b>
#5/ #6	30	95 °C / 30 seconds
	30	60°C /30 seconds
	30	70 °C/1 minutes
	pause	10°C/

**Table 3.2 Summary of single round HCV PCR conditions used by Roche Discovery laboratory.**

### 3.12.1 Southern blot analysis

Following electrophoresis the gel was soaked in denaturation solution for 30 minutes, washed in distilled water, after which it was soaked in neutralisation solution for 30 minutes. The gel was then capillary blotted overnight using 6 X SSC onto Hybond N (+) membrane, which was presoaked in 6 X SSC. On completion of blotting the membrane was dried and the DNA was crosslinked to the membrane using a stratalinker (Stratagene). The membrane was prehybridised at 60 °C for 30 minutes using 30ml prehybridisation mix containing: 100µl 10% SDS; 0.5ml 20X Block solution (ECL kit); 2ml 50 X Denhardts solution; 100µl 10 mg/ml sheared salmon sperm DNA; and 5ml 10 X SSC in a final volume of 10ml. The denatured fluorescein-labeled probe was added to the prehybridisation mixture and allowed to hybridise overnight at 60°C.

The probe was made using the random prime ECL kit with the linearised plasmid (pBSK9 containing 5'UTR, Core, E1 and E2 regions of HCV (Nucleotides 1-2545), using SP6 RNA polymerase, denatured for 5 minutes at 95°C and snap cooled for 2 minutes. The membrane was washed twice with two stringency washes: 1XSSC, 0.1 SDS and 0.1% XSSC, 0.1% SDS and blocked with blocking solution (ECL kit) for one hour, followed by a 30 minute incubation with 20µl of α-

fluorescein-HRP conjugated antibody (ECL kit). It was then wrapped in cling film with 20mls of detection mix (ECL kit) and exposed for autoradiography for 30 minutes at room temperature.

## CHAPTER 4

### Results and Discussion

#### 4.1 Background

The severe combined immunodeficient (SCID) mouse is capable of accepting xenografts from a range of species including humans and has been used to develop a number of human mouse chimeras (Krensky, 1997).

The aim of this project was to establish a reliable animal model for hepatitis C virus (HCV) by using human hepatocyte SCID chimera mice. The SCID human model for exoerythrocytic stages of *Plasmodium falciparum* was the basis for such a model (Sacci *et al*, 1992). Transplantation of human liver derived cell-lines or primary hepatocytes into SCID mice and infecting them either by *in vitro* or *in vivo* routes with HCV positive serum was the approach taken. The rationale rests on the hypothesis that these cells would attain a biologically functional state of differentiation *in vivo*, resulting in an increased sensitivity to HCV infection and acting as targets for HCV replication in an *in vivo* culture system. This would be assisted by a partial hepatectomy to stimulate primary hepatocyte proliferation (Michalopoulos, 1997). or inhibit excessive tumour development (Shiota *et al*, 1996). Intrasplenic transplantation of hepatocytes would allow transplantation of large numbers of cells where translocation of these cells would be localised to the portal triad of the liver. Other sites of transplantation were both renal capsules. The animals would then be sacrificed at weekly intervals or after six weeks, and tested for HCV at these time points.



There are a number of permissive liver derived cell-lines that have the potential for becoming infected with HCV under suitable *in vitro* culture conditions (chapter 1.2.1a). Of these, HepG2, Huh7 and WRL68 cell-lines were selected in addition to primary human hepatocytes taken from normal donors or HCV positive liver transplant recipients to develop this model. Tumour cell growth post-transplantation was verified histologically using haematoxylin and eosin (H&E) staining. Human albumin detection by *in situ* hybridisation and cytokeratin 18 staining with immunocytochemistry were used to confirm the presence of human derived liver cells after transplantation.

#### **4.2 Preparation of cells for transplantation**

To obtain a viable primary hepatocyte cell suspension, donor's liver tissues (left lobe) were perfused with University of Wisconsin solution prior to transportation. Perfusion was performed within 24 hours from explantation, with 4 different buffers (chapter 2.6.1).

Table 4.1 presents the viability of the primary hepatocytes prior to cryopreservation, estimated by trypan blue exclusion.

The number of cells obtained from cirrhotic HCV positive livers was reduced by one to two logs, however, their viability determined by trypan blue exclusion was relatively comparable to that obtained from normal livers. The tissue received from donor 4 was cirrhotic and difficult to perfuse. The low yield of cells from HCV positive donors could be attributed to cirrhosis of the tissue. This was also reported by Kusano *et al*, where the number of primary hepatocytes isolated from a cirrhotic liver was reduced with enzyme digestion perfusion method (Kusano *et al*, 1997). Cells isolated from donors 1, 2, 3 and 5 tested positive for HCV RT PCR and cytoplasmic preparations of these cells were positive for human albumin expression.

Donor	Donor HCV serology	Cell Viability (%)	Number of cells obtained	Method of freezing
1	Positive	92%	10 <sup>6</sup> /ml	NF
2	Positive	85%	10 <sup>6</sup> /ml	NF
3	Positive	95%	10 <sup>6</sup> /ml	C.F
4	Positive	Not Processed	Not Processed	Not Processed
5	Positive	87%	10 <sup>6</sup> /ml	C.F
6	Negative	90%	10 <sup>8</sup> /ml	C.F
7	Negative	98%	2.0x10 <sup>8</sup> /ml	C.F
8	Negative	98%	10 <sup>8</sup> /ml	C.F
9	Not done	99%	2.5x10 <sup>7</sup> /ml	C.F

Cell Freezer method: C.F

Nalgene freezing box: NF.

**Table 4.1 Perfused primary hepatocytes isolated from donor tissue, their viability, method of freezing and number of cells obtained.**

### 4.3 Surgery and Anaesthesia

In our hands a 40% hepatectomy had a perioperative mortality of 5% (9 of 192 animals). This was comparable to Doer *et al's* 4% mortality. Additionally, some animals died from anaesthetic exposure prior to surgery (2%), this was within the acceptable constraints of the animal project licence.

## 4.4 Transplantation and engraftment

### 4.4.1 HepG2 cells

HepG2 cells are hepatoblastoma cells that secrete 17 of the major human plasma proteins into cell culture supernatant; these include albumin, alphafetoprotein and plasminogen. They resemble liver parenchymal cells morphologically and are chromosomally abnormal  $\bar{X} = 55(50-56)$  with a distinctive rearrangement of chromosome 1 (Knowles *et al*, 1980).

A suspension of *in vitro* cultured HepG2 cells was either transplanted under the renal capsule and intrasplenically at a concentration of  $10^6$  cells per site or grown on inert silicon disks *in vitro* 3 days before transplantation. The disks were then implanted in the peritoneal cavity adjacent to the liver. The animals were inoculated with 50 $\mu$ l of (neat) HCV positive serum intraperitoneally (IP) one-week post transplantation. The results are presented in table 4.2 .

Transplantation route	Number of transplanted animal		HCV PCR result
	PH <sub>x</sub>	No PH <sub>x</sub>	
Spleen and kidney	18	1	Negative
Disk	11	ND	Negative

PH<sub>x</sub>: Partial hepatectomy      ND: Not done

**Table 4.2**      **Number of animals transplanted with HepG2 cells via two routes of cell transplantation and HCV RT PCR result.**

Of the 33 transplanted animals (including 3 controls), 29 had a partial hepatectomy. None of the animals were transplanted with *ex vivo* infected HepG2 cells. The controls for this experiment were two HepG2 transplanted animals

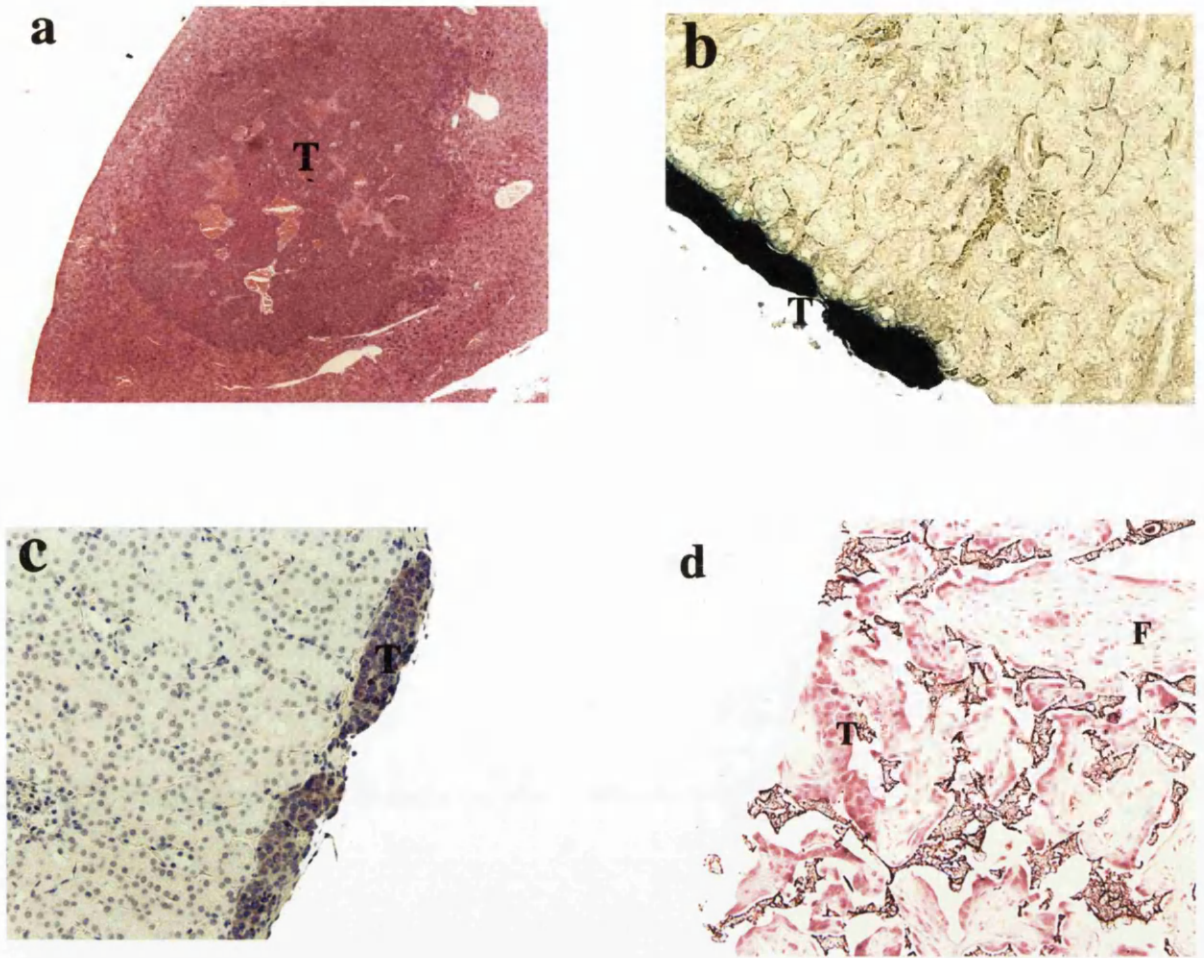
injected IP with negative serum (one did not have a partial hepatectomy) and one animal implanted with a blank disk.

None of the RNA isolated from all the transplanted animals was HCV RT PCR positive. Overall HCV RT results will be discussed in detail in section 4.4 (a).

#### **4.4.1(a) Pathology and histology**

Of the 19 animals directly transplanted with HepG2 cells, 17 developed well-defined tumour masses demonstrated by H & E staining under the renal capsule. All animals developed tumors in the spleen with satellite tumors generated around vessels and under the capsule in the liver. These were vascular, encapsulated and often “lobated”. Some tumors were haemorrhagic and necrotic. (figure 4.1a). The cells expressed albumin and were cytokeratin 18 positive; the former suggests that they had not dedifferentiated after transplantation, the latter indicating that they are epithelial in origin (figure 4.1b, 4.1c). The tumor cells resembled liver cells with prominent large nuclei that had a “ground glass” appearance. No metastasis was observed in the lungs or the myocardium in the transplanted animals; this is consistent with Victor *et al*'s observations in nude mice injected IP with HepG2 cells (Victor *et al*, 1999).

Transplanted inert silicon disks containing HepG2 cells became vascularised and in some animals were attached to the liver mass. The tumor cells had infiltrated the disk crevices which in turn, became infiltrated with the animal's fibrous tissue (figure 4.1d). However, HepG2 cells were not detected in any other tissue examined from these mice. None of the blank transplanted disks became vascularised and no mouse fibrous tissue was observed.



**Figure 4.1 HepG2 tumours in a transplanted SCID mouse**

- a) H&E staining of liver containing a tumour mass (T) with focal haemorrhage (x40 magnification).
- b) Subcapsular renal tumour mass stained by albumin *in situ* hybridisation (x100 magnification).
- c) Subcapsular renal tumour mass stained by cytokeratin 18 immunocytochemistry (x100 magnification).
- d) H&E staining of a silicon disk after implantation showing SCID fibrous tissue (F) and tumour infiltration (x200 magnification).

#### 4.4.3 WRL68 cells

The WRL68 cells are derived from human foetal, normal liver. They were registered as a patent (USA patent number 3 935066 in 1976) of human epithelial heteroploid liver cells by Apostolov (Apostolov1976). The patent stated: “that these cells form individually separated islands on discrete clumps when cultured in growth medium they have a morphology closely resembling human hepatocytes and their generation time is no more than 24 hours. They also increase production of glycogen in the presence of 1% glucose medium and could support viruses.” (Gutierrez-Ruiz, *et al*, 1994).

Gutierrez-Ruiz *et al* found that *in vitro* cultured WRL68 cells morphologically resemble hepatic primary cultures, secreting albumin and alpha-feto protein and preserving the activity of specific liver enzymes that include: alkaline phosphatase, gamma-glutamyl transpeptidase, aspartate amino transferase and alanine amino transferase (Gutierrez-Ruiz, *et al*, 1994).

The WRL68 cells used for transplantation were obtained from the European Collection of Animal Cell Cultures at passage number 92, stocks of this cell line were made and cryo-preserved. A suspension of these cells was transplanted intrasplenically and under the renal capsule at a concentration of  $10^6$  cells per site with or without performing a partial hepatectomy. Table 4.3 summarises the number of animals transplanted with WRL 68 cells, route of infection and RT-PCR results.

Of the 41 transplanted animals, 10 animals were transplanted with *in vitro* inoculated HCV cells and 22 animals were inoculated IP after transplantation. A partial hepatectomy was performed in 24 animals prior to transplantation. The RNA extracted from the spleen, kidney and liver was HCV RT-PCR negative.

##### 4.4.3 (a) Pathology and histology

Gross post mortem examination of two animals of 39 one-week post transplantation revealed tumour dissemination throughout the peritoneal cavity, distributed on the surfaces of organs. This was more pronounced in animals that had not been hepatectomised. Microscopic examination of the myocardium from 3 of 39 animals sacrificed 5 weeks post transplantation showed evidence of metastasis (figure 4.2 d).

Infection route	Number of animals		HCV PCR result
	PH <sub>x</sub>	No PH <sub>x</sub>	
<i>In vitro</i>	5	5	Negative
IP	14	8	Negative
Uninfected cells	5	4	Negative

PH<sub>x</sub>: Partial hepatectomy

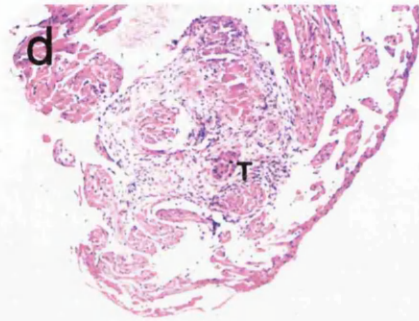
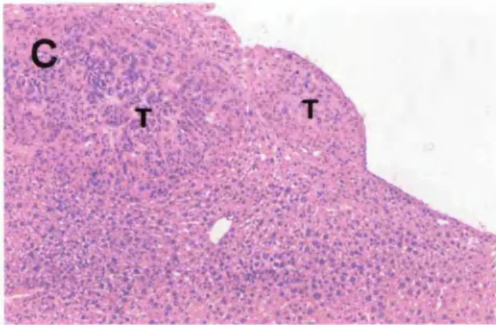
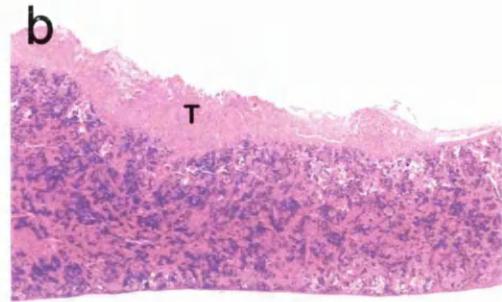
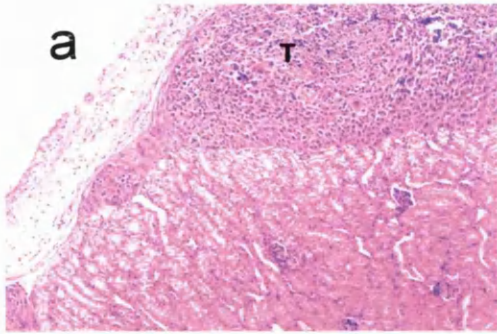
IP: intraperitoneal

**Table 4.3** Number of animals transplanted with WRL68 cells, route of infection and HCV RT PCR result.

Further, in most of the animals the tumour mass occupied around 20-30% of the spleen (figure 4.2 a) and 10-20% of the kidney (figure 4.2 b), spreading internally from the sup-capsular space. In the liver, the tumours were encapsulated with several satellite tumours (figure 4.2 c). The animals had swollen abdomens and were sacrificed before the final experimental time point (six weeks).

Tumours in all transplanted animals expressed cytokeratin 18, but failed to express human albumin or alpha-fetoprotein. Similarly, cytopsin preparations made from WRL68 cell suspensions did not stain for human albumin. The latter contradicts Gutierrez-Ruiz, *et al's* observations (Gutierrez-Ruiz, *et al* 1994). It is possible that our cells derived from a higher cell passage had become dedifferentiated; alternatively, our cells expressed these proteins in minute amounts and as a result were not detected.

Histologically, the tumour cells had a polygonal to spindle arrangement, exhibiting prominent round or oval nuclei. In some cells the cytoplasm appeared granular and dense.



**Figure 4.2 WRL tumours in a transplanted SCID mouse**

- a) H&E staining of WRL tumour spreading beneath the renal capsule (x100 magnification).
- b) H&E staining of a spleen with a large tumour (x40 magnification).
- c) Two WRL tumours in the liver (x40 magnification).
- d) WRL tumour metastasis in the myocardium (x40 magnification).

T: WRL tumour



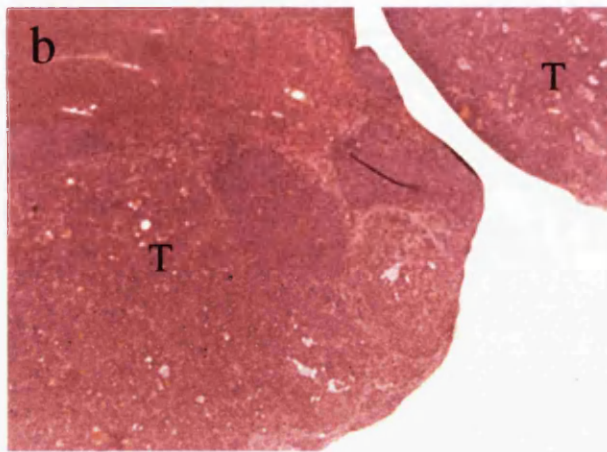
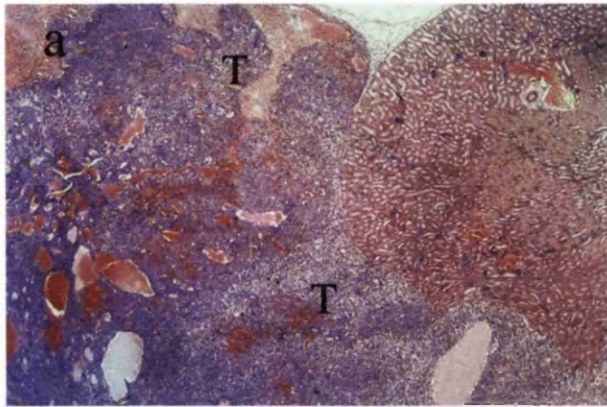
#### 4.4.4 Huh7 cells

The Huh7 cell-line was established from well-differentiated hepatocellular carcinoma tissue. The cells express a number of plasma proteins *in vitro*, including alphafetoprotein and albumin (Nakabayashi *et al*, 1982). When transplanted into nude mice through the portal vein, liver metastases were present in 80% of the animals (Shiota *et al*, 1996).

A suspension of these cells was transplanted intrasplenically and under the renal capsule at a concentration of  $10^6$  cells per site with or without performing a partial hepatectomy. However, no infection experiments were performed because the animals became unwell. Of the 20 animals transplanted 11 were sacrificed one week posttransplantation and the remaining animals were sacrificed 10 days post-transplantation.

#### 4.4.4 (a) Pathology and histology

The development of extremely large haemorrhagic tumours in the abdominal cavity was substantiated by gross postmortem examination of the animals. Microscopic examination of these tumour masses revealed large vascular, encapsulated tumours, which were haemorrhagic with extensive necrosis (figures 4.2 a, 4.2 b) occupying 50% of the organs examined, with a large number of mitotic figures. No metastasis was observed in the lungs or the myocardium. The cells resemble liver parenchymal cells morphologically with large prominent nuclei; they also expressed human albumin and cytokeratin 18.



**Figure 4.3 Huh7 tumours in a transplanted SCID mouse**

- a) H&E staining of a SCID kidney with large haemorrhagic and necrotic tumours (to the left of the figure) (x40 magnification).
- b) H&E staining of a SCID liver with 2 large tumours (x 40 magnification).

T: Huh7 tumour

#### 4.4.5 Primary hepatocytes

Hepatocytes are anchorage dependent cells, which are structurally and functionally polarized *in vivo*. When cultured on plastic, they attach poorly with a gradual loss of hepatocyte specific functions, such as albumin synthesis, and as a consequence soon die. Provision of an extracellular matrix would allow prolonged survival, continued expression of differentiated functions and preservation of a more normal, near-cuboidal cell shape. This could be achieved by transplantation of primary hepatocytes within the liver extracellular matrix, which intervenes between fenestrated endothelial cells and hepatocyte epithelium, thus allowing interaction between transplanted hepatocytes and non-parenchymal cells for example Kuppfer cells. A progressive decrease of albumin production *in vitro* in pure populations of hepatocytes has been observed; however, when co-cultured with endothelial cells, albumin production was maintained (Morin and Normand, 1986).

Syngeneic and allogeneic transplantation of primary hepatocytes in rodents has been successful. For example, syngeneic transplanted primary hepatocytes can survive up to 2 years in the spleen of a rat (Sawa, 1997). In the malaria mouse model, primary human hepatocytes can become infected with sporozoites of *Plasmodium falciparum* 4 months after transplantation (Sacci *et al*, 1992).

Hepatocytes used for transplantation were either from HCV positive donors or from normal donors. The spleen and renal capsules allow the transplantation of a large number of cells; this increases the chances of engraftment. The short-term elevation of hepatocyte growth hormone levels after a partial hepatectomy allows initial cell proliferation (Michalopoulos, 1997).

Perfused cells from donor 1 were cryo-preserved using a Nalgene freezing box. Their viability was comparable to that obtained after perfusion (90%). Cells obtained from donor 2 were transplanted within one hour after perfusion. All animals were partially hepatectomised prior to transplantation. The animals were sacrificed 6 weeks post transplantation; RNA was extracted from whole blood, and samples taken from the spleen, liver and kidney. The remaining tissues from

these organs were processed for human albumin, cytokeratin 18 and H & E staining.

Table 4.4 outlines the number of animals transplanted with HCV positive hepatocytes obtained from donors 1 and 2:

Donor No	No of transplanted animals	Histology results	HCV PCR results
1	6	Negative	Negative
2	4	Negative	Negative

**Table 4.4** Number (No) of animals transplanted with fresh HCV donor hepatocytes, or with cryo-preserved hepatocytes

No cells were detected in the spleen or the renal capsules in all animals examined and no HCV was detected. As a result, it was decided to transplant fresh hepatocytes after perfusion (up to 20 hours post perfusion) and sacrifice the animals at weekly intervals. Further, primary HCV negative hepatocytes would be transplanted after *ex vivo* infection with HCV positive serum (Roche Discovery), or after mixing with HCV negative serum. Two further animals transplanted with cells from donor 7 were not hepatectomised; the cells were either treated with negative or positive HCV serum. The effects of mixing with freezing medium and storing for 10 hours at 4 °C post-transfusion on cell viability was tested by transplantation of hepatocytes from donor 8. The remaining perfused cells were cryo-preserved using a cell freezer.

Table 4.5 summarises the number of animals transplanted with freshly perfused HCV negative primary hepatocytes obtained from donors 6,7, and 8.

Donor No	No of transplanted animals	Time of transplantation (Hrs) post-perfusion	Sacrifice time Intervals	No with PHx	Ex Vivo treated primary hepatocytes No of animals		
					HCV Positive serum	HCV Negative serum	Freezing medium
6	6	>10	1 week*	6/6	3	3	0
7	6	10	1 week *	4/6	3	3	0
8	4	>20	1 week or 10 days **	4/4	0	0	4

Duration of experiment:

\* 3 weeks

\*\* 10 days

PHx Partial hepatectomy

**Table 4.5** Number (No) of animals transplanted with fresh HCV negative donor hepatocytes, after treatment with either HCV positive serum, HCV negative serum or freezing medium

Donor Number	Number of transplanted animals	Time of transplantation (Hrs) post-perfusion	Sacrifice time	Number with PHx
3	3	3	1 week	3/3
3	3	24	1 week	3/3

PHx Partial hepatectomy

**Table 4.6** Number of animals transplanted with fresh HCV positive donor hepatocytes

No primary hepatocytes were detected in these animals from all donor livers and HCV PCR results were negative.

Fresh HCV positive primary hepatocytes were transplanted either at 3 or 24 hours post perfusion, and all the animals were hepatectomised (Table 4.6).

RNA was extracted from whole blood, and samples from the spleen, liver and kidney, were then sent to Roche laboratories for HCV RT-PCR testing (discussed in section 4.4 b). The remaining tissues from these organs were presented for H & E, human albumin and cytokeratin 18 staining.

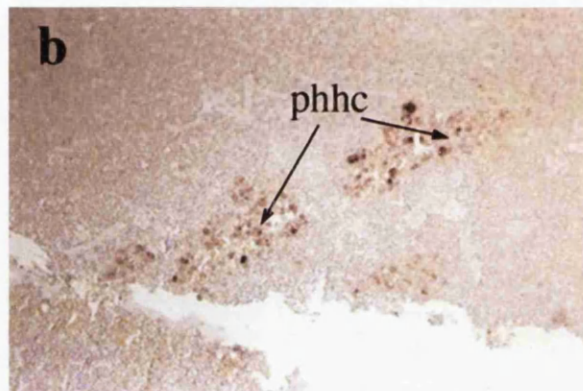
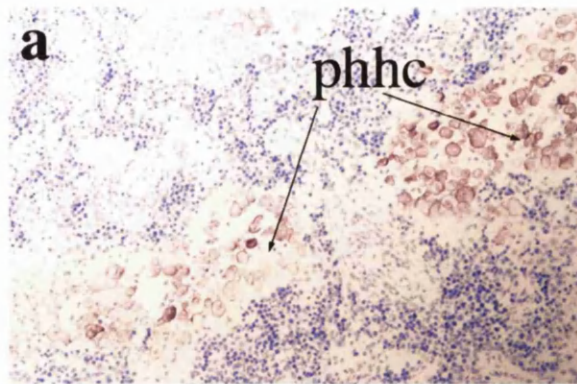
#### **4.4.6 Cause of primary hepatocyte absence**

The absence of primary hepatocytes in the spleen or under the renal capsule, one week post transplantation was investigated by addressing the following possibilities:

##### **4.4.6 a) Needle size and its effects on cell viability**

The question arose as to whether the gauge of the needle may be damaging the cells, thus leading to cell lysis during the transplantation procedure. A needle size of 27G had previously been used in the successful transplantation of WRL-68 and HepG2 cells; using larger gauge (> 25G) needles could be considered unacceptable as it could lead to excessive bleeding in the mouse.

An *in vitro* experiment to address this phenomenon was undertaken, where frozen aliquots of primary hepatocytes from donor 7 were thawed; their viability was >90%. Cells were passed through 27G, 25G and 23G needles (27G < 25G < 23G), incubated at 37°C in PBS and their viability tested again at times 0, 1 hour and 3 hours. Results summarized in Tables 4.7a, 4.7b and 4.7c



**Figure 4.4** Primary hepatocytes one hour posttransplantation in a SCID mouse spleen.

- a) Transplanted primary hepatocytes (phhc) stained by cytokeratin 18 immunocytochemistry (x200 magnification).
- b) Transplanted primary hepatocytes stained by albumin *in situ* hybridisation (x100 magnification).



Sample Treatment with needle	Cell Number/50 $\mu$ l	% Viability
Control (no treatment)	$3.8 \times 10^5$	87
27G	$2.2 \times 10^5$	82
25G	$2.2 \times 10^5$	90
23G	$2.2 \times 10^5$	89

**Table 4.7 (a) Viability of primary hepatocytes at time 0 after treating the cells with different size needles**

Sample Treatment with needle	Cell Number/50 $\mu$ l	% Viability
Control (no treatment)	$4.0 \times 10^5$	91
27G	$2.5 \times 10^5$	61
25G	$2.8 \times 10^5$	92
23G	$2.7 \times 10^5$	92

**Table 4.7 (b) Viability of primary hepatocytes after 1 hour**

Sample Treatment with needle	Cell Number/50 $\mu$ l	% Viability
Control (no treatment)	$2.4 \times 10^5$	88
27G	$0.9 \times 10^5$	66
25G	$2.0 \times 10^5$	87
23G	$2.3 \times 10^5$	81

**Table 4.7 (c) Viability of primary hepatocytes after 3 hours**

All the cells had a comparable viability after passing them through the different needles prior to incubation, however, the viability of cells treated with needle size of 27G was reduced to 61% one hour post-incubation, suggesting possible cell lysis. After 3 hour incubation all the cell viabilities remained relatively comparable to those after one hour incubation.

To assess the cell's response to different needle gauges *in vivo* an experiment was also undertaken where cryo-preserved HepG2 and cryo-preserved hepatocytes from donor 7 were transplanted in 5 mice, using 27G (only used in one animal), 25G or 23G needles. The mice were sacrificed 1 hour after surgery. Tissues were stained for H & E, albumin and cytokeratin 18.

The transplanted primary hepatocytes were only detected in the spleens of each animal by cytokeratin 18 and albumin staining (Figures 4.4 a and 4.4 b); irrespective of the needle used. Staining of the kidneys for albumin and cytokeratin 18 was negative. It is possible that some of the transplanted hepatocytes might have leached out during transplantation under the renal capsule. Both the spleen and kidneys stained positive for albumin and cytokeratin 18 in the mice transplanted with HepG2 cells; the 23G needle was not used to transplant HepG2 cells.

#### **4.4.6 b) Transport medium: PBS versus medium.**

Hepatocytes are metabolically active cells that require growth factors and hormones to survive. Epidermal growth factor (EGF) has wide ranging mitogenic activities on cells in culture. Richman *et al* were the first to show that EGF, especially in combination with insulin and glucagon, could stimulate DNA synthesis in isolated hepatocytes maintained in monolayer culture (Richman *et al.*, 1976).

As all cells had previously been transported in PBS and the surgery can take up to 4 hours, we decided to test whether medium (DMEM, 1%, Bovine serum albumin 0.02µg/ml Epidermal growth factor, 10U/ml Insulin) rather than PBS would better sustain the cells prior to transplantation. Cryo-preserved cells from HCV negative donors were suspended in medium or PBS and incubated for 5 hours at 37°C (Table 4.8). The cell viability was reduced in the presence of PBS after a 5 hour incubation.

Primary hepatocyte source	% Cell viability in	
	PBS	medium
donor 6	27	>90
donor 9	40	>90
donor 7	0	88
donor 8	0	86

**Table 4.8 Viability of different Primary hepatocytes suspended in either PBS or medium after a 5 hour incubation**

#### 4.4.6 c) Survival of cryo-preserved primary hepatocytes *in vivo*.

To investigate the influence of transport medium on *in vivo* survival time, 8 mice were transplanted with primary hepatocytes from donor 9 using a 25G needle. They were sacrificed at 1, 3, 24, 48 hours and 1-week intervals; the spleen, liver and kidneys were stained for cytokeratin 18 and human albumin, results are summarized in Table 4.9

Cells from animals sacrificed one and three hour post transplantation were positive for albumin and cytokeratin 18 staining in both kidneys. Cells from animals sacrificed one hour post transplantation were also detected in the spleen. However, in animals sacrificed at 24, and 48 hour post transplantation, primary hepatocytes only stained for cytokeratin 18 in the kidney (Figure 4.5). The absence of cells in the spleen could be due to the cells being translocated to the liver. The lack of albumin staining could either be attributed to the fact that the cells are in an acute phase response resulting in down regulation of albumin production (Selden *et al*, 1999), or that mRNA has degraded because the cells are dying. Cytokeratin 18 stain is more robust than albumin as it is a cytoskeletal stain, explaining it's detection at 48 hours posttransplantation. However, no primary human hepatocytes were detected in the animals sacrificed at one-week.

No. of mice	Time of sacrifice (Hrs)	Cytokeratin 18 (ICC)			Albumin (ISH)		
		Spleen	Liver	Kidney	Spleen	Liver	Kidney
1	1	Pos	Neg	Pos	Pos	Neg	Pos
1	3	Neg	Neg	Pos	Neg	Neg	Pos
2	24	Neg	Neg	Pos	Neg	Neg	Neg
2	48	Neg	Neg	Pos	Neg	Neg	Neg
2	168	Neg	Neg	Neg	Neg	Neg	Neg

Hr Hours

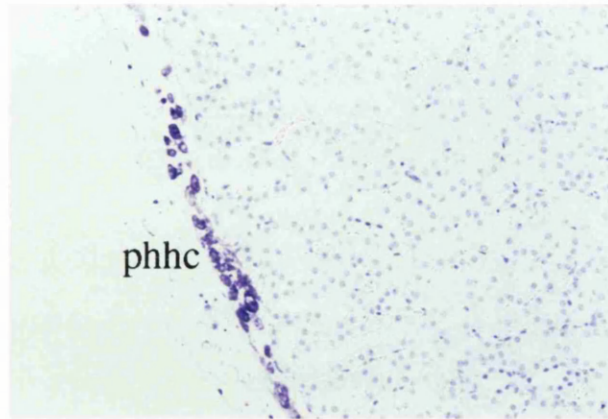
ICC Immunocytochemistry

Pos Positive

ISH *In situ* Hybridisation

Neg Negative

**Table 4.9 Summary of albumin and cytokeatin 18 staining of primary hepatocytes from donor 9**



**Figure 4.5** Primary hepatocytes 48 hours post transplantation in a SCID mouse

Cytokeratin 18 immunocytochemistry of primary hepatocytes (phhc) transplanted in the kidney subcapsule (x200 magnification).

To test the survival rate of cryo-preserved hepatocytes obtained from different donors (5, 6 and 7,) a similar experiment was conducted using 8 animals, the animals were sacrificed at 1, 3, 24, 48 hours 4 and 6 day intervals.

Similar results to those above were observed in animals transplanted with cells from donor 7. Surprisingly, animals transplanted with cells from donors 5 and 6 were negative for both albumin and cytokeratin 18, suggesting that these cell's viability was somehow compromised after transplantation

#### **4.4.6 d) *In vivo* survival of transplanted human hepatocytes after macrophage depletion**

Macrophages have been implicated as the main effector cells, as judged by F4/80 immunocytochemistry, in xenograft rejection of porcine islet cell clusters after transplantation under the kidney capsule of normal C57BL6 mice (Karlsson-Parra *et al* 1996). Lin *et al* elucidated the pathogenesis of delayed xenograft rejection in the transplantation of hamster heart to leflunomide-treated rats and attributed to macrophage activation by xenoantigens in the absence of NK cells and xeno-antibodies (Lin *et al* 1997). Further, previous unpublished data have indicated that primary foetal hepatocytes are destroyed by macrophages in nude mice (personal communication, F. Prince, the Lindsley F. Kimball research of New York Blood Centre). Thus, macrophage ablation may enhance the survival of primary human hepatocytes in this model.

Macrophage depletion with the purpose of facilitating engraftment of allogeneic and xenogeneic bone marrow grafts has been demonstrated with silica (Lotzova *et al.* 1974), where silica abrogates or weakens resistance to parental, allogeneic and rat marrow grafts. Recently, *in vivo* macrophage depletion has been achieved by the administration of liposomes containing dichloromethylene diphosphate (DMDP). Such liposomes are injected by macrophages and after disruption, their contents will kill these cells effectively. The route of administration of the liposomes will determine the population of macrophages

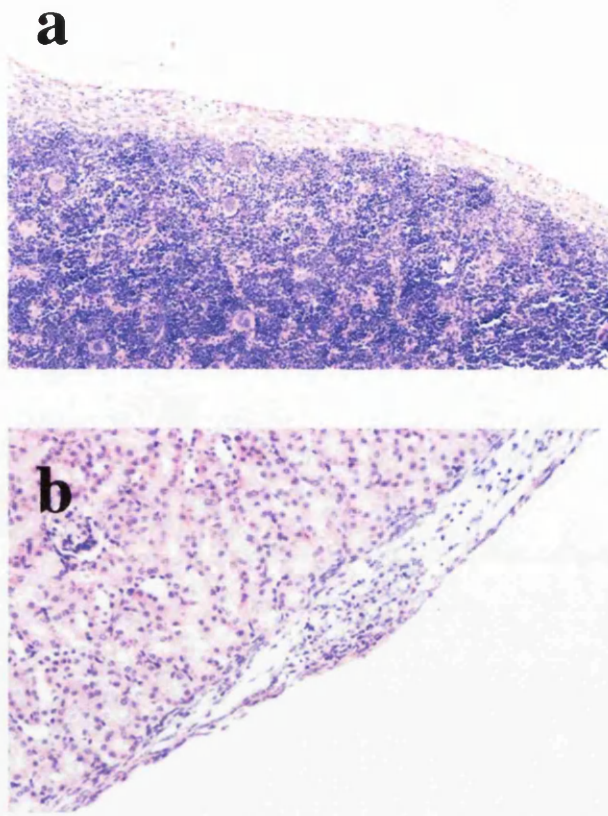
removed; for example: intravenous injection mainly eliminates phagocytic cells in the spleen and the liver (Terpstra *et al*, 1997).

DMDP is more effective than silica, which does not appear to completely eliminate the macrophages. Unfortunately, it is highly toxic to the mice, so increasing the severity band of the project license. Therefore macrophage depletion by DMDP was not attempted.

To test the effectiveness of silica, 8 animals were used and treated with or without silica. After partial hepatectomy and transplantation of cryo-preserved primary hepatocytes (donor 8), 0.2ml (38.5mg/ml) of silica was administered to the peritoneal cavity of a group of 4 mice. To compare its effect, a control group of 4 untreated mice was sacrificed concurrently at 24, 48 (one mouse from each group at each time point) and remaining two mice from each group, 72 hour intervals post-transplantation. Tissues were stained for H & E, albumin and cytokeratin 18; and to detect the presence of macrophages, the F4/80 antibody was used.

Surprisingly, no primary hepatocytes were detected in both groups at 24 hours post transplantation, as determined by albumin and cytokeratin staining. However, gross examination of the abdominal organs showed thickening of the capsular wall compared to normal mice. This was confirmed microscopically, with H&E staining showing “thickening” of the capsular membrane in the spleen and kidney in the silica treated animals indicating a possible inflammatory response to the silica (figures 4.6a, 4.6b).

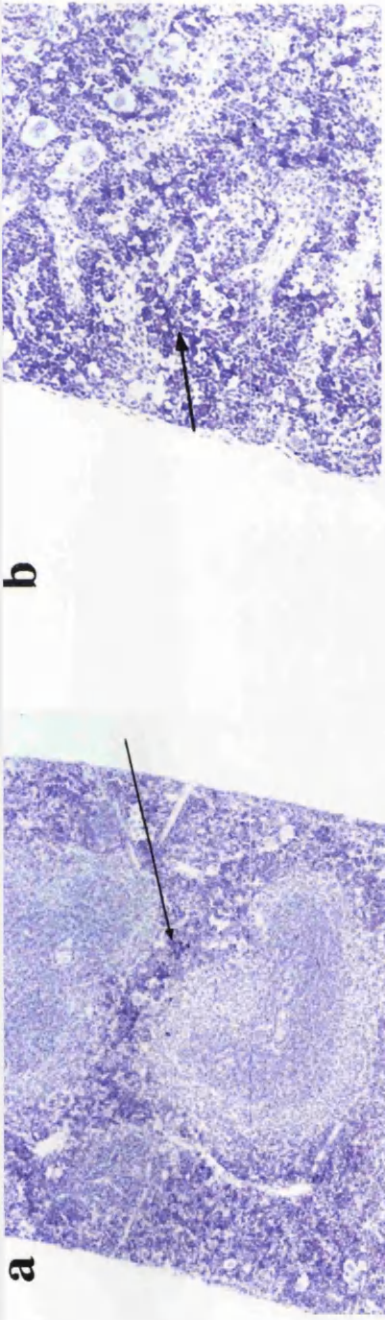
The F4/80 antibody staining, which will identify red pulp macrophages (Austyn and Gordon 1981), is demonstrated in normal mouse spleen (Figure 4.7a) and SCID mouse spleen (Figure 4.7b). In the animals treated with silica and sacrificed 24 and 36 hours post transplantation there was substantial abolition of macrophages in the spleen (4.7c). However, after 72 hours, the macrophages reappeared (4.7d). The application of silica therefore did not affect the unsuccessful outcome of transplantation of primary hepatocytes from donor 8.



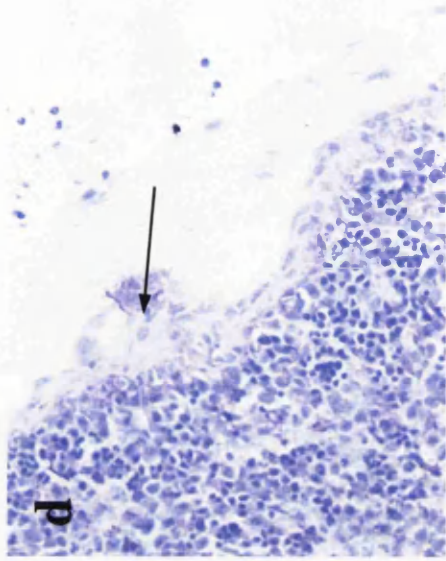
**Figure 4.6** "Thickening" of the capsular membranes of spleen and kidney post silica treatment of a transplanted SCID mouse

- a) H&E staining of a spleen from a SCID mouse treated with silica (x100 magnification).
- b) H&E staining of a kidney from a SCID mouse treated with silica (x200 magnification).

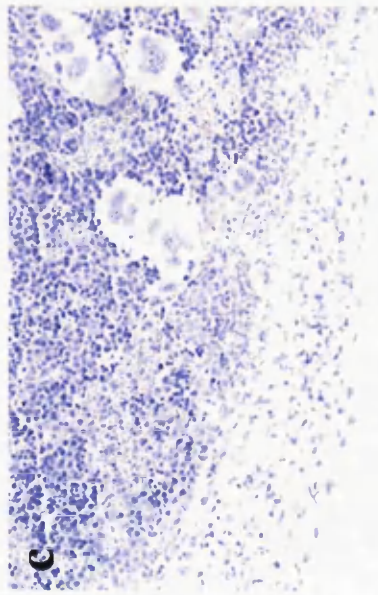




**a**



**b**



**c**

### Figure 4.7

#### Macrophage depletion in silica treated mice

- a) F4/80 immunocytochemistry of a normal mouse spleen (x40 magnification).
- b) F4/80 immunocytochemistry of a SCID mouse spleen (x100 magnification).
- c) F4/80 immunocytochemistry of a SCID mouse spleen 24 hours post silica treatment (x200 magnification).
- d) F4/80 immunocytochemistry of a SCID mouse spleen 72 hours post silica treatment (x400 magnification).

Arrow indicating macrophage position.

#### 4.4.6 e) Growth stimulants: priming before implantation

The rationale for performing a partial hepatectomy was to stimulate liver regeneration in the mouse thus releasing active hepatocyte growth factor (HGF) and other mitogens including interleukin-6 (IL6) (chapter 1.3), into the blood stream and stimulating DNA synthesis in the transplanted hepatocytes by binding to the *c-met* receptor. This in turn would allow the transplanted cells to become established. However, the question arose as to whether there were sufficient growth stimulants to sustain the transplanted primary hepatocytes and maintain them in a biologically functional state of differentiation.

The administration of recombinant HGF by IP injection could resolve this problem, but it would be too costly, in the region of £1000/mouse.

The use of an agonist against *c-met* receptor that will activate it and evoke DNA synthesis and cell proliferation is another approach. Prat *et al* demonstrated agonistic activities of monoclonal antibodies against the extracellular domain of HGF-R (*c-met*) triggering biological responses required for invasive growth (Prat *et al.* 1998). They postulated that ligand-induced oligomerisation was the most likely mechanism responsible for activation of growth factor receptors (Prat *et al.* 1998).

An antibody that had been raised against human HGF-R, which does not cross react with mouse HGF-R, which would preferentially stimulate the transplanted primary human hepatocytes was made available by Dr Ken Hillan from Genentech, Inc., South San Francisco, California, to test in this model. 50µg of anti-*met* antibody was mixed with  $3 \times 10^6$  cells from donor 8 prior to transplantation and a group of 8 mice were sacrificed at intervals of 3, 6, 10 days, 3, 4 and 5 weeks; (Table 4.10).

Unfortunately, no primary hepatocytes were detected in all the transplanted animals. This could be attributed to lack of binding of the antibody to donor 8 cells, or that the animals macrophages prevented engraftment.

No. of animals	Time of sacrifice	Albumin (ISH)	Cytokeratin 18 (ICC)
2	3 days	Negative	Negative
2	6 days	Negative	Negative
2	10 days	Negative	Negative
2	3 weeks	Negative	Negative
2	4 weeks	Negative	Negative
2	5 weeks	Negative	Negative

ISH *In situ* hybridisation

ICC immunocytochemistry

**Table.4.10 Summary of the effects anti-*met* antibody in SCIDS**

Priming the cells with interleukin-6 (IL6) in the presence of EGF prior to transplantation, and combining this with the anti-*met* antibody, was another approach adopted, for IL6 results in hepatocyte proliferation *in vitro* (Kuma *et al* 1990), and might encourage cell proliferation prior to transplantation. To address the possibility of macrophage inhibition, silica was used during transplantation. Using a 25G needle and in the presence of DMEM with EGF (0.02µg/ml), primary hepatocytes from donor 7 were mixed with either anti-*met* antibody, IL6 (1000 U/10<sup>7</sup>cells) or silica; the animals were sacrificed at either 5 or 10 day intervals (Table 4.11). However, despite of the use of silica no hepatocytes were detected in the transplanted animals.

Number of animals/group	Treatment	Time of sacrifice days	Cytokeratin and albumin staining
2	anti- <i>met</i> antibody, IL6 and silica	5&10	Negative
2	anti- <i>met</i> antibody, IL6 without silica	5&10	Negative
1	anti- <i>met</i> antibody, and silica	5	Negative
1	anti- <i>met</i> only	5	Negative

IL6 interleukin-6

**Table 4.11 Summary of the effects Interleukin-6 and anti-*met* antibody in the presence of silica in SCIDS transplanted with hepatocytes from donor 7**

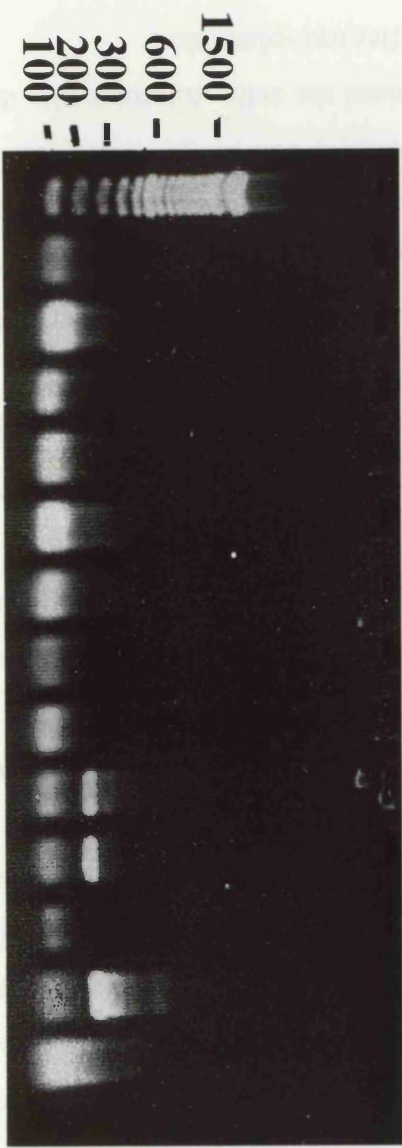
## 4.5 RNA and RT-PCR analysis

### 4.5.1 Nested RT PCR

For consistency, Chomczynski and Sacchi's method for RNA extraction was used (Chomczynski and Sacchi, 1987), on all samples. The RNA of tissue samples was quantified spectro-photometrically and 1µg was used to synthesise HCV cDNA with the Vtaq primer for the 5'UTR region; but for 200µl serum/plasma samples, 3 µl of extracted RNA was used. The 5'UTR region was chosen because it is highly conserved among different HCV isolates (Smith *et al* 1995). To test the sensitivity of the extraction and nested RT PCR methods, the Pelispy HCV-RNA run control at two dilutions, 1/5 and 1/10 were used. The Pelispy sample, is a weak positive plasma standard for the validation of HCV-RNA amplification test runs. It contains 3800 genome equivalents /ml and was obtained from Central Laboratories of the Netherlands Red Cross Blood Transfusion service. Alternatively, HCV positive serum at a 10<sup>-4</sup> dilution was used. Additionally, RNA was extracted from 10<sup>6</sup> primary HCV positive hepatocytes with each extraction run. Figure 4.8 is an example of a typical RT PCR run. All SCID tissue RNA was negative, both the Pelispy dilutions were positive; thus the sensitivity of the nested PCR run was around 100 genome equivalents per reaction.

### 4.5.2 Single round PCR

This was performed as part of a collaboration with Roche Discovery Laboratories, Welwyn Garden City (Adina Ochert) RNA samples from experiments in section 4.3 were tested. Extraction controls were included; an RNA sample from 10<sup>6</sup> HCV positive primary hepatocytes and an HCV positive serum (10<sup>4</sup> dilution). The sensitivity of this method was around 200 genome equivalents/ reaction as estimated by titration of plasmid RNA. However, only 3 of the 10 positive controls were positive, suggesting that the results were at the limits of this method's detection.



- 100bp DNA ladder
- HCV negative serum
- SCID (LV sample)
- SCID (SP sample)
- SCID (KD sample)
- HCV negative serum
- SCID (WB sample)
- PCR negative control
- HCV negative serum
- Pelispy Control (1/10) dilution
- Pelispy Control (1/5) dilution
- PCR negative control
- HCV positive primary hepatocytes
- HCV negative control

**Figure 4.8**  
**Ethidium bromide stained 1.5% (1X TBE) agarose gel illustrating nested HCV RT PCR (5'UTR region)**

RNA was extracted from: liver (LV), spleen (SP), kidney (KD), and whole blood (WB) of an animal implanted with HCV positive primary hepatocytes obtained from donor 1.  
 The positive controls were 2 Pelispy sample dilutions and HCV positive primary hepatocytes.

Since there was no evidence of human material transplanted in the SCID mice, the PCR data are in accordance with the histology data.

#### 4.6 Summary of results

The liver derived cell lines used in this model were successfully transplanted in SCIDs with out the loss of albumin production, and by inference loss of differentiation. However, these cells have several drawbacks, for some produce extremely large metastatic tumours that are hemorrhagic resulting in the animals death. Inhibition of these tumours by the production of HGF as a result of a partial hepatectomy was ineffectual. Allied to this, these cells were not susceptible to HCV infection in an *in vivo* culture system, irrespective of the route of infection, viral titre ( $10^7$  genome equivalents/ ml of serum) and source of the infectious serum. Consequently, they are not suitable candidates for the production a HCV animal model. Using inert silicon disks as a means of introducing cells *in vivo*, in theory, offers an alternative replacement. For, in this model these disks became vascularised with infiltration of host fibrous tissue. But the cell's proliferation was not as pronounced as direct cell transplantation over the experimental time scale. As a consequence this was not a realistic substitute. Primary human hepatocytes (PHHC) did not successfully engraft after transplantation. To date PHHCs were not detected one week post transplantation however, similar results have recently been demonstrated in nude mice by Kusano *et al* (Kusano *et al*, 1997). Unlike the malaria mouse model (Sacci *et al*, 1992), PHHCs survive for only 48 hours post transplantation as was demonstrated by cytokeratin 18 staining. Results from the anti-*met* antibody, IL6 and silica experiments should in theory demonstrate an increase in the survivability and proliferation of the PHHCs *in vivo*, but this was not the case. It is possible that the perfusion and freezing processes might have some how compromised the cells. Alternatively, the animal's NK cells may have destroyed the cells after transplantation.

## CHAPTER 5

### General Discussion

The difficulties associated with establishing an *in vivo* HCV model are, to a certain extent, linked to the availability of a reliable source of cells that would support HCV replication. *In vivo* transplantation of hepatocyte derived cells may allow them to attain a “biologically functional” state of differentiation, increasing their susceptibility to HCV. Ideally, a non-metastatic cell-line with attributes comparable to primary hepatocytes should be used. This could be achieved by, for example, simian virus 40 (SV40) transformation (Nakamura *et al* 1997). The absence of a cheap and accessible HCV animal model, and the lack of a suitable *in vitro* repeatable culture system are reasons for attempting this project.

Several approaches were attempted at developing an animal model for HCV. The following could explain the unsuccessful outcome of this project:

#### 5.1 Serum infectivity and hepatocyte derived cell susceptibility *in vivo*

The absence an HCV PCR signal in transplanted animals could partly be attributed to a low viral titre or a lack of infectious virus in the inoculum. But the sera used in this model were from two HCV RNA positive patients, one of whom, had chronic HCV, the other was a Sacramento blood donor with cryoglobulinemia / macronodular liver disease. The latter also had a titre of  $10^7$  genome equivalents /ml of serum and produced a positive strand HCV RNA signal after *in vitro* inoculation of Huh7 and WRL68 cells and for the duration of 4 weeks (personal communication, Adina Ochert, Roche Discovery, Welwyn Garden City). However, negative strand RT PCR was not performed on these cultured cells or supernatant to confirm viral replication.

Non-infectivity of RNA positive sera has been previously reported. Lanford *et al* screened a number of sera from chronically infected individuals,



using chimpanzee primary hepatocytes and found many non-infectious. Further, their infectivity did not correlate with PCR titres (Lanford *et al*, 1994). Similarly, Fournier *et al* detected both negative and positive strand HCV RNA in 10 of 33 primary hepatocyte cultures infected with 33 different HCV positive sera. They also noted a high titre in the inoculum is necessary but not sufficient for *in vitro* infection of primary hepatocytes (Fournier *et al*, 1998). Rumin *et al* (1999) reported that different virus variants can replicate in primary hepatocytes irrespective of the viral titre, but sera displaying low anti-E2 antibodies or no neutralisation of binding titres may be more infectious *in vitro* (Rumin *et al*, 1999). Therefore, the presence of neutralising antibodies to specific domains in the viral envelope region could abrogate the infectivity of an inoculum and may explain this anomaly. Alternatively, the sera may have contained lymphotropic HCV isolates. Tropism to lymphocytes though controversial, has been reported (Chapter 1.2.1), and might explain the absence of infectivity of the two sera used in this animal model.

Intrinsic resistance to HCV infection of the transplanted cells could also explain the absence HCV replication. HCV replication was only achieved in the presence of DMSO or and normal human serum, both favouring long-term maintenance of highly differentiated hepatocytes (Rumin *et al*, 1999). Virus attachment and subsequent replication might be dependent on the cellular replicative cycle. For example, reovirus infection of murine hepatocarcinoma cells *in vivo* is correlated with cellular division, as virus antigen is only present in dividing cells (Taterka *et al*, 1994). On the other hand hepatitis B virus replication in transfected hepatoblastoma cells, is enhanced in quiescent hepatocytes (Ozer *et al*, 1995). The transplanted hepatocyte derived cells in this model metastasised and did not support HCV. It is possible therefore, that HCV will only attach and replicate in quiescent cells. However, HCV core protein localization in the cytoplasm of transfected HepG2 cells, is not cell cycle dependent (Barba *et al*, 1997). Ideally a panel of HCV positive sera should have been tested for the production of negative strand HCV RNA on the selected cell-lines in an *in vitro* culture system. This was not attempted. Further, these cells could behave

differently *in vivo*, de-differentiating and losing their ability to sustain HCV. The expression of the albumin gene mRNA, a liver specific marker of functional activity and differentiation, was sustained in for example the HepG2 cells after transplantation, but this is only one gene of a potentially large number confirming liver specific activities. Because CD81 has been implicated as the site of HCV binding, the lack of expression for this possible HCV receptor on the tumour cells *in vivo*, might also explain the lack of a PCR signal. Interestingly, the cell-line Huh7 expresses CD 81 receptor while the cell-line HepG2 lacks the expression of this receptor *in vitro* (personal communication from Dr Arvin Patel at the Institute of Virology, MRC Virology Unit, Glasgow 1999).

## 5.2 *In vivo* primary hepatocyte viability and engraftment

Albumin expression mRNA in sequential histological sections from the transplanted animals was used to establish cell viability and successful engraftment in this model. However, perfusing primary hepatocytes and separating them from the cell matrix could lead to an acute phase response, resulting in down regulation of albumin production (Selden *et al*, 1999) and might explain the loss of the albumin signal *in vivo* as early as 24 hours post-transplantation (Chapter 4.35 c). Cytokeratin 18 staining was used to confirm the presence of primary hepatocytes and display their morphology. However, a more precise measurement of hepatocyte viability, *in vivo* and after cryo-preservation was warranted.

One approach could involve testing the maintenance of the hepatocyte enzyme system, cytochromes P450, which serve as electron acceptors and mono-oxygenases in electron transport systems (Wang *et al* 1997). This could be performed on *in vivo* extracted mouse RNA samples using an RT-PCR method to test the mRNA for three P450 cytochrome enzymes: 2E1, 1A2 and 3A4 (Finnstrom *et al* 1999). This could also be used on the cryo-preserved hepatocyte batches, retrospectively acting as a back up for the trypan blue exclusion test and as a result might help define the reasons for the absence of cells 72 hours post-transplantation. An initial selection on the basis of viability could be performed

prior to transplantation, allowing transplantation of robust cells. Confirmation of retrospective successful engraftment of transplanted cells can also be achieved with PCR amplification of human *Alu* Sb2-containing loci using primers R16A/6 corresponding to the 5' end of the *Alu* consensus sequence and the INS2 Sb2 specific oligonucleotide primer (Ziekiewicz *et al*, 1994).

### **5.3 Possible mechanisms of primary hepatocyte rejection in this model**

The maximum survival time of primary hepatocytes in this model was limited to 72 hours post-transplantation; this was irrespective of the pre-treatment of the cells prior to transplantation (Chapter 4.3.5). Similar results have recently been demonstrated in nude mice by Kusano *et al* suggesting a reduction in growth factor production especially, hepatocyte growth factor 72 hours post partial hepatectomy (Kusano *et al*. 1997), (Chapter 1.3). The use of albumin-urokinase (Alb Upa) transgenic mice, in which a functional liver deficit created by hepatocyte-targeted expression of a hepatotoxic transgene, and results in a chronic stimulus for liver growth, was, explored (Rhim *et al* 1994). But most Alb Upa colonies are infected with *Helicobacter hepaticus* (personal communication from Mr Colin Hughes, Biological services, University of Glasgow), which is a naturally occurring pathogen causing chronic hepatitis and liver cancer (Ihrig *et al* 1999).

Treatment of the animals with silica to nullify or weaken the impact of the mouse's macrophage cells, though successful was ineffectual in our model in protecting transplanted human hepatocytes.

Peripheral natural killer (NK) cells have been implicated in marrow allograft rejection in SCID mice (Murphy *et al*.1987) .The fact that liver NK effector cell functions become temporarily suppressed during liver regeneration will, in theory, protect regenerating hepatocytes (Vujanovic *et al*. 1995) but only in the liver. Since the sites of transplantation were the spleen and the renal capsules, it is possible that peripheral NK cells could have contributed to the primary hepatocyte lysis; via apoptosis and primary necrosis *in vitro*. Interleukin -2 activated rat NK cells were shown to be fully equipped to induce both

apoptosis and necrosis in primary hepatocyte (Blom *et al*, 1999). *In vivo* depletion of NK cells by anti-NKR-P1 monoclonal antibody could remove these cells augmenting liver regeneration following a partial hepatectomy (Vujanovic *et al*. 1995). Alternatively, SCID/Beige mice could have been used for these animals intrinsically have low NK cell activity (Mosier *et al*, 1993). The latter approach was not pursued, as it was difficult to obtain these animals in the time constraints of the project.

One other explanation for hepatocyte rejection was that host granulocytes lyse the cells after transplantation. Olszewski *et al* found host granulocytes responsible for the rapid destruction of transplanted hepatocytes in syngeneic and allogeneic recipient rats. As granulocytes were found surrounding intraperitoneally infused hepatocytes after 2 hours, where the hepatocytes had a pyknotic appearance; 24 hours later all the hepatocytes were lysed. A similar reaction was observed in subcutaneously transplanted hepatocytes. Additionally, granulocytes were found to be cytotoxic to hepatocytes *in vitro* (Olszewski *et al*, 1996). This could offer a plausible explanation for the rapid disappearance of the transplanted hepatocytes in our model, as Olszewski *et al* postulated that isolating cells from a solid organ, like the liver, might damage their membrane during the isolation procedure. This in turn and the release of intracellular chemical substances could act as a “chemo-attractant”, mobilizing the host granulocytes (Olszewski *et al*, 1998). Circumvention of transplanted primary hepatocytes from the animal’s immune system is therefore essential to allow prolonged survival of these transplanted cells.

Encapsulation of these cells in semi-permeable membranes is one approach, for it allows isolation from the host’s immune system while permitting metabolic exchanges. This has been demonstrated by Wen *et al* where human hepatocytes were encapsulated in semi-permeable hydrogel based hollow fibres and implanted in the peritoneal cavity of Lewis rats. Human albumin presence was demonstrated in rat sera as assessed by Western blot and by ELISA. Immuno histochemical staining for albumin of the encapsulated cells was also demonstrated after 28 days (Wen *et al*, 1998). Micro-encapsulation of hepatocyte

derived cell-lines could also protect the host from the metastatic attributes of these cells. This is one approach that has not been attempted in SCID mice and might be a valuable tool for the development of a HCV animal model.

To date there has not been a successful SCID/ human chimeric HCV animal model developed using either PHHC or hepatocyte derived cell line's transplantation techniques. However, Ohashi *et al* recently demonstrated the successful transplantation and subsequent infection of human primary hepatocytes in non-obese diabetic (NOD)/ SCID mice with HBV and hepatitis delta virus (HDV). The hepatocytes were transplanted in a matrix (matrigel), under the kidney capsule and an agonistic antibody against *c-met* was administered IV every 2 weeks. The cells were infused with  $10^8$  HBV genome equivalents by injection of equal amounts into the transplant and intravenously. HBV infection was demonstrated by Southern blot analysis of HVB DNA replicative forms, immunohistochemical staining for HBsAg and HBcAg of the transplanted cells and by serial inoculation of other transplanted mice after 10 weeks from the original inoculation. Immunohistological examination of these samples showed both HBsAg and HBcAg positive staining. Superinfection with HDV was achieved by infecting with HBV and injecting them 60 days later with HDV positive serum. HDV RNA became detectable in the serum 10 days post infection and remained so for 4 weeks.

Ohashi *et al's* approach differed from this project's in that the transplanted hepatocytes were supported by a matrix and the agonistic antibody was administered IV at weekly intervals with out the performance of a partial hepatectomy. Thus, the hepatocytes were stimulated continually and as a result would have retained their differentiated state, allowing their infection by these viruses. Ohashi *et al's* approach therefore has potential for the development of a HCV animal model.

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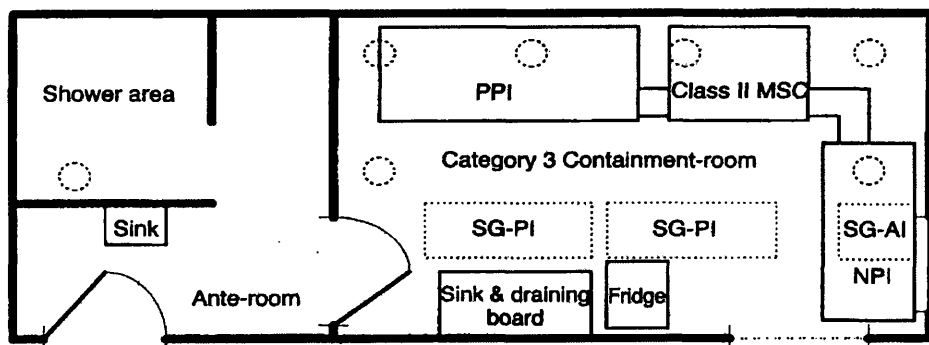


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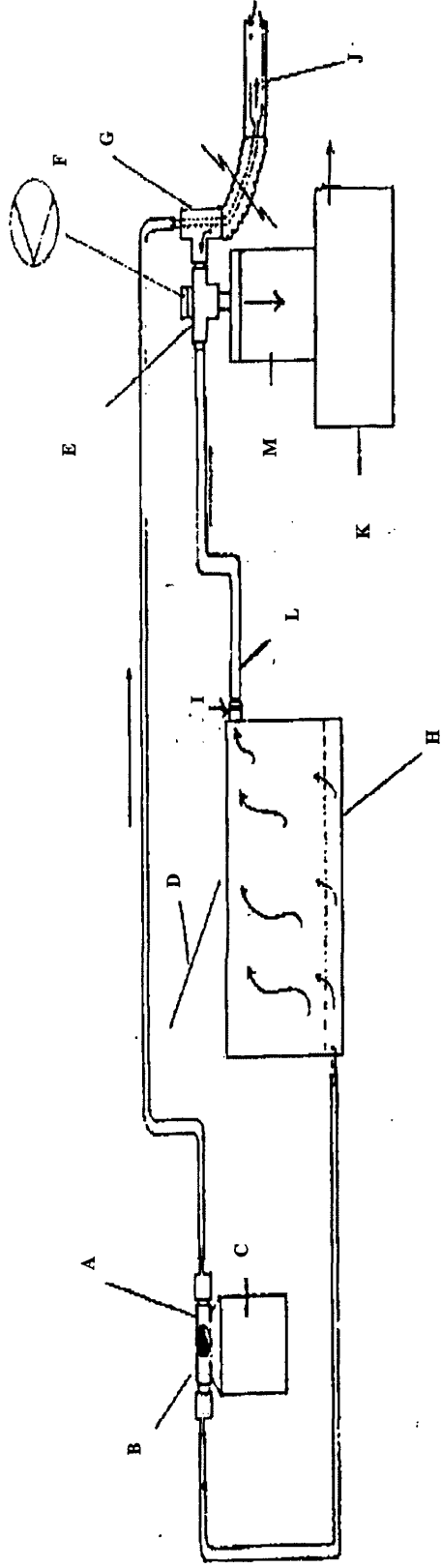
## Appendix I



**Outline of the Category III laboratory**

PPI, positive pressure isolator; NPI, negative pressure isolator; SG-AI, supply grill auxiliary inflow; SG-PI, supply grill primary inflow; ○ extractor grill (Osborne *et al* 1999)

## Appendix II



**Diagram illustrating circuit for anaesthetic induction chamber and maintenance mask**  
(As demonstrated in Fluovac system outline, International market supplies catalogue, 1999)

- A Directa-valve with tube mount
- B Vapouriser outlet
- C Vapouriser
- D Lid
- E Directa-valve with tube mount
- F Knob on top of directa-valve
- G Fluovalve mask unit
- H Induction chamber
- I Tube mount and flow control
- J Mask
- K Fluosobe

## Appendix III

### Processing tissue to paraffin wax

Overnight schedule		
Container	Fluids	Time Minutes
1	10% formalin	0
2	70% Alcohol	30
3	95% Alcohol	30
4	100% Alcohol	30
5	100% Alcohol	60
6	100% Alcohol	60
7	100% Alcohol	60
8	100% Alcohol/Xylene	30
9	Xylene	60
10	Xylene	120
11	Wax	150
12	Wax	240

**Automated processing schedule using an enclosed processor (Tissue-TEK VIP machine, Miles Scientific). This was used to process the tissue removed from the mice (Anderson and Gordon 1996)**

