THE DEVELOPMENT OF

A HUMAN PRIMARY HEPATOCYTE MODEL FOR INVESTIGATING THE PATHOGENESIS OF THE HEPATITIS C VIRUS

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RESEARCH IS THE ACT OF GOING UP ALLEYS TO SEE IF THEY ARE BLIND

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ABSTRACT

The blood-borne virus, hepatitis C (HCV), is causing an increasing burden of chronic and terminal liver disease, world-wide. The development of successful drug treatments for this infection has been hampered by the lack of an efficient and physiologically relevant *in vitro* model of viral pathogenesis. The recent characterisation of the JFH1 clone of HCV, which is capable of both infection and replication in some types of cell lines, has revolutionised the potential of *in vitro* HCV research. Yet very few studies have been able to investigate the pathogenesis of HCV in normal, healthy hepatocytes, and none has examined the effects of such infection on other human liver cells.

This thesis presents the techniques and results of work to optimise human primary liver cell cultures, in order to permit investigation of the JFH1 clone of HCV. A protocol was developed for the isolation of healthy human hepatocytes from surgically resected liver tissue. Methods for the non-viral transfection of primary hepatocytes were then optimised and compared. Finally, the expression of a JFH1 replicon (incorporating the luciferase marker gene) was assessed in human primary hepatocytes, both in monoculture and in three-dimensional co-culture with hepatic stellate cells (HSCs).

The level of expression of the JFH1 replicon in human primary hepatocytes was considerably lower than that found in the human hepatoma Huh7 cell line, as expected, and highly dependent upon the batch of primary cells used. Hepatocytes which were grown in co-culture with HSCs showed some evidence of a greater capacity to support the translation and replication of JFH1. Luciferase was largely undetectable by 48 hours, particularly in hepatocyte–HSC co-cultures, suggesting that innate anti-viral mechanisms are preserved in these cultures. Further studies, to examine the intriguing dialogue between these models and JFH1, now have the potential to provide unique insights into the pathogenesis of HCV in the human liver.

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ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
ALU	arbitrary light units
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CMV	cytomegalovirus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ECM	extra-cellular matrix
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
Geq	genome equivalent
GFP	green fluorescent protein
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC	hepatic stellate cell
Huh7	human hepatoma cell line 7

IDU	intravenous drug user
IRES	internal ribosome entry site
IRF-3	interferon regulatory factor 3
JFH1	Japanese fulminant hepatitis clone 1
kb	kilobase
l	litre
LDL-R	low density lipoprotein receptor
Luc	luciferase
ml	millilitre
mm	millimetre
mRNA	messenger ribonucleic acid
µl	microlitre
NANBH	non-A, non-B hepatitis
NF-κB	nuclear factor κB
NICE	National Institute for Clinical Excellence
nm	nanometre
NTR	non-translated region
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
R^2	regression coefficient of determination
RIG1	retinoid-inducible gene 1

RNA	ribonucleic acid
RSD	relative standard deviation
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SGR	subgenomic replicon
TLR-3	toll-like receptor 3
TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
UK	United Kingdom
UV	ultraviolet
WHO	World Health Organization

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1. INTRODUCTION

1.1 The hepatitis C virus

1.1.1 Clinical aspects of hepatitis C virus infection

1.1.1.1 Epidemiology and natural history of HCV

Hepatitis C virus (HCV) was identified in 1989 as the leading cause of non-A, non-B hepatitis[1]. Humans are the only natural hosts. Infectious particles of hepatitis C virus are circulated in the blood of infected individuals. The virus may therefore be transmitted by procedures such as blood transfusion, haemodialysis, intravenous drug use, tattooing or invasive medical procedures using inadequately sterilized instruments. In the UK, blood products have been screened for evidence of HCV since 1991 and high standards of medical practice have virtually eradicated iatrogenic transmissions of HCV. Recreational intravenous drug use is now the most common route of transmission in the UK. In developing countries however, blood transfusions and the re-use of medical instruments remain common routes of infection with HCV.

Vertical transmission, from mother to baby, is also possible and is most likely to occur through contact with bodily fluids during the perinatal period. Sexual transmission has been documented but appears to be a far less frequent mode of transmission, epidemiologically[2,3]. There have also been outbreaks of acute HCV infection in men who have sex with men (MSM), sometimes associated with pre-existing HIV infection[4-6]. Following the discovery of HCV, screening of donated blood has substantially reduced the transmission risk from blood products and intravenous

drug use is now the most common risk factor for transmission of the virus in the UK[7].

During the acute phase of an infection, around 20% of people who contract the virus will successfully eliminate it from their circulation. The remaining majority become chronic carriers of HCV[8]. The mechanism of natural HCV eradication is unclear. It is largely unknown why only a minority of those infected with the virus avoid persistent infection. A number of studies have found that female gender correlates with higher rates (42% vs. 20%) of spontaneous clearance, perhaps due to an association with oestrogen hormone levels[9]. There is also some evidence that a very young age at time of acquisition is associated with higher levels of HCV clearance[10]. Co-infection with chronic hepatitis B correlates with a 3 to 4-fold higher rate of HCV clearance, purportedly due to due to reciprocal inhibition of viral replication[10], whereas co-infection with HIV is known both to worsen the outcome of HCV infection[11], and to reduce the likelihood of treatment-related HCV clearance[12,13]. Interestingly however, a recent study of an HIV-infected cohort has found that those who acquire HCV by heterosexual transmission are more likely to clear the infection than if it is contracted by another route (odds ratio = 2.81[14]).

One common finding is that patients who develop symptoms and/or signs of an acute hepatitis after acquiring HCV are more likely to clear the virus (relative risk = 1.7[9]); usually within 3 months of the onset of the illness[15]. A vigorous early immune response may therefore be responsible for both the acute illness and the subsequent recovery from infection[9] and the finding that strong CD4+ and CD8+ T-cell responses increase rates of HCV clearance corroborates this hypothesis[16,17]. The specific major histocompatibility (MHC) class II alleles HLA-DRB1*1101 and HLA-

DQB1*0301 are associated with HCV eradication and this is thought to be due to more effective antigen presentation[18,19].

Despite the critical role of this early response to hepatitis C infection, acute infection is usually asymptomatic and, even in those in whom a chronic infection is established, no symptoms may be apparent for 10-20 years. During this period however, the patient remains viraemic and therefore their blood and, to a lesser extent other bodily fluids, may infect others during this time, through percutaneous or mucous membrane exposure. Furthermore, increasing levels of liver inflammation and fibrosis may develop in response to the infection during this asymptomatic phase, meaning that by the time noticeable symptoms cause the patient to present for investigation, significant and sometimes irreversible liver disease has often already occurred.

Many of those with chronic HCV infection will eventually die of non-liver related causes but, if untreated, approximately 20% are estimated to develop cirrhosis in the 20 years after acquisition. Of these, 25% will progress to liver failure or hepatocellular carcinoma (HCC) within a further 5 years[20]. The natural progression of chronic HCV infection is further outlined in Figure 1.1.

Many different HCV strains have been identified by examination of their genomes. These strains can be grouped into six main types of HCV virus, named genotypes 1-6. The geographical distribution of the different genotypes varies widely, as shown in figure 1.2. In the UK, genotype 3 strains have been the most prevalent historically, followed by genotypes 1 and 2[21]. Infection with HCV genotype 3 is particularly associated with the build-up of excessive fat in liver cells, known as hepatic steatosis, although this can occur as a result of any HCV infection[22].

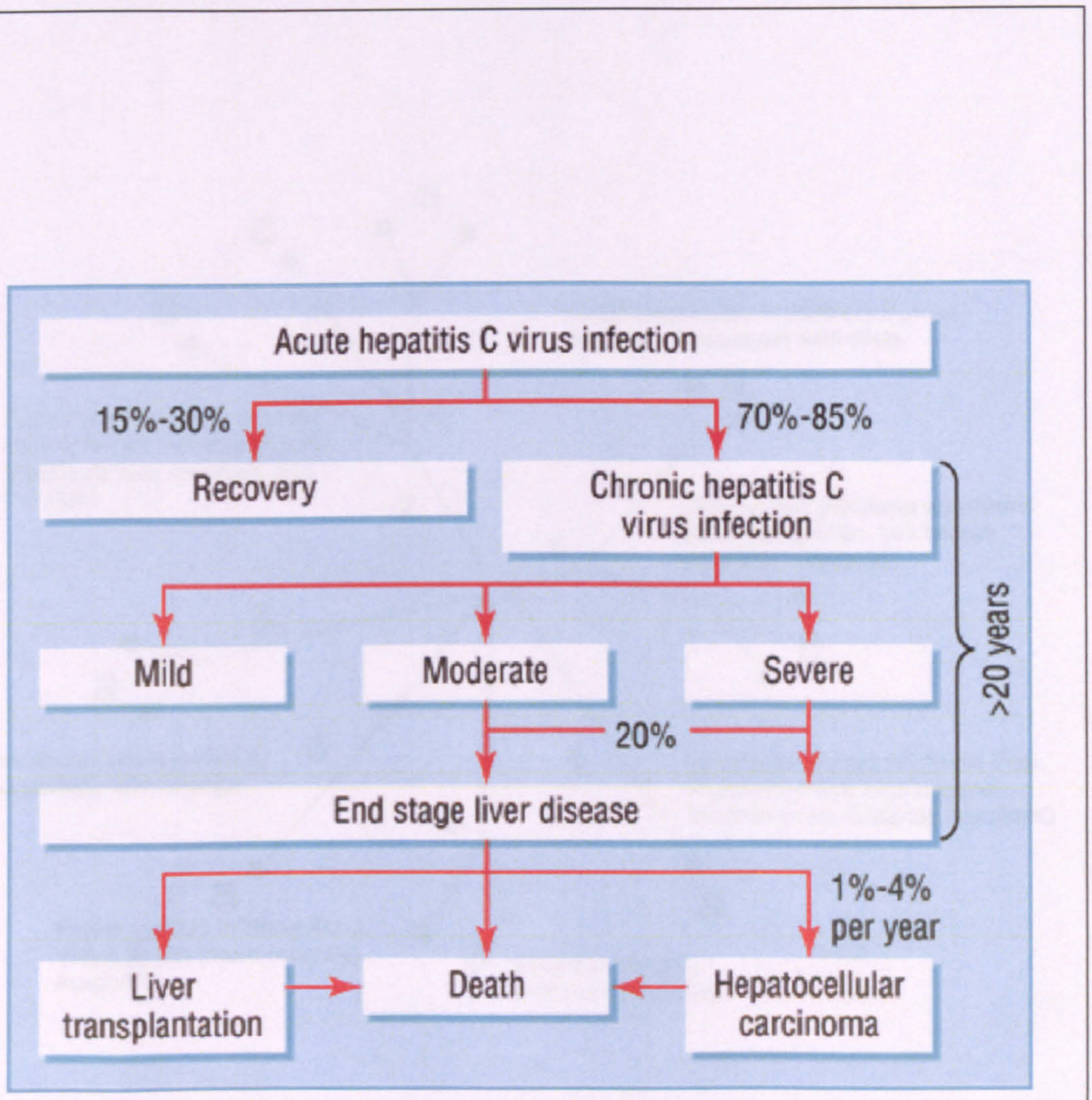

Figure 1.1: Flow chart showing the progression of disease in patients who acquire hepatitis C infection (from Patel et al.[23])

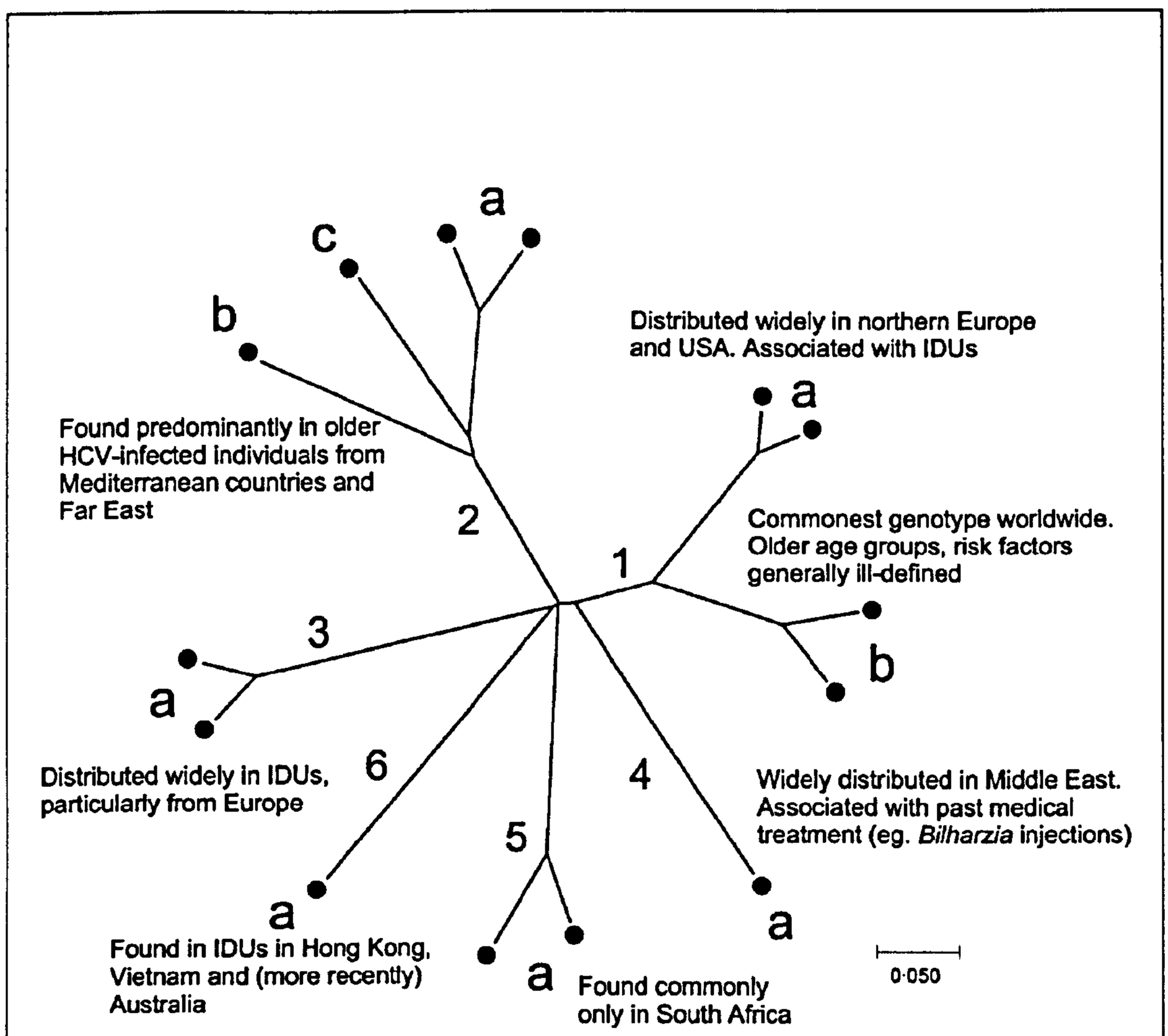

Figure 1.2: *Evolutionary tree of the principal genotypes of HCV that are found in industrialized countries and their main epidemiological associations – distance from the centre point indicates amount of genomic sequence change (and thus proportional duration of evolution). Scale indicates number of nucleotide substitutions per site (from Simmonds et al.[24]).*

1.1.1.2 The burden of chronic hepatitis C infection

Hepatitis C virus (HCV) can cause chronic hepatitis, cirrhosis, liver failure and hepatocellular carcinoma (HCC). The last three sequelae are associated with significant mortality rates. The longer term effects of chronic infection with the virus mean that hepatitis C is an increasingly serious and costly global healthcare problem.

The World Health Organization estimates that around 3% of the population (170 million people) are chronically infected worldwide (see also figure 1.3). In England and Wales, diagnoses of HCV increased by 10% during 2007[25]. As screening rates for HCV have increased, so have the medical and financial burdens attributable to the virus. It is likely that the prevalence of the virus, as well as the prevalence of its diagnosis, will continue to rise, thus increasing HCV-related healthcare costs around the world.

In England and Wales, studies have shown that around 0.4-0.7% of the population has been infected with the hepatitis C virus[7,8]. The majority of an estimated 250,000 chronically infected people are likely to be unaware of their status, although a high percentage may already have mild liver disease[26]. The costs of treating decompensated liver cirrhosis due to chronic hepatitis C infection are predicted to increase enormously if this "silent epidemic" remains undiagnosed and untreated[26] and the Department of Health is running a national campaign ("FaCe It") to increase public and medical awareness of the risks of HCV infection[27].

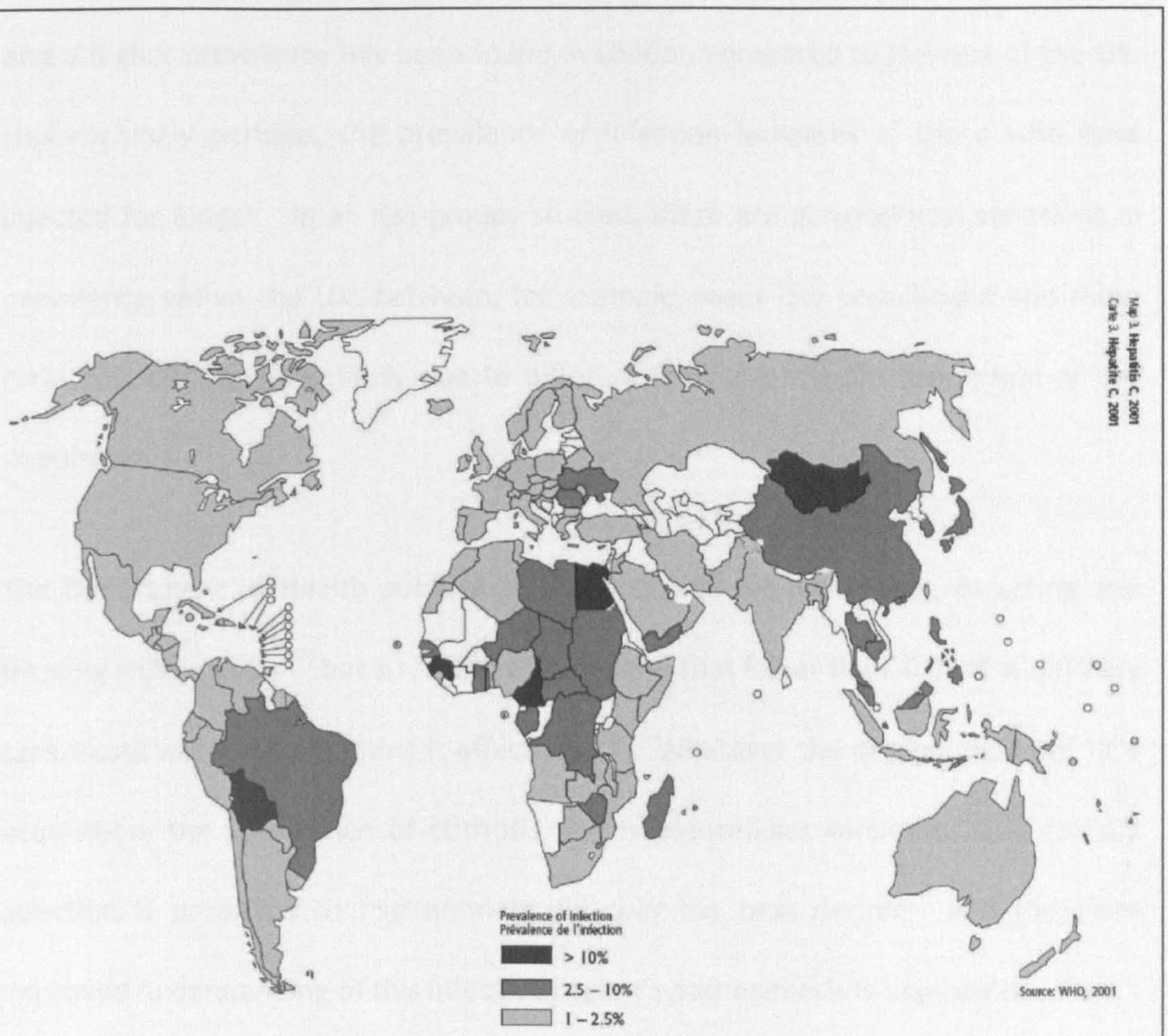

Figure 1.3: Global population prevalence of hepatitis C, 2001, as estimated by reported detections of HCV antibodies. Circles indicate the prevalence in the indicated islands. Unshaded areas indicate those for which no data was available (from the World Health Organization[28])

Furthermore, in high risk groups of active intravenous drug users the rate of infection has been found to be between 30 and 50%[29]. Rates are evidently higher in males and a higher prevalence has been found in London compared to the rest of the UK. Unsurprisingly perhaps, the prevalence of infection increases in those who have injected for longer. In all risk-groups studied, there are geographical variations in prevalence within the UK: between, for example, inner city populations and more rural populations, most likely due to differences in the migrant proportion of the population.

The Department of Health published an action plan for preventing, detecting and treating HCV in 2004[27] but a recent audit showed that fewer than 40% of all primary care trusts were implementing it effectively[25]. Whatever the original mode of HCV acquisition, the prevalence of cirrhosis and hepatocellular carcinoma due to HCV infection is predicted to rise dramatically over the next decade[7] and therefore improved understanding of this infectious agent's pathogenesis is urgently required.

1.1.1.3 Current treatment strategies

No preventative or curative vaccine is available for HCV. Current treatment for HCV aims to reduce inflammation and slow or halt progression of liver damage by eradicating the virus. The current recommended treatment strategy in the UK uses combination therapy with pegylated recombinant alpha-interferon and oral ribavirin. Interferons occur naturally in the body and can directly inhibit viral replication, whilst also having a number of beneficial effects on the immune response. Pegylation (the attachment of a large molecule called polyethylene glycol (PEG)) of interferon is thought to protect it from degradation and so confers prolonged bioavailability and therefore enhanced efficacy. Ribavirin is a purine nucleoside analogue. Its

mechanism of action is unclear but it may act to inhibit the NS5B RNA-polymerase. Increasing the doses and/or the duration of treatment generally increases the chance of achieving a sustained virological response (defined as the absence of detectable HCV RNA in the peripheral blood, sustained for 6 months after the end of treatment) but, equally, often decreases treatment compliance due to an increase in side effects such as haemolysis and depression[30].

Standard durations of treatment are usually between 6 and 12 months depending on the HCV genotype and quantified viral response, as determined by the viral concentration in peripheral blood. A rigorous clinical assessment, sometimes including liver biopsy, is required before treating HCV infection (described further in the NICE guidelines[31] and by Strader *et al.*[32]). Significant and persistent side effects of treatment are common and include fatigue, malaise, myalgia, psychiatric disturbance and blood disorders. The absence of either a simpler care pathway or a better-tolerated treatment regimen may contribute significantly to the fact that only around 20% of diagnosed chronic HCV carriers ever receive treatment, whilst not all of this minority will complete the course. Treatment can render the virus undetectable in up to 90% of people with genotypes 2 and 3 and up to 50% of people with genotype 1[33].

New possibilities for anti-HCV therapy include new interferon adjuvants (for example human serum albumin), which reduce the frequency of doses required, and interferon inducers (such as the toll-like receptor (TLR-) agonists CPG10101 and isatoribine, and the antiprotozoal agent nitazoxanide), which stimulate both the innate and humoral immune response pathways. A number of pharmaceutical companies are working to design small molecule HCV protease inhibitors (such as

telaprevir which inhibits NS3-4a and is currently in phase III trials) and NS5B polymerase inhibitors (either nucleoside analogues such as R1626 or non-nucleoside inhibitors such as VCH-759), which have potential to improve both the effectiveness and the specificity of HCV treatment[30]. Caspase inhibitors, to reduce hepatocyte apoptosis, and therapeutic vaccines against HCV envelope glycoproteins are also under investigation[33].

The relatively non-specific agents currently used in the treatment of hepatitis C infection are testament to a lack of understanding of the effects of the virus on host cells. An immense amount of research using hepatoma-derived cell lines has provided many, sometimes conflicting, insights into the viral life-cycle of HCV. These discoveries are beginning to enable the development of new therapeutic agents, but the *in vivo* response to these drugs is currently unpredictable.

1.1.2 An overview of hepatitis C virus research

1.1.2.1 Discovery and molecular structure of the hepatitis C virus

From the late 1970s, researchers had been searching for a causative agent for non-A, non-B hepatitis (NANBH). A blood-borne aetiology seemed clear but nothing could be isolated in cell cultures or reliably visualised using electron microscopy. Finally, Choo *et al.*[1] constructed a cDNA library from the serum of a patient with NANBH using random primers. One clone was found to be associated with other NANBH infections and it encoded a virus with genetic similarity to flaviviruses. Ultimately, this newly discovered agent was assigned to its own genus, Hepacivirus, within the Flaviviridae family.

As a single-stranded RNA virus, HCV is prone to uncorrected replication errors which lead to a virus population with multiple genome variants, also known as a quasi-species*, within each infected patient. Nonetheless, consensus sequences have been derived which show there to be six main genotypes. The genome is now known to consist of a 9.6 kb positive strand of RNA which encodes a single polyprotein. Translation of the genome is mediated by an internal ribosome entry site contained in the well-conserved 5' non-translated region (NTR). The resulting polyprotein is processed by cellular and viral proteases to yield mature, functional viral proteins (see figures 1.4 and 1.5).

Core protein forms the nucleocapsid of HCV and is perhaps the most studied of all the individual HCV proteins. It has been shown to interact with the endoplasmic reticulum (ER) during maturation cleavage by signal peptide peptidase (see figure 5) and subsequently associates with lipid droplets for transport within the cell[34]. Further work suggests that this association of HCV core protein with lipid is essential for the production of infectious virus from the host cell[35].

In addition to its structural function enclosing the viral RNA, core protein has also been found to affect various aspects of cellular signalling, lipid metabolism, transcription and apoptosis[36]. Many findings have been derived from single gene transfection studies and some have yielded conflicting or heterogeneous results. For example, it has been shown that HCV core protein derived from genotype 1a virus represses nuclear factor κB (NF-κB; a transcription factor involved in inflammatory signalling and cell proliferation), whereas core protein from other genotypes of HCV

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* In virological terms, quasispecies describes a population of genomic mutants that becomes distributed, over time, around the master sequence during an RNA virus infection.

did not have this effect[37]. A number of studies have also investigated the effect of HCV core protein on the tumour suppressor p53. One such study found that the two proteins co-localise and that low levels of core protein increase the activity of p53 but that, conversely, over-expression of HCV core protein inhibited it[38].

The E1 and E2 genes encode two envelope glycoproteins which form glycosylated heterodimers during cellular processing before becoming the key transmembrane proteins on the mature virus envelope. These glycoproteins have been shown to be important for virus attachment and binding during initiation of infection, associating with CD-81, SR-B1 and claudin-1 cell surface receptors[39,40]. A hypervariable domain (HVR1) at the 3' end of E2 is the site of many HCV mutations and therefore the source of most of the heterogeneity between quasispecies. Furthermore, this variability must be a key factor in evading a neutralising antibody response.

A very small polypeptide, p7, is encoded between the main structural and non-structural genes of HCV, with a largely uncertain role in the viral life cycle. It has been shown to form hexamers, with ion channel function, and may function as a viroporin to confer infectivity. This role in facilitating the entry of HCV into the host cell means that the p7 protein is also a potential target for antiviral drugs such as amantadine[41].

At the N-terminal end of the non-structural segment of the HCV polyprotein, NS2 has been found to be cleaved from NS3 by an auto-protease mechanism and its subsequent activity, if any, is unknown. In contrast, NS3 has more than one function. The N-terminal portion of NS3 possesses serine-specific protease activity and may also inhibit the signalling for the innate immune response, whereas the C-terminal

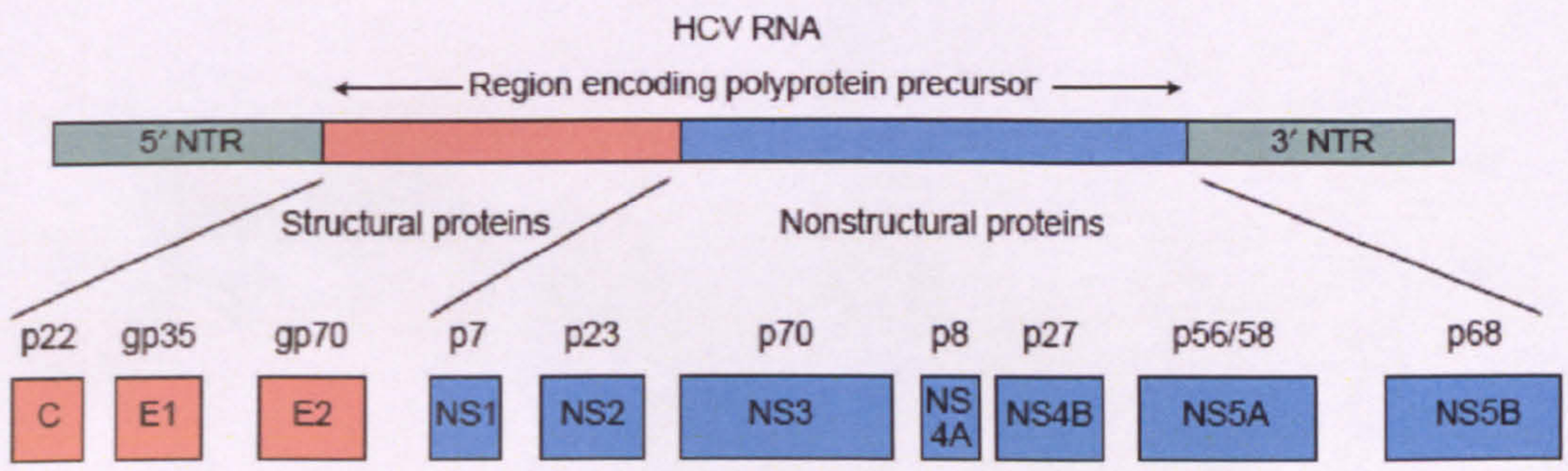

Figure 1.4: *Diagram showing the genome map and polyprotein structure of HCV. NTR = non-translated region; gp = glycoprotein; p = protein. (from Anzola and Burgos[42])*

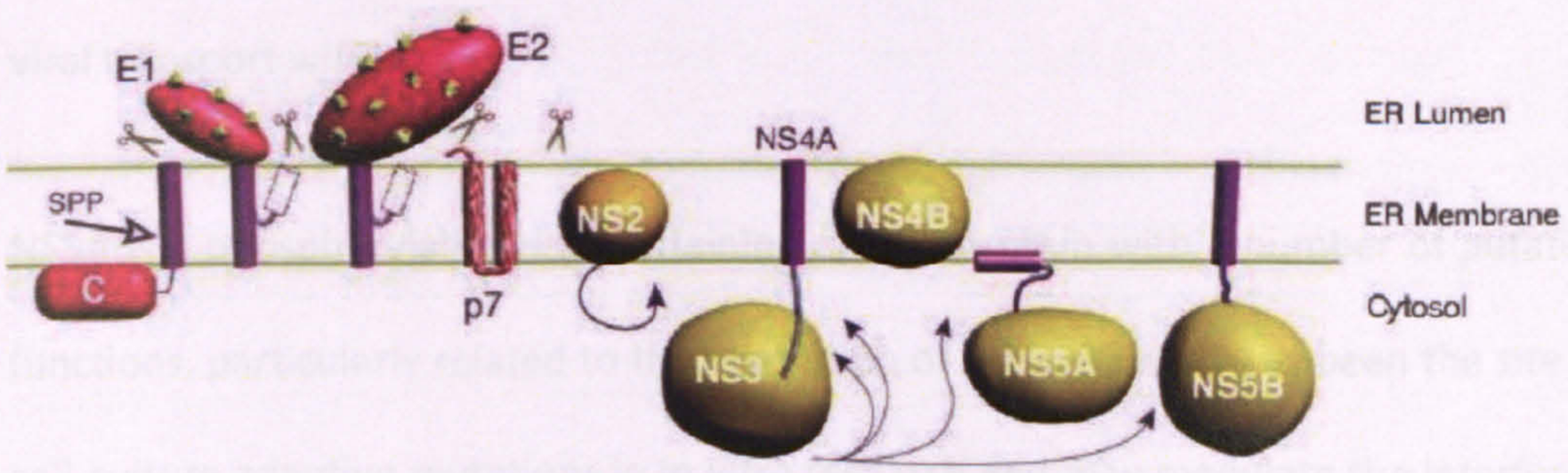

Figure 1.5: Schematic representation of the processing, interactions and cellular distribution of the proteins derived from the HCV genome. The proteins are closely associated with the cellular membranes during their post-translational processing and/or subsequent functionning (from the European HCV Database[43]).

functions as an RNA helicase, which is therefore essential for virus replication. These functions ensure that NS3 is of great interest as a target for new antiviral agents.

NS4A remains closely associated with NS3 (see figure 1.5), and seems to be required as a cofactor for the serine-specific protease function. It may also be responsible for association of the non-structural proteins with cellular membranes. NS4B, a very hydrophobic protein, is known to localise to the ER and may alter membrane conformation during HCV replication. It might also be associated with lipid-mediated viral transport within cells.

NS5A is a phosphorylated zinc-containing metalloprotein with a number of putative functions, particularly related to the regulation of replication. It has been the site of cell-culture adaptive mutations in *in vitro* research and may modulate the interferon response. It, too, possesses a membrane-associating domain which presumably anchors it within the viral replication complex.

Finally, NS5B plays a key role in viral replication, being an RNA-dependent RNA polymerase. A complementary negative strand is synthesized and used as a template for genomic RNA replicates. Despite this key function, sequence variability in the NS5B gene is common. This diversity means that the NS5B gene is often one of the main regions used to determine the genotype of a patient's infection.

As has been noted, much of the early research into the pathogenesis of HCV focused on exploring the effects of single genes, and their proteins, on the function and signalling of tumour cell lines (most notably the hepatoma cell lines HepG2 and Huh7). The relationship between these *in vitro* effects and the *in vivo* pathogenesis of an intact virus on normal human liver cells is inadequately understood. For this

reason, the conclusions drawn from such research are, at best, putative and, at worst, unclear and even misleading. Even with more recent approaches to HCV research, described below, the processes underlying viral replication, assembly and release remain poorly understood.

1.1.2.2 The biology of the hepatitis C virus

Although the HCV genome has been well characterised, understanding of the virus life cycle (as pictured in figure 1.6) is far from complete. HCV can be found in hepatocytes and peripheral blood mononuclear cells. Differentiated adult hepatocytes express a number of receptors which are associated with lipids and have also been associated with HCV infection. CD81 is a common tetraspanin protein with many functions and is expressed on almost all cell types. It has been hypothesised that CD81 binds to an HCV surface protein but this may not be sufficient to allow infection on its own. Scavenger receptor B1 (SR-B1) functions to transport both high and low density lipoproteins across the hepatocyte cell membrane and has also been shown to bind an HCV envelope protein. The low density lipoprotein receptor (LDL-R) functions, as its name suggests, to bind and endocytose lipoproteins and has also been found to act as an HCV receptor[39]. More recently another family of cell-surface proteins, the claudins, has also been demonstrated to mediate the entry of HCV into host cells. Claudins are highly conserved tetraspanin proteins that are essential in the formation of tight junctions between cells. At least twenty isotypes exist, of which claudins-1, -6 and -9 have been found to act as co-receptors for HCV[40]. Many researchers now speculate that HCV is associated with lipoprotein complexes in the peripheral circulation and that this facilitates both immune evasion and the infection of naive cells[39].

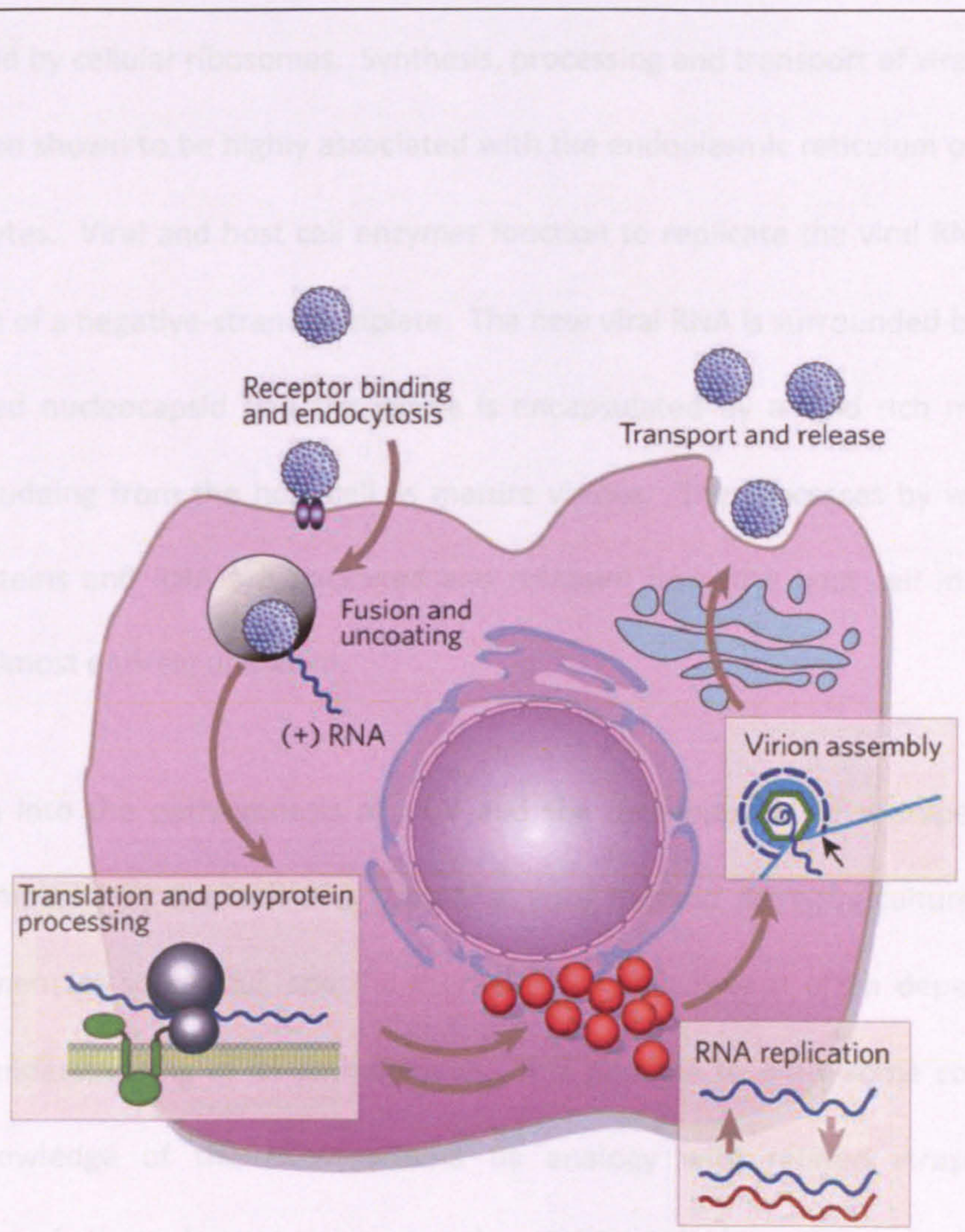

Figure 1.6: *Schematic diagram of the HCV life-cycle, showing its entry into, processing within, and release from the host hepatocyte (from Lindenbach and Rice[44]).*

After release from the endosome and uncoating of the viral envelope and capsid, through mechanisms which are unclear, HCV RNA functions directly as mRNA and is translated by cellular ribosomes. Synthesis, processing and transport of viral proteins have been shown to be highly associated with the endoplasmic reticulum of infected hepatocytes. Viral and host cell enzymes function to replicate the viral RNA via the synthesis of a negative-strand template. The new viral RNA is surrounded by a newly assembled nucleocapsid and the whole is encapsulated by a lipid rich membrane before budding from the host cell as mature virions. The processes by which new viral proteins and RNA are packaged and released from the host cell in this way remain almost entirely unknown.

Research into the pathogenesis of HCV and the development of therapeutics has been hampered by the lack of a robust, *in vitro* method for virus culture[45]. The development of successful, specific therapies for any disease often depends on a precise understanding of its pathogenesis. It is possible to draw some conclusions from knowledge of the HCV genome by analogy with related viruses. The chimpanzee is the only permissive animal model for HCV but ethical considerations severely limit the use and scope of this model for research. Evidence of the outcome of chronic HCV infection is easily available by histological study of clinical liver specimens. However, to understand the mechanisms behind such damage, studies of cellular responses to viral entry, protein expression and replication are required.

1.1.2.3 Cell culture models for hepatitis C

Since HCV is thought to replicate mainly in hepatocytes, these cells are the obvious choice for investigating the viral life cycle. However, the low percentages of infected hepatocytes, the presence of relatively few genomes per cell, and the error-prone nature of reverse transciption polymerase chain reaction (RT-PCR) means that reliably-representative liver-derived virus is hard to isolate and clone for further investigation[46,47]. Another problem is that the availability of primary human hepatocytes is limited; as is their durability in *in vitro* culture conditions (further discussed in chapter 3).

Liver-cell lines, principally Huh7 and derived-clones, have been the main cell type using for investigating the pathogenesis of HCV. The Huh7 cell line was derived in 1982 from a well-differentiated human hepatocellular carcinoma[48]. These cells proliferate easily in culture with a doubling time of around 35-40 hours[48]. Huh7s are relatively well differentiated, producing various plasma proteins such as albumin, ceruloplasmin and alpha-1 antitrypsin as well as a few carbohydrate-metabolizing enzymes such as glucose-6-phosphatase (G6Pase) and fructose 1,6-diphosphatase (FDPase)[48]. Unfortunately, although the phenotype of the original tumour is well maintained, these cells are not fully representative of normal healthy hepatocytes. Huh7 cells lack cytochrome P450 function and their ready proliferation *in vitro* is uncharacteristic compared to primary hepatocytes. For the same reasons, they may not retain normal mechanisms or levels of signalling when challenged or cultured with other cells.

1.1.2.4 Replicons of the hepatitis C virus

Serum-derived HCV comprises a heterogeneous and unique mixture of genomic variants within the quasi-species; a disadvantage in the search for reproducible results and broadly-applicable conclusions. Early research therefore sought consensus sequences of HCV from patients with very high viral titres. These sequences were used to generate full length HCV RNA clones, such as H77, which were infectious by inoculation into chimpanzees but still failed to replicate to any detectable level in unmodified human liver cell-culture systems[49].

Replicons are RNA or DNA sequences that replicate from a single starting point. Wild-type HCV RNA essentially functions as a replicon but, since it appeared to lack the ability for robust replication *in vitro*, modified replicons (some with alternative or additional internal ribosome entry site (IRES) sequences) were created. Subgenomic replicon systems for HCV were developed first[49], allowing examination of viral RNA synthesis and the functions of viral proteins. First generation replicons carried selectable neomycin-resistance genes on a bicistronic sequence: where translation of the resistance marker was initiated by the HCV IRES and translation of the non-structural genes was mediated by an inserted encephalo-myocarditis virus (EMCV) IRES, as shown in figure 1.7 below.

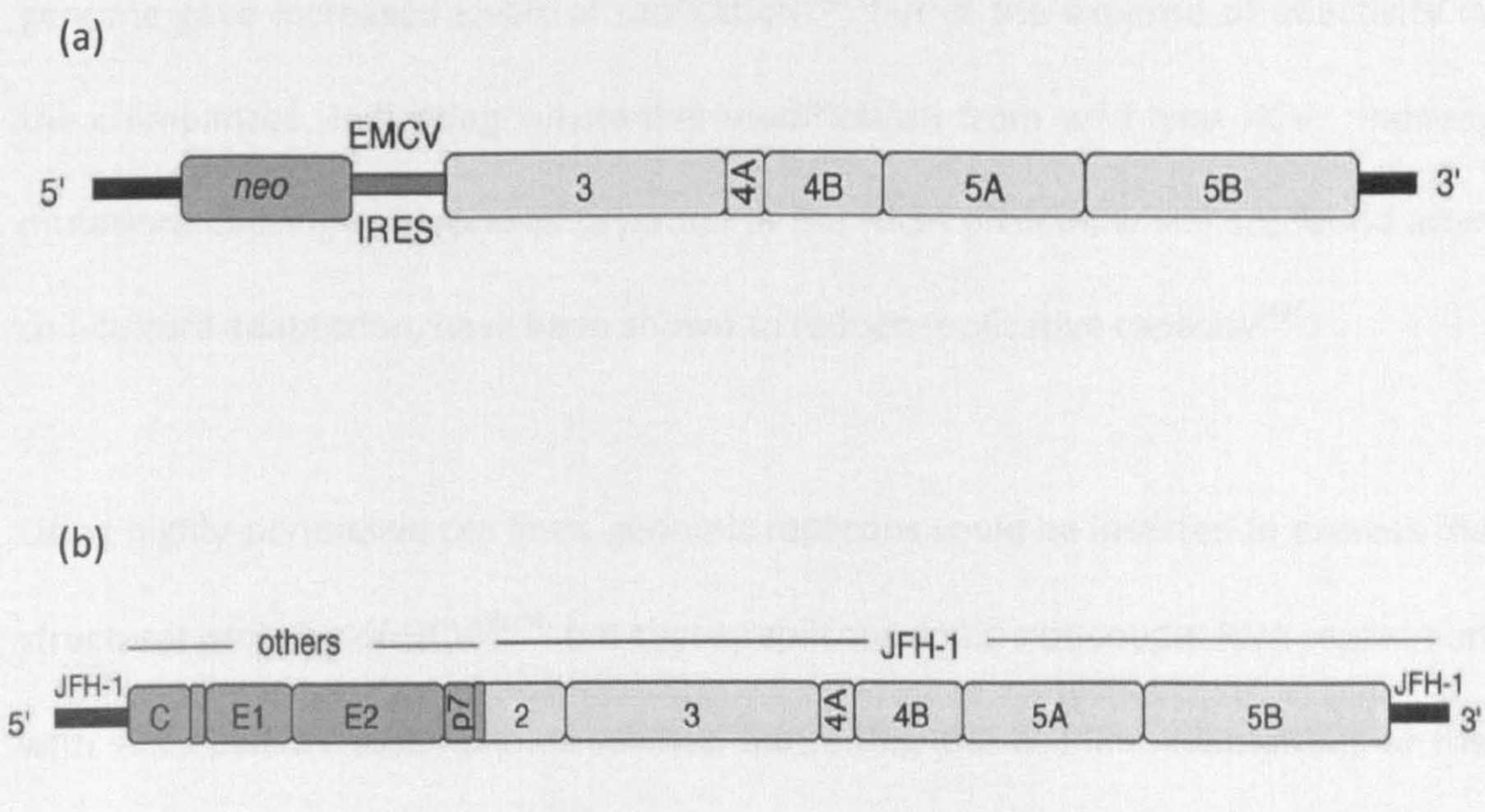

Figure 1.7: *General structure of (a) sub-genomic replicons of HCV and (b) the full length infectious replicon JFH1 (from Bartenschlager[49] and Pietschman et al.[50]). Further examples of replicons are given in chapter 5.*

After producing stably transfected cell lines, a few neomycin-resistant cell colonies were isolated and found to contain replicating RNA[51,52], though this was most successful with selected highly permissive clones of Huh-7 cell lines. The spontaneous development of cell culture-adaptive mutations within the viral genome gave increased levels of replication[53], but at the expense of infectivity in the chimpanzee, indicating substantial modification from wild-type HCV. Indeed, mutations causing hyperphosphorylation of the NS5A protein, which are found after cell-culture adaptation, have been shown to reduce replicative capacity[49].

Using highly-permissive cell lines, genomic replicons could be inserted to express the structural proteins of HCV[50,54] but these replicons could not couple RNA replication with virus particle assembly. It seemed that either the cell-line adaptations or the changes to the replicative efficiency were responsible for the failure to produce infectious virus.

So the generation, from the JFH1 clone of HCV, of a novel replicon which replicates without cell culture-adaptive mutations in the Huh7 cell line was a highly important development[55,56]. Wild-type genotype 2a virus was isolated from a Japanese patient who presented with acute fulminant hepatitis. The consensus sequence of this virus was designated Japanese fulminant hepatitis clone 1 (JFH1). A subgenomic replicon was initially derived from this consensus sequence and was shown to replicate at high levels in unmodified Huh-7 cells[55,56].

1.1.2.5. Infectious hepatitis C virus in cell culture

The JFH1 clone has been modified by the addition of structural genes, as shown in figure 1.7, and this full-length genome permits generation of infectious virus particles and also retains infectivity in the chimpanzee[57]. Several successful chimeric genomes have also been produced, most of which produce even higher titres of infectious HCV virus in culture than JFH1. More importantly, these chimeras enable the study of more common genotypes including genotype 1a, 1b and 3a, although so far only the JFH1 non-structural genes support efficient virus production.

These constructs have all been developed and investigated in Huh7 cells. However, as previously noted, these tumour-derived cells do not retain all the functional characteristics of *in vivo* hepatocytes. In addition, continuous cell lines cannot offer a physiologically reliable model in which to examine the mechanisms by which HCV infection affects the cell cycle and cell death. A number of obstacles therefore still hamper those who seek a robust culture system of greater relevance to the *in vivo* situation.

1.2. Primary hepatocyte culture systems

1.2.1. The human liver

1.2.1.1. An introduction to hepatocytes

The human liver is a large organ which receives nutrient-rich blood from the portal vein and oxygenated blood from the hepatic artery. A membrane, known as Glisson's capsule, surrounds a vascular tissue mass which is comprised of several types of cells. Up to 80% of the total mass is made up of parenchymal cells called hepatocytes. These cells perform a wide range of complex functions, including synthesis of proteins, lipoproteins and bile salts; modification of carbohydrates; storage of various substances; and they are also responsible for the processing and excretion of numerous endogenous and exogenous molecules. Hepatocytes therefore exhibit large nuclei and large amounts of intracellular storage granules, endoplasmic reticulum and mitochondria are evident on microscopy. A high level of both active and passive cell transport is thus required for the cells' excretory and metabolic functions and this is supported by a specific histological architecture.

Liver cells are normally organised within a three-dimensional polygonal unit called a lobule (figure 1.8). Within the lobule, single-cell layers of cuboidal hepatocytes are arrayed in a radial pattern around a central venule. Each layer is adjacent to a sinusoid, which carries blood and is bounded by a fenestrated epithelium. Microvilli on the sinusoidal surface of hepatocytes project into the sinusoid and facilitate efficient nutrient and oxygen transfer. On the non-sinusoidal faces of each hepatocyte, bile canuliculi form and transport excreted substances back towards the bile duct in the portal triad.

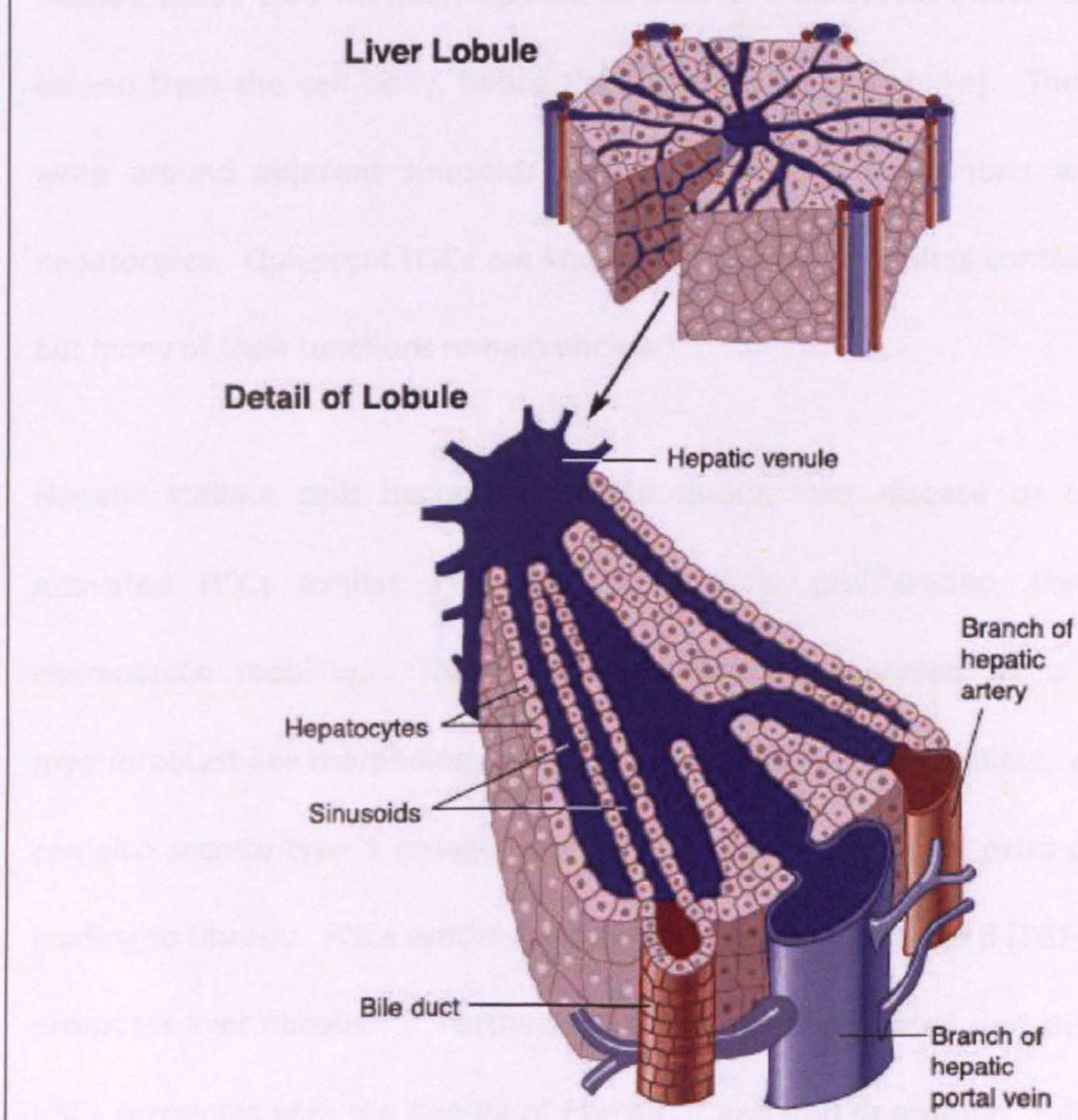

Figure 1.8: Schematic diagram of the three-dimensional structure of the human liver lobule, showing its blood supply and the cords of cuboidal hepatocytes (from Cunningham and Van Horn[58])

1.1.1.1. An introduction to hepatic stellate cells

Hepatic stellate cells (HSCs), also called Ito cells or fat-storing cells, comprise around 5-8% of cells in the normal healthy liver. HSCs are of mesenchymal descent and in healthy tissue they normally appear to exist in a quiescent state. Long processes extend from the cell body, hence the name stellate (star-like). These projections wrap around adjacent sinusoids and are also in close contact with numerous hepatocytes. Quiescent HSCs are known to store lipid droplets containing vitamin A but many of their functions remain unclear.

Hepatic stellate cells become activated during liver disease or other damage. Activated HSCs exhibit a marked increase in proliferation, contractility and chemotactic mobility. These processes are accompanied by a change to a myofibroblast-like morphology and loss of cytoplasmic lipid droplets. Activated HSCs can also secrete type 1 collagen and remodel the surrounding extra-cellular matrix, leading to fibrosis. HSCs synthesise transforming growth factor β (TGF-β), which also promotes liver fibrosis[59]. Further, it has been demonstrated that the activation of HSCs correlates with the degree of fibrosis[60] and iron deposition[61] during hepatitis C infection. As fibrosis is such a key event in the pathogenesis of chronic HCV infection, HSCs are of great interest in any study investigating the cellular and molecular basis of the fibrotic process.

In addition, HSCs have been found to adhere to, and encourage proliferation of, hepatocytes during liver regeneration after hepatic injury[62], probably through the exchange of growth and chemotactic factors. HSCs can exert both positive and negative influences on hepatocyte proliferation, including production of hepatocyte growth factor (HGF), which is up-regulated after liver injury[63].

1.2.2. *In vitro* culture of primary liver cells

1.2.2.1 Isolation, culture and functional assessment of primary hepatocytes

Primary adult hepatocytes are fairly large cells with a naturally cuboidal morphology and contain numerous organelles. They are complex cells whose membranes are readily susceptible to damage by physical or chemical means. As such they are difficult to isolate successfully and reliably. The number, quality and viability of the isolated cells are all dependent on a number of factors, such as the vascular architecture of the tissue used and the expertise and speed with which the isolation procedure is performed. The quality, quantity and structure of the liver tissue used also impacts upon the yield and viability of hepatocytes obtained from each tissue preparation. The further confounding factor of donor heterogeneity, which is particularly marked when obtaining human cells, adds an additional and significant source of variation (discussed further in chapter 3).

Isolation of primary hepatocytes was first carried out in the 1960s using mechanical and enzymatic digestion of rat liver[64]. The technique was modified, most notably by Seglen *et al.*[65], to become a two-step collagenase perfusion technique that is widely used as a basis for most hepatocyte isolation work today. The existing vasculature of the liver lobe or segment is cannulated and a series of buffer solutions is perfused through the tissue. The first buffer solution usually performs the dual function of flushing the tissue of blood and disrupting cell-cell junctions by means of a calcium chelating agent. The second buffer solution contains a collagenase (usually tissue-specific) which digests much of the extra-cellular matrix. This then enables the hepatocytes to be gently liberated from the treated tissue with a minimum of mechanical force. Generally the capsule can be teased apart using forceps and the

liver cells flushed from any undigested tissue using warmed culture medium. Following a mesh filtration step to isolate a suspension of single cells, several low-speed centrifugation steps are generally used to isolate and purify the parenchymal cell fraction.

The hepatocytes obtained by these methods will rapidly suffer decreases in both viability and differentiated function if not cultured in an appropriately supportive environment. A number of methods for prolonging both viability and function exist; however, most require conditions that are both physically supportive and hormonally enriched. Substances which mimic the support of the *in vivo* extra-cellular matrix are commonly used, along with serum, insulin, steroid hormones and growth factors. Few hepatocytes will spontaneously proliferate in *in vitro* culture conditions and, if grown on standard tissue culture plastic-ware, they will usually die within one week. The physical fragility of hepatocytes also makes them intolerant of removal (or passage) into new culture environments after they have adhered to the initial culture surface. Specialized media and growth matrices can in some cases maintain viability and function for several months. Unfortunately a dilemma exists in that additives which promote proliferation tend to encourage dedifferentiation, whereas substances which promote the maintenance of differentiated hepatocyte function also minimise progress through the cell cycle.

In vitro hepatocyte function can be measured in several ways. Albumin production and urea metabolism can be measured easily but are maintained even in relatively poorly differentiated cell cultures. Demonstration of cytochrome P450 function is a far more sensitive measure of continued hepatocyte-specific function. The cytochrome P450 (CYP450) protein family comprises a large number of iron-

containing enzymes which metabolise thousands of different substrates. CYP450 enzymes are mainly associated with mitochondrial or endoplasmic reticulum membranes and, in hepatocytes, act on diverse substrates including bilirubin, ethanol, steroid hormones, and exogenous drugs. Furthermore, their activity can be induced or inhibited by some exogenous compounds. Differentiated hepatocytes are therefore of great interest to the pharmaceutical industry for investigating drug metabolism and toxicities.

1.2.2.2. Isolation and culture of primary hepatic stellate cells

Hepatic stellate cells (HSCs) can also be isolated from liver tissue by digestion. Due to the more robust nature of these mesenchymal-derived cells, isolation protocols can make use of more aggressive enzymatic or mechanical digestion techniques than those used for hepatocyte isolation, while still yielding viable cells. Mincing and stirring may therefore be used after perfusion and digestion of tissue. This may help to maximise the numbers of HSCs obtained as, by their nature, they are likely to be closely associated with the extra-cellular matrix.

Most methods developed for the isolation of hepatic stellate cells employ sequential perfusions of the tissue and treatment of the resulting cell suspension, using collagenases and a protease called pronase, in order to digest the tissue fully. Deoxyribonuclease is also used to digest DNA released from lysed cells, which can otherwise cause clumping of cells in the suspension. Unfortunately, these methods preclude the concurrent isolation of hepatocytes as they cause too much parenchymal cell damage. Some protocols do exist to isolate the HSC fraction after the hepatocytes have been extracted[66]; the advantage being that the same tissue can yield both cell types, thus maximising its potential.

The second, and equally important, stage of HSC isolation is fractionation of the non-parenchymal cell suspension obtained by any of the above methods. Due to their relatively high lipid content, HSCs have the lowest density of any hepatic cell type and can be purified by density gradient centrifugation. Following isolation, HSCs usually must be cultured *in vitro* for at least a week, to allow proliferation and generation of sufficient numbers of cells for use in studies. Rat HSCs can be passaged and maintained for up to one year (Amanj Saeed, University of Nottingham, personal communication). Unlike hepatocytes, HSCs tend to proliferate readily in *in vitro* culture and are tolerant of passage onto other culture surfaces.

1.2.3. HCV replication in three-dimensional co-cultures of liver cells

The characteristics of *in vivo* hepatocytes, in particular their conformation and expression of cell surface moieties, may be significantly altered by the process of the two-step isolation procedure. Ongoing culture in the physiologically most relevant conditions possible is therefore essential to reconstitute normal phenotypic traits. Media constituents and culture substrate are most important for prolonging viability and function of primary hepatocytes in conventional single-layer plating conditions. However, culture in a three-dimensional "spheroid" formation has been found to confer additional benefits: increased longevity and the maintenance of differentiated function and both intra- and extra-cellular structures[67].

It is known that liver-specific functions are somewhat stabilised in hepatocytes co-cultured with non-parenchymal cells[68,69]. Use of a novel *in vitro* hepatocyte culture method has demonstrated that static co-culture of rat hepatocytes and hepatic stellate cells leads to the formation of self-organising three-dimensional spheroids[70,71]. It has been shown that the *in vitro* co-culture of hepatocytes with

HSCs can significantly prolong hepatocyte-specific cytochrome P450 function[72]. Proliferation also seems to be enhanced[73], in contrast with "normal" *in vitro* hepatocyte culture, in which the ready proliferation of *in vivo* cells is lost. Related studies have also demonstrated that, in 3D co-cultures of hepatocytes and HSCs, significant histological similarities to *in vivo* liver architecture can be seen[71]. Most interestingly, a supportive extra-cellular matrix of collagen and fibrin develops around these organised aggregates of hepatocytes. This feature of the hepatocyte-HSC co-culture system is likely to make it particularly useful for investigating fibrotic processes. These functions of HSCs, both *in vivo* and *in vitro*, make them a logical cell type to study in parallel with hepatocytes when investigating the pathogenesis of HCV in primary liver cells.

Three-dimensional cultures of hepatocytes are thus of interest in the study of hepatitis C infection for several reasons. Firstly, as already documented, they retain more of the functional and structural characteristics of *in vivo* cells and any finding should therefore have greater relevance to the clinical situation. Secondly, spheroids have also been shown to be more permissive to HCV infection, presumably due to improved generation or retention of cell surface receptors and cellular functions that confer susceptibility to HCV. Thirdly, cells in a three-dimensional structure are likely to develop and maintain cell polarity, cell-cell interactions and therefore cell-cell signalling, to a far greater extent than plated cells.

1.2.4. Using HCV replicons in a human primary liver cell culture model

A small number of studies have evaluated the susceptibility of human primary hepatocytes to HCV infection or replication *in vitro*. Wild-type virus in patient serum samples has been shown to infect primary hepatocytes in monoculture and the cells can subsequently support replication of HCV RNA[74,75]. Importantly, consistent susceptibility was seen between hepatocyte preparations from different donors, supporting a theory of universal susceptibility of human primary hepatocytes to HCV infection[75]. However, virus replication varied in both duration and extent depending upon genotype and quasispecies and was, in most cases, detectable only between 3-5 days after inoculation[74]. In a later study the average number of HCV RNA copies ranged from between 0.18 to 36 genome copies per hepatocyte[75].

Low density lipoproteins (LDL) and soluble forms of the low density lipoprotein receptor (LDL-R) have been shown to inhibit or enhance, respectively, the ability of HCV to infect primary hepatocytes[76,77], reinforcing the evidence from Huh-7 studies which identified LDL-R as a possible HCV receptor. Modulation of LDL-R expression similarly modulated the efficacy of wild-type HCV infection. Again, there was good concordance of findings between different preparations of primary cells but, interestingly, these findings do not corroborate evidence from studies using HCV pseudoparticles (HCVpp; in which HCV envelope glycoproteins are wrapped around a retroviral core) or insect-derived virus-like particles (VLPs; in which HCV envelope and core proteins are combined into non-infectious particles lacking the viral RNA), which have found that LDL-R is not required for infection to occur[78,79]. HCV replication in primary hepatocytes has also been shown to be susceptible to interferon-a, as it is *in vivo*[80]. It has more recently been reported that primary

hepatocytes exposed to proliferative cytokines supported productive infection with wild-type virus but that such treatment sometimes led to selection of viral quasispecies[81].

Alternative approaches have involved: *in vitro* culture of primary hepatocytes from chimpanzees or humans with chronic HCV infection[74]; or infection of fetal primary hepatocytes or adult hepatocyte cell lines, using infected patient serum[82]. All of these systems are disadvantaged either by functional dissimilarities with primary adult cells or by the unavoidable variability of virus found in patient serum. Further detailed discussion of some of these model systems and previous studies which have utilised HCV replicons is presented in section 5.1.

1.3. Research questions

Cultivation of a defined HCV clone in primary human hepatocytes is desirable for a number of reasons. Firstly, it is important to confirm (or indeed refute) data on the life cycle and pathogenesis of HCV which have been derived from studies in less-differentiated cell lines, such as the Huh7 clones. Secondly, it is anticipated that *in vitro* studies of normal primary hepatocytes which are exposed to HCV replication and/or infection would mimic, more closely than Huh7s, the pathophysiology seen *in vivo*. Such a model would thus be expected to enable unique evidence about the cellular effects of HCV infection to be obtained. In particular, it is hypothesised that highly differentiated and durable co-cultures of primary hepatocytes and HSCs will provide a superior system in which to examine the mechanisms of HCV-induced hepatocellular damage and cell death. Further, a hepatocyte-HSC co-culture model would allow additional examination of the effects of HCV on cell-cell signalling, HSC activation and the fibrotic remodelling of the extra-cellular matrix, all of which are of relevance to the pathogenesis of HCV, as previously discussed. Ultimately, it is hoped that establishing replication and/or infection of HCV in differentiated hepatocytes would enable more physiologically relevant evaluation of both the antiviral and the inductive functional effects of new anti-HCV compounds.

Originally, the intention of the present studies was to investigate how expression of the HCV core protein affected the function of primary hepatocytes and HSCs in 3D co-culture. However, during the first year of work it became evident that experimental work using the newly developed JFH1 replicon would, in many cases, be of more importance and greater interest to the research community than studies based on single-gene transfection. In addition, as the majority of other research

teams began to use this new clone in established cell-line culture systems, it was important to assess alternative cell culture models within this contemporaneous context.

Following the generation of the highly replication-competent JFH1 replicon[55], the research questions were therefore altered to the following:

i. To what extent does JFH1 replicate in monocultures of human primary hepatocytes?

ii. To what extent does JFH1 replicate in three-dimensional co-cultures of human primary hepatocytes with hepatic stellate cells?

iii. To what extent, and by what mechanisms, does the translation and/or replication of JFH1 modulate the viability and functional capacity of human primary hepatocytes, in mono-culture and co-culture?

The ambition was also to examine whether primary human hepatocytes are permissive to infection by the full-length JFH1 HCV virus and, if so, whether they would sustain viral replication and the production of viral particles.

1.4. Aims

The aims of this project were to:

- establish and optimise the techniques required for reliable isolation of fresh human primary hepatocytes from resected liver tissue;
- optimise at least one method for non-viral transfection of primary hepatocytes;
- investigate the levels of expression and replication of subgenomic JFH1 replicons in human primary hepatocytes, both in monoculture and in three-dimensional co-culture with hepatic stellate cells.
- measure the effect of JFH1 expression and replication on the viability, hepatocyte-specific function and lipid content of human primary hepatocytes, both in monoculture and in three-dimensional co-culture with hepatic stellate cells.

1.5. Thesis outline

This thesis reviews the work carried out to establish reliable and effective methods of human hepatocyte isolation (Chapter 3) and of primary hepatocyte transfection (Chapter 4). A more detailed introduction to each of these subjects is presented at the beginning of each of these chapters.

Chapter 5 presents a detailed review of current methods used for examining and measuring the replication of HCV *in vitro*. The methods and results of experiments to examine the evidence for expression, and replication, of the JFH1 replicon in monocultures and co-cultures of human primary hepatocytes, are presented and compared with those obtained using the Huh7 cell line.

Finally, chapter 6 contains a summary and appraisal of all of the work presented, along with a discussion of the opportunities created for further studies.

2. GENERAL MATERIALS AND METHODS

2.1. General materials

All chemicals were sourced from Sigma Aldrich© unless otherwise stated.

2.1.1. Buffers for isolating rat hepatocytes

Hanks' HEPES buffer, stock solution (10X)

A 10X stock solution was made using 1 l autoclaved de-ionised water containing: NaCl (1.37 M, 80 g/l); KCl (54 mM, 4 g/l); KH_2PO_4 (4.4 mM, 0.6 g/l); $Na_2HPO_4.12H_2O$ (3.6 mM, 1.2 g/l); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (200 mM, 47.6 g/l); NaOH (100 mM, 4 g/l), pH 7.4 (target value). Hanks' HEPES 1X buffer was prepared as required by diluting the 10X solution with autoclaved de-ionised water.

Glucose-bicarbonate solution

Autoclaved de-ionised water (50 ml) containing: $NaHCO_3$ (0.74 M, 3.1 g/50 ml); D-glucose (0.28 M, 2.5 g/50 ml); L-methionine (0.1 M, 0.75 g/50 ml) (Gibco©).

25 mM EGTA solution

1 M NaOH (2.5 ml) and 0.48 g EGTA were dissolved in 25 ml 1X Hanks' HEPES 25 ml, and then made to 50 ml with 1X Hanks' HEPES buffer.

250 mM $CaCl_2$ solution

Autoclaved de-ionised water (50 ml) containing 1.84 g $CaCl_2$.

Buffer A comprised 400 ml 1X Hanks' HEPES buffer with 8 ml glucose-bicarbonate solution and 4 ml EGTA solution (pH 7.4).

Buffer B comprised 200 ml 1X Hanks' HEPES buffer with 4 ml glucose-bicarbonate solution, 2 ml $CaCl_2$ solution and, added just before use, 20000 Units type IV collagenase (C-5138 Sigma-Aldrich©) (pH 7.4).

All solutions were sterilised before use, by filtration using 0.05 µm filters.

2.1.2. Cell culture media for liver cells

Medium 1

William's Medium E (Gibco©) (500 ml), supplemented by the addition of 50 ml fetal calf serum (PAA Laboratories©), 2 mM L-glutamine (Gibco©), 0.2 U/ml penicillin (Gibco©), 0.2 µg/ml streptomycin (Gibco©), 0.5 ng/ml amphotericin B (Gibco©), 5 mM nicotinamide and 10 µg/ml bovine pancreas insulin (final concentrations of supplements indicated in each case).

Medium 2

William's Medium E (Gibco©) (500 ml), supplemented by the addition of 2 mM L-glutamine (Gibco©), 0.2 U/ml penicillin (Gibco©), 0.2 µg/ml streptomycin (Gibco©), 0.5 ng/ml amphotericin B (Gibco©), 5 mM nicotinamide and 10 µg/ml bovine pancreas insulin (final concentrations of supplements indicated in each case).

Medium 3

Dulbecco's Modified Eagle's Medium (Gibco©) (500 ml), supplemented by the addition of 50 ml fetal calf serum Gold (PAA Laboratories©), 2 mM L-glutamine (Gibco©), 0.2 U/ml penicillin (Gibco©), 0.2 µg/ml streptomycin (Gibco©), 0.5 ng/ml amphotericin B (Gibco©) (final concentrations of supplements indicated in each case).

Medium 4

Dulbecco's modified Eagle's medium (Gibco©) (500 ml), supplemented by the addition of 50 ml fetal calf serum (PAA laboratories©), 2 mM L-glutamine (Gibco©), 0.2 U/ml penicillin (Gibco©), 0.2 µg/ml streptomycin (Gibco©), 0.5 ng/ml amphotericin B (Gibco©) (final concentrations of supplements indicated in each case).

Medium 5

William's Medium E (Gibco©) (500 ml), supplemented by the addition of 50 ml fetal calf serum (PAA Laboratories©), 2 mM L-glutamine (Gibco©), 0.2 U/ml penicillin (Gibco©) and 0.2 µg/ml streptomycin (Gibco©) (final concentrations of supplements indicated in each case).

Medium 6

William's Medium E (Gibco©) (500 ml), supplemented by the addition of 2 mM L-glutamine (Gibco©), 0.2 U/ml penicillin (Gibco©), 0.2 µg/ml streptomycin (Gibco©), 5 mM nicotinamide and 10 µg/ml bovine pancreas insulin (final concentrations of supplements indicated in each case).

2.1.3. Collagen coating of plastic cell culture plates

Cell culture plates (Nunclon© Primeria®) were coated with collagen as follows: a working solution of 0.05 mg/ml type I rat-tail collagen (Upstate Cell Signalling Solutions©) was prepared in phosphate-buffered saline. Working solution (1 ml) was added to each well of a 6-well tissue culture plate and incubated for 2 hours at room temperature, or overnight at 4°C. The collagen solution was then discarded and the culture wells rinsed with phosphate buffered saline prior to use.

2.1.4. Poly-DL-lactic acid coating of plastic cell culture plates

Single 3 cm diameter plastic cell culture wells (Nunclon©) were coated with poly-DL-lactic acid (PLA) in order to create a low-adhesion and hydrophobic surface for spheroid cell culture. For each well to be coated, 1.5 mg anhydrous PLA (Sigma) was dissolved in 1 ml trifluoroethanol (TFE). This solution was added to the well and the plate was placed in an oven at 50°C until all liquid had evaporated. The wells were then sterilised by exposure to ultraviolet light for 30 minutes and stored in anhydrous conditions at -20°C until use. Just prior to use, each well was rinsed using sterile phosphate buffered saline.

2.1.5. Other cell preparation materials

Lockertex® polyester gauze mesh (PE/MO/64/45) was purchased from Clarcor UK.

10X Hank's Buffered Salt Solution and Percoll® (Amersham Biosciences©) were mixed in the ratio 1:9 to form an isotonic solution with a density of 1.07 g/ml and stored at 4°C until use.

Trypan Blue dye was purchased ready for use in a 0.4% (w/v) solution.

Phosphate buffered saline (PBS) was prepared by dissolving 1 PBS tablet in 200 ml of deionised water to produce a 1X PBS buffer (0.01 M phosphate, 0.0027 M potassium chloride and 0.137 M sodium chloride, adjusted to pH 7.4). The buffer solution was autoclaved prior to use.

2.1.6. Microscopy

Except where stated, cell cultures were examined using a Leica© DM IRB Microscope, with EBQ100 UV lamp, and photographed using a QImaging QICAM 1384 camera and QCapture image capture software.

2.2. General cell preparation and culture methods

2.2.1. Primary rat liver cells

2.2.1.1. Isolation of primary rat hepatocytes

To obtain fresh rat primary hepatocytes, male rats (Wistar strain) between 180 g and 500 g in weight were first killed by cervical dislocation. The abdomen was rapidly dissected and the liver removed. The two largest liver lobes were placed on a sintered glass platform and their main vessels cannulated in parallel for perfusion with the buffers, which had been pre-warmed to 37°C. Buffer A was perfused for 10 minutes, during which accurate placement of the cannulas was checked by examining for warming, swelling and blood clearance from the lobes. Buffer B was then perfused, with recirculation of the buffer solution, for 15-20 minutes until the tissue was judged to appear sufficiently digested. The lobes were then removed to a Petri dish containing medium 1 at 37°C and the Glisson's capsule gently teased apart using forceps. The tissue was then flushed with further medium 1, both to halt the action of the collagenase in the digestion solution and to release the maximum numbers of hepatocytes. Liberated cells in suspension were then separated from any remaining undigested tissue by filtering through a polyester gauze mesh with 64 µm apertures.

The liver cell suspension was decanted into two conical ended centrifuge tubes and a total of approximately 100 ml was centrifuged for 5 minutes at low speed (50*g*), with minimum acceleration and deceleration forces, to pellet the hepatocyte-rich fraction. The supernatant was removed and retained for isolation of hepatic stellate cells. The pellets were re-suspended in further medium 1 and the centrifugation step repeated,

to rinse the cells. Following this step, the hepatocyte pellets were re-suspended in 20 ml of medium 1 and combined in a single conical-ended centrifuge tube. An equal volume of 90% Percoll solution was added and the tube was inverted, gently, to mix the contents. This mixture was centrifuged again, under the same conditions, in order to enrich the proportion of viable hepatocytes obtained.

The pellet obtained from this final centrifugation step was resuspended in medium 1 or 2, depending on the requirements of the ongoing experiment, and hepatocyte viability and numbers were determined using a Trypan Blue exclusion method[83], as follows. Briefly, 20 µl of the cell suspension was added to 20 µl of Trypan Blue dye in a sterile capped tube and inverted briefly to mix. This mixture was introduced, by capillary action, into the chamber of an Improved Neubauer haemocytometer and the chamber was examined by light microscopy at 100x magnification. Total hepatocyte numbers were counted in the entire 1 mm delineated field of the haemocytometer (X). Hepatocytes which showed evidence of the blue dye in their cytoplasm and nucleus (and were therefore assumed to have damaged cell membranes) were counted as dead cells (Y). The proportion of live hepatocytes was calculated as:

$$\frac{X-Y}{X}\times 100\%$$

The concentration of viable hepatocytes, per ml of cell suspension, was calculated as:

$$(X-Y)\times 20{,}000$$

2.2.1.2. Culture of rat primary hepatocytes

For standard control monoculture, hepatocytes were plated into collagen-coated 6-well plates at a target density of $10^5/cm^2$. Each culture well was washed with phosphate buffered saline prior to use, then approximately 900,000 viable hepatocytes were seeded to each well in a total of 1.5 ml medium 1. After 2 hours incubation (37°C in 5% CO_2), to allow cell attachment, the medium was aspirated and replaced with medium 2 for ongoing incubation. Thereafter, medium changes were carried out every 48 hours.

2.2.1.3. Isolation of rat primary hepatic stellate cells (HSC)

The supernatants obtained at each stage of hepatocyte preparation, described above, were combined and centrifuged twice more at 50 *g*, discarding the pellet on each occasion. Finally, the remaining supernatant was centrifuged at 250 *g* to pellet an HSC-rich fraction. The pellet was resuspended in medium 3 prior to culture.

2.2.1.4. Culture of rat primary hepatic stellate cells

The HSC-rich suspension was plated into a 75 cm^2 tissue culture flask with a total of 20 ml of medium 3. The flasks were incubated at 37°C in 5% CO_2 and the medium was exchanged after overnight incubation. Thereafter, the medium was changed every 3 days. When 80-90% confluent, the cells were either used in co-culture as described in chapter 5, or passaged to encourage proliferation and activation, as follows:

The cell-culture medium was removed and 5 ml EDTA-trypsin solution (pre-warmed to 37°C) was added to each flask. The flask was incubated at 37°C for about

5 minutes or until visible cell detachment began to occur. Pre-warmed medium 3 (5 ml) was added and the cells detached and separated using a cell scraper. The resultant cell suspension was centrifuged for 5 minutes at 250 *g*. The pelleted cells were re-suspended in medium 3, with half of the volume plated into each of two further 75cm^2 tissue culture flasks, for repeat culture as previously described.

2.2.2. Huh7 cell line

2.2.2.1. Culture of the Huh7 cell line

Standard tissue-culture plastic-ware was used, with no additional treatment. Huh7 cells were cultured and manipulated in a containment level 2 laboratory.

Huh7 cells were cultured in monolayers on 75 cm^2 flasks with filter lids, using medium 4. The medium was changed after overnight incubation and at twice-weekly intervals thereafter. Huh7 cells were passaged weekly, to provide cells at 80-95% confluence for experiments where required. To provide overnight control cultures after transfection experiments, approximately 1 x 10^5 cells/cm^2 were seeded into 75 cm^2 flasks and the media changes carried out as above.

2.2.2.2. Proliferating the Huh7 cell line

The culture medium was removed and 5 ml of pre-warmed trypsin-EDTA solution was added. The flask was re-incubated at 37°C for 2-3 minutes, until signs of cell detachment were visible. The flask was tapped to loosen adherent cells; then 10 ml of pre-warmed medium 4 was added and washed repeatedly over the culture surface of the flask to detach and separate the cells. The resulting cell suspension was then

centrifuged for 5 minutes at 150*g*, the supernatant discarded and the cell pellet re-suspended in medium 4 before adding to the required ongoing cultures.

2.2.2.3. Cryopreservation and thawing of Huh7 cell line

A cell suspension was obtained by the same procedure as for passaging, above. The cells were counted using a haemocytometer. The Huh7s were then centrifuged again under the same conditions and resuspended in a 9:1 mixture of medium 4 and dimethyl sulfoxide (DMSO) for cryoprotection. Sufficient medium was added to produce a cell concentration of 10^6/ml, and the suspension was sealed into cryostorage vials, in 1 ml aliquots. Working quickly, and on ice, the vials were filled and removed to a freezer at -80°C. After overnight freezing, one vial was removed and re-cultured to check that viability and cell numbers had been maintained; the remaining aliquots were transferred to liquid nitrogen cryostorage.

2.3. Statistical methods

Numerical data were tabulated in the spreadsheet package Microsoft Office Excel 2007 and standard equations within the software were used to obtain values for the mean, standard deviation(SD), and standard deviation relative to the mean (RSD) where applicable. Comparisons of group data were perfomed using the online calculators available at http://www.graphpad.com/quickcalcs/index.cfm. A probability of ≤5% ($p \leq 0.05$) that the null hypothesis was incorrectly rejected was chosen to indicate statistical significance, by scientific convention. Unpaired, 2-tailed student's T-tests were used to obtain p-values and confidence intervals for differences in continuous data values, unless otherwise stated in the text. Graphical representations of data were produced using Microsoft Office Excel 2007.

3. ISOLATION AND CULTURE OF PRIMARY HUMAN HEPATOCYTES

3.1. Introduction

3.1.1. Challenges encountered when isolating human, rather than rat, hepatocytes

Rat primary hepatocytes are widely used in many areas of research. Methods for the isolation of rat hepatocytes have been refined since the first protocols were developed in the 1960s and have been comprehensively described[65,66]. Although they require experience and extreme care in their execution, these methods can generally be relied upon to produce predictable quantities of viable hepatocytes and other liver cells. In contrast, the isolation of primary hepatocytes from human liver tissue is much less widely practised or reported. There are a number of important differences between rat and human hepatocyte isolation, and some specific difficulties in obtaining suitable human tissue for cell isolation.

The most obvious difference between the rat liver and a human liver is that of size. There are also significant dissimilarities in the number of lobes and their disposition. On a microscopic level, the lobular architecture is relatively similar between the species, however the human capsule is significantly thicker and more difficult to disrupt.

Another significant difference involves the physical practicality of working with liver tissue of the two species. Starting with a complete rat liver, the worker can perfuse several whole, encapsulated lobes. In contrast, human liver tissue is most commonly obtained as a by-product of partial hepatectomy or liver resection operations. For

this reason only one or two segments of a lobe are most commonly removed, rather than a complete, intact lobe. The human tissue therefore frequently has a disrupted capsule and may have inadequate intact vasculature for perfusion of the whole tissue segment.

The quality and quantity of hepatocytes obtained from any species is affected by the underlying health and vasculature of the liver tissue, the composition of buffer solutions and media, the time (and any mechanical force) used for digestion and variations of buffer solution temperature during perfusion. However, other factors, which are avoided when using laboratory animals, affect the viability and functionality of human cells obtained as a by-product of surgical procedures, such as the underlying operative indication, age of the donor, the presence of liver disease and the warm ischaemic time. These issues are discussed further below.

3.1.2. Sourcing human liver tissue

Advances in medical care, and growing numbers of patients with complex co-morbidities, are driving increasing needs for the development of bioartificial liver devices and model systems for the assessment of drug toxicity, and for further *in vitro* research into the processes underlying liver tissue regeneration. There is therefore an essential requirement for primary human cells to use in these applications. Human liver cells can be sourced from cadaver tissue which has been rejected, or resized, for use in transplantation. However, the reasons for rejection may also influence the subsequent function of the isolated cells in culture; additionally, tissue is rarely available from such sources. More commonly therefore, cells are harvested from the tissue discarded during surgical liver resections.

The most common reason for liver resection in the UK is removal of metastases of colonic carcinoma, although, due to the source of its blood supply, the liver is susceptible to tumour metastasis from most sites in the gut. Primary hepatocellular carcinomas (HCC) and certain benign tumours may also be resectable. Depending on the location of the tumour(s) within the resected lobe, surrounding healthy tissue may not always be needed for histological confirmation that sufficient tumour margins have been excised. This tissue may thus be used to obtain cells for research, if the patient consents.

Colorectal cancer is the second commonest cause of cancer-related death in the UK. Around 32000 cases are diagnosed each year and more than half of these patients will have liver metastases, either at the time of diagnosis or in the years following removal of their primary colonic tumour. This currently equates to around 18000 cases per year in the UK. Once detected, and without treatment for this secondary disease, median survival is only 8 months and 5-year survival rates are extremely low. In contrast, the 5-year survival rate after liver resection is reportedly up to 44%[84]. Operative mortality ranges from 0-7%, depending on a variety of pre- and intra-operative factors. However, due to pre-existing morbidities and the individual characteristics (and spread) of their liver metastases, only approximately 3600 patients per year will be considered suitable for liver resection surgery[84]. At Nottingham University Hospitals NHS Trust, around 50-100 patients are listed for this operation each year.

Open surgical resection, to remove the affected part of the liver, has been the standard treatment for patients with localised colorectal liver metastases and HCC. Benign liver tumours are usually treated only if they are causing symptoms. The

standard resection procedure is performed through a large incision across the abdomen and is a major operation, usually taking several hours.

However, a number of alternative or additional treatments are now available, which may increase the likelihood of a successful outcome and/or reduce post-operative morbidity. Most notably, laparoscopic ("keyhole") surgery may sometimes be possible. In this procedure, a number of small incisions are made in the abdominal wall, to provide access for the laparoscope and surgical instruments. The resected liver is enclosed in a bag and removed through another relatively small incision. The National Institute for Clinical Excellence (NICE) reports that post-operative hospital stay is generally significantly shorter after laparoscopic liver resection (mean stay ranged from 4 to 15 days) than after open liver resection (mean stay ranged from 8 to 22 days)[85]; but not all metastases are sufficiently isolated to be amenable to this technique. Other possible treatments include direct ablation of the tumour (using thermal, radiowave or microwave energy). Pre-operative procedures, to shrink the tumour(s) using chemotherapy or embolization of the portal vein branches, may also be of benefit. Whilst beneficial for the patient, these procedures now reduce the likelihood that resected liver tissue will be available, or suitable, for the preparation of hepatocytes for *in vitro* culture.

3.1.3. Ethical issues and consent

Patients scheduled to undergo hepatic resection may be requested to donate, for research purposes, those parts of the resected tissue which are not required for diagnostic tests. The use of such specimens poses relatively few ethical problems, since the research work does not affect the patient's care in any way. However, in general this patient group has been rapidly progressed to face major surgery

following a, perhaps unexpected, diagnosis of malignancy and consequently the patient must be approached with sensitivity and respect, to ensure that proper informed consent is obtained. Furthermore, it is important that the surgical procedure and its outcome are not altered by participation in the study.

3.1.4. Inherent problems in obtaining suitable human liver tissue

There are many practical and ethical constraints and obstacles to obtaining suitable human liver tissue for successful hepatocyte isolation.

The opportunities for obtaining resected hepatic tissue are necessarily limited by the number of operations carried out. Further, a significant number of resection procedures are unfortunately delayed, or cancelled, due to lack of availability of intensive care beds for post-operative care. Alternatively, operations may be curtailed due to inoperability; usually because of peritoneal spread. In addition, there may be tissue-specific factors (including cirrhosis, steatosis and the location of metastases) which may cause the liver material to be unsuitable or unavailable for research purposes. All of the above hurdles make obtaining suitable human liver tissue highly unpredictable. For projects requiring primary cells which, generally, do not replicate *in vitro* these logistical problems may cause major and unavoidable delays.

Primary human hepatocytes are optimally isolated from fresh, healthy *post-mortem* or *ex-vivo* liver tissue. Cells obtained from fatty or steatotic livers are difficult to purify, due to their variable and unpredictable density and even greater than usual fragility, usually resulting in a lower yield. There is also a significant inverse correlation between donor age and the viability of isolated hepatocytes[64,86]. Other

researchers report that cirrhosis, cholestasis and increased intra-operative clamping (Pringle) time negatively affect the yield[64,86,87]: the last of these factors presumably depressing yield due to both warm ischaemia and blood coagulation in the capillary beds.

Warm ischaemia is defined as the interruption of blood supply to tissue which remains at body, or room, temperature. The temperature at which ischaemia occurs is vitally important as it determines the overall metabolic status of the cells and therefore the scale of detrimental effects. Warm ischaemia is probably the main factor adversely affecting the viability of hepatocytes obtained from resected tissue but is, at certain stages, unavoidable during resections. Intra-operative interruption of the blood supply by clamping is frequently employed and, even after anatomical separation of the resected portion, there is usually some delay before the liver tissue is removed from the abdominal cavity. Unfortunately, during laparoscopic resections (which are favoured where possible due to faster recovery times), the warm ischaemic time of the resected tissue is generally increased by the additional time required for this technically demanding procedure. Post-operatively, liver cells deteriorate rapidly, so that samples must be processed without delay. Cold ischaemic time has also been found to affect the viability and yield of liver cells but in a less predictable manner[64,86].

Due to ethical issues and the limited routes by which it may be obtained, human liver tissue for research purposes is not readily available to most workers. For this reason, relatively few methods for the extraction of human liver cells have been published, and none of them can be considered well established, compared with the techniques available for extracting the liver cell populations from rodents. Some researchers

using human liver cells *in vitro* purchase their cells from commercial companies, whose methods of extraction are considered commercially confidential. However, a few workers have described methods for cell extraction, from which protocols may be derived.

3.1.5. Overview of primary human hepatocyte isolation techniques

Donated tissue is collected in the operating theatre and processing should start without delay, which can create logistical problems if the tissue becomes available at or beyond the end of a normal working day. If the tissue must be stored or transported before perfusion it should be maintained on ice or in an ice-cold preservation solution[88-91]. It is also important to flush the tissue with a buffer or preservation solution before storage, to remove blood from the capillary vasculature before coagulation (and blockage) occurs[92,93]. Where processing can begin within one hour of obtaining the tissue segment, published methods suggest simply transporting the sample to the laboratory in ice-cold saline or preservation solution, as quickly as possible[91,94].

Once the sample is in the laboratory, perfusion can be started, as the first step in the process of separating the cells. Tissue weights of between 50 g and 100 g yield the optimum numbers of cells per gram[91]. Smaller tissue fragments may lack suitable blood vessels for sufficient perfusion, whereas larger specimens will be exposed to proportionately less collagenase activity during the perfusion time and will not be optimally digested. Ideally, the tissue segment should have only one cut edge and be otherwise encapsulated by Glisson's capsule, so that the perfusion fluid is relatively well contained. Most workers seal unused cut blood vessels on the tissue edge with either glue or sutures[64,91].

A modified two-stage perfusion is usually performed. The perfusion buffers used vary[88,89,94,95] but are almost always pre-warmed to 37°C. The first stage is to flush the blood from the vasculature before coagulation and blockage occurs. The perfusion buffer used may contain a calcium-chelating agent, to disrupt the tight junctions between cells. The second stage involves a pre-warmed buffer containing collagenase or similar enzymes, to disrupt and digest the extracellular matrix. In some cases, an additional perfusion step has been performed between stages one and two, in order to remove chelating agent from the vasculature before introducing the (calcium-dependent) collagenase enzyme[94].

The buffer composition, buffer volumes, perfusion rate, duration of perfusion, and temperature of the first buffer have varied between reporting laboratories[64,89,91,94,95]. The type and concentration of digestion enzymes is also far from standardised, with some researchers having recirculated the second stage buffer and others not having done so. Furthermore, as collagenase is a mixture of proteolytic enzymes derived from bacteria, its efficiency and toxicity varies according to the source, and also over time, making its effects unpredictable[64]. In the absence of universally adopted automated equipment, all laboratories which prepare primary cells from liver tissue are likely to have differing perfusion circuits, through which the buffers are circulated, and the circuit structure, capacity and temperature are rarely described by authors. The optimal conditions for human liver tissue digestion are therefore unknown.

The period of time allowed for tissue digestion is, like any enzymatic reaction, affected by several factors:

- concentration (and location) of the enzyme
- concentration (and location) of the substrate
- environmental conditions (for example pH, temperature, presence of ions and other co-factors or inhibitors)

A process of continuous perfusion of liver samples facilitates maintenance of the correct temperature and provides a constant presence of fresh enzyme mix. However, the amount of substrate (the extra-cellular matrix, ECM) and its distribution relative to the route of enzyme delivery (the liver vasculature) is widely variable and unpredictable in the sample obtained. If the second stage of perfusion is too short, there will be insufficient exposure to the enzymes and consequently less digestion of the tissue. Any extra mechanical force which is then required to harvest the cells from an insufficiently loosened ECM will increase physical damage to, and breakage of, the isolated cells. On the other hand, the proteases used for digesting the liver's extra-cellular matrix will also damage the parenchymal cells, if present for too long or at too high a concentration. Furthermore, as mentioned previously, the warm ischaemia necessitated by the digestion period also leads to hepatocyte damage and death.

Following tissue digestion, the capsule surrounding the lobe is incised or broken using sterile instruments, at which point any dissociated cells are released. Some workers suggest that the tissue should be minced at this stage and subjected to a

further incubation in a collagenase solution to release the hepatocytes from the extra-cellular matrix[95]. The liberated hepatocytes are separated from the resultant suspension of mixed cells by low-speed centrifugation, with or without use of a density-gradient centrifugation step to purify the viable hepatocyte fraction[64,90]. The non-parenchymal fraction remains within the supernatant and can be subjected to further, higher speed centrifugation to enrich the hepatic stellate cell fraction.

Mean hepatocyte viabilities achieved by various authors ranged from 70 - 83%[64,86,90]. Mean yields, per gram of digested tissue, ranged from 0.12 - 83 million viable hepatocytes[90,93,96]. The average human adult liver, *in vivo*, is thought to contain approximately 10^8 hepatocytes per gram of tissue[97].

The remainder of this chapter presents the rationale for, and results obtained during, refinement of a method for the isolation of human primary hepatocytes from locally-obtained resected liver tissue. By this method, viable human hepatocytes could be reliably obtained for the first time in the University of Nottingham laboratory and made available for ongoing experiments.

3.2. Outline of the procedure for transporting and processing human liver tissue to obtain primary hepatocytes

In brief, the steps required to obtain hepatocytes from resected liver tissue were as follows.

- Planned resections were reviewed, to identify patients with the potential to provide suitable tissue, and patients were interviewed to obtain informed consent.

- A perfusion circuit was constructed in the laboratory and readied for processing samples.

- The resected liver segment(s) was taken from the donor's body cavity in the operating theatre and a distal piece was removed for hepatocyte isolation.

- The donated tissue was prepared for transport and conveyed to the research laboratory.

- The liver tissue was established on the perfusion circuit and perfused with buffers and enzymes to digest the liver structure.

- A crude suspension of liver cells was liberated from the tissue and filtered to obtain a single-cell suspension.

- The cell suspension was washed and centrifuged to obtain a hepatocyte-rich fraction.

3.3. Materials and methods

3.3.1. Materials

Marshall's hypertonic citrate solution (marketed as Soltran Kidney Perfusion solution) was obtained from Baxter Healthcare, UK. Gibco™ Liver Perfusion Medium and Gibco™ Liver Digest Medium were purchased from Invitrogen™. Other materials were obtained and prepared as described in chapter 2.

3.3.2. Obtaining liver tissue samples

3.3.2.1. Evaluation of patients and obtaining informed consent

Approval to enrol patients in the current study was sought and gained from the Local Research Ethics Committee (LREC) and from the local Trust's Research and Development Department. Patients who were due to undergo some form of surgical liver resection were seen for medical review at the surgical pre-assessment, around 1-2 weeks prior to the scheduled operation date. At this time, those who were expected to have an open (rather than a laparoscopic) procedure, and who were not known to have generalised liver disease or damage, were given information about the study and asked whether they would consent to donate their resected tissue to the research team. Patients had the opportunity to ask further questions and signed a written consent form if they agreed to participate. It was a condition of the study that no patient data were collected and that the sources of specimens were anonymous to the research team (other than the consenting doctor).

During the period September 2004 to September 2006, hepatic resections were scheduled for operation approximately once every two weeks in the local centre (Nottingham University Hospitals NHS Trust). Over this period, a total of 109 patients

attended the Surgical Pre-assessment Clinic around 1-2 weeks prior to their operation date, for a baseline medical assessment and anaesthetic review, and their cases were assessed for potential inclusion in the present study. During these appointments, a total of 39 patients were approached for consent to donate resected liver tissue to the study. Patients were not included in the study if they were known to have hepatic fibrosis, fatty liver disease, extensive metastases or were planned to have tissue removed by laparoscopic or radio-ablative means. The study protocol and its aims were well accepted by the majority of patients approached for the study and, of the 39 patients approached, 38 consented to donate resected hepatic tissue for the research. The patient information sheet and consent form are contained in Appendix 8.1.

3.3.2.2. Co-ordination and logistics for sampling

On the day of operation, the theatre sister and/or consultant surgeon were contacted at the time the operation was expected to start, to ascertain whether the procedure would be carried out as planned. Unfortunately, about half of the 38 planned resections, in which patients had consented to donate tissue, were cancelled or postponed due to patient factors or the unavailability of post-operative care facilities. Once verbal confirmation was received that an operation would go ahead, it was then essential to maintain good communication and co-ordination with operating theatre staff, to optimise the potential for acquiring good quality samples and to ensure that resected tissue specimens were not inadvertently discarded, damaged, or subjected to undue bacterial contamination. Therefore, once surgery was underway in the remaining cases, contact with the theatre staff was maintained by telephone and pager until the final stage of resection was started (usually around

3-5 hours after the patient entered theatre). From this point on it was necessary to be present in the theatre, as the tissue could be made available at any time (although tissue procurement could take up to a further 3 hours, depending on intra-operative factors).

3.3.2.3. Sampling

Liver tissue was removed from the donor patient's abdominal cavity as soon as possible after its separation from the remaining *in vivo* liver, in order to minimise the warm ischaemic time. Following dissection of the resected tissue to identify the diseased segment(s), the lead surgeon then removed an apparently healthy portion of the resected tissue for use in hepatocyte isolation (Figure 3.1). However, due to the varying location(s) of tumours or cysts within the resected tissue, samples for hepatocyte isolation were sometimes unavailable, smaller than required or poorly encapsulated. From the 38 potential sources of samples, a total of only 27 samples suitable for use in the present study were obtained. Where possible, this was an encapsulated end wedge of tissue distal to the tumour site. The median weight of the tissue samples was 70 g, although individual sample weights were highly variable (range = 8 – 251 g). Taking into account the rather high failure rate, due to factors beyond the control of the study, an average of approximately 5 hours intensive work was required to obtain each of the samples considered suitable for further processing, using currently available technology. Future improvements in operative and patient treatment technologies may decrease the frequency with which samples, of the size and status required for the present study, can be obtained.

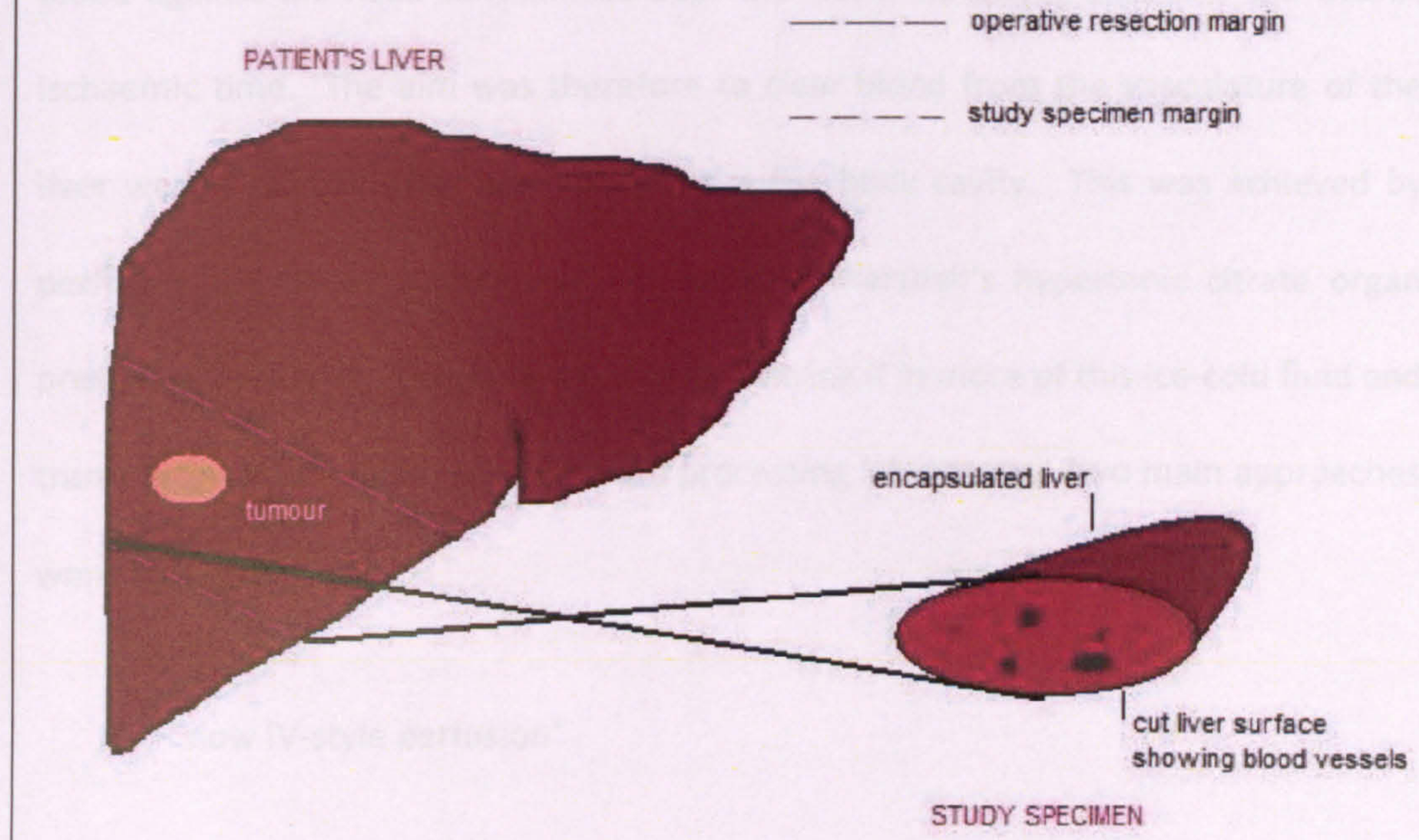

Figure 3.1: Example diagram showing the anatomical source of a well perfused and encapsulated liver tissue specimen for in vitro isolation of hepatocytes.

3.3.2.4. Preparing and transporting the liver tissue sample from the operating theatre to the laboratory

Two methods for preparing and transporting the liver tissue to the laboratory were assessed. It was necessary to balance the need to clear the tissue of coagulating blood against the need to minimise both the warm ischaemic time and the overall ischaemic time. The aim was therefore to clear blood from the vasculature of the liver wedge directly after its removal from the body cavity. This was achieved by perfusing the tissue vasculature with ice-cold Marshall's hypertonic citrate organ preservation solution (Soltran), prior to immersing it in more of this ice-cold fluid and transporting the sample, on ice, to the processing laboratory. Two main approaches were assessed:

a. "slow IV-style perfusion"

b. "rapid syringed perfusion"

A total of 4 samples were treated using the first method (a). An intravenous fluid giving set and cannula was attached to a pre-refrigerated bag of Soltran solution (mounted on a drip-stand) and each visible vessel on the cut surface(s) of the tissue was manually perfused in turn, until no further blood flowed from the tissue. However, even if an inflatable cuff was used around the solution bag to increase the pressure and hence the rate of flow, this process was found to take up to half an hour and usually resulted in only incomplete clearance of blood. Moreover, due to conditions and facilities in the operating theatre, the specimen and fluid could not be kept optimally chilled during this procedure.

In a total of 23 subsequent experiments, tissue specimens were cleared of blood and simultaneously chilled by the following, alternative method (b). A 50 ml Luer-lock syringe was filled with pre-chilled Soltran solution and the nozzle applied directly to accessible vessels in the sample. The tissue was rapidly perfused by firmly depressing the plunger and the process repeated for each vessel until no further blood flowed from the tissue. The second method (b) was adopted for two reasons. Firstly, the tissue reached the laboratory (and hence the start of the isolation procedure) more quickly and ischaemic time was therefore minimised. Secondly, both the Soltran solution and the cooled tissue sample were subject to less environmental warming in the operating theatre.

Following either method of perfusion, the tissue sample was placed in a sterile 1 L plastic screw-capped pot containing a known amount of pre-chilled Soltran solution and placed on ice in an insulated box for transport to the laboratory. Some further blood was generally passively washed from the sample during carriage; the remainder was rapidly cleared from the sample during the initial phase of perfusion with chelating buffer (as described further below).

3.3.3. Constructing and preparing liver tissue perfusion circuits

The requirements of a perfusion circuit for the digestion of human liver tissue are broadly as follows:

a. The perfusion buffers must be retained at around 37°C. Although this creates a period of warm ischaemic time for the hepatocytes, this temperature is required for the optimal activity of collagenase or other enzymes used to digest the tissue. Two pumps are thus required: one to circulate the buffer fluids through the tissue and another to circulate warm water around the circuit and bottles of buffer fluid in order to keep them at 37 ± 1°C.

b. There should be the facility to switch between running the perfused fluid to waste or to recirculate it back through the tissue. Whilst chelating buffer becomes quickly contaminated with blood, and therefore must be discarded, it may be desirable to recycle digestion buffers (to minimise costs).

The perfusion circuitry used for these experiments was mainly determined by the availability of existing hardware. Two pumped circuits were created, as shown in figure 3.2.

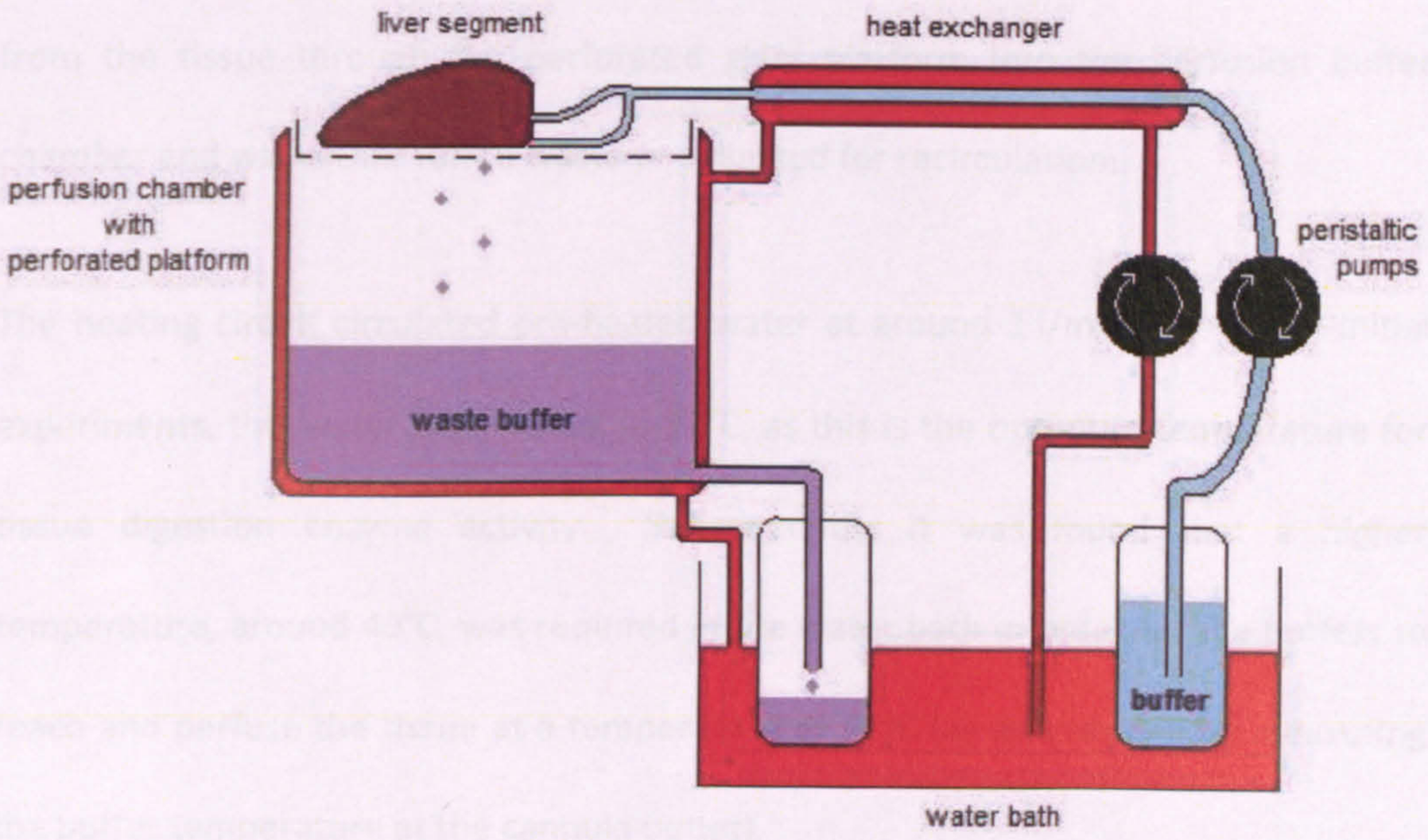

Figure 3.2: Schematic diagram of the perfusion circuit used to perfuse liver tissue specimens obtained during the present work.

One circuit (shown in red in figure 3.2 and hereafter referred to as the heating circuit) was used to circulate pre-heated water from a water bath through the outer chambers of a glass, coiled tube, heat exchanger and a glass perfusion vessel. The second circuit (shown in blue and purple in figure 3.2 and hereafter referred to as the buffer circuit) used a second peristaltic pump to circulate perfusion buffers through the heat exchanger and into the cannulated liver tissue, which was suspended in the perfusion vessel on a perforated glass platform. Buffer was then allowed to drain from the tissue through the perforated glass platform into the perfusion buffer chamber and was either run to waste or collected for recirculation.

The heating circuit circulated pre-heated water at around 1 l/minute. In the initial experiments, this water was heated to 37°C, as this is the optimum temperature for tissue digestion enzyme activity. Subsequently, it was found that a higher temperature, around 40°C, was required in the water bath in order for the buffers to reach and perfuse the tissue at a temperature of 37°C (as determined by measuring the buffer temperature at the cannula outlet).

The buffer circuit was used to pump the perfusion buffers through the heat exchanger and into the liver tissue segment. After passage through the heat exchanger, a horizontal Y-connector split the flow equally to allow the tissue to be perfused through two blood vessels simultaneously. It was found that a flow rate of 21 ml/min per cannula was normally required to ensure a constant forward flow of buffer through the tissue samples without causing any perceptible damage to the tissue (other than the intended digestion) or undue buffer loss. The buffer circuit was rinsed with fresh, sterile phosphate-buffered saline immediately prior to use, as shown in figure 3.3, below.

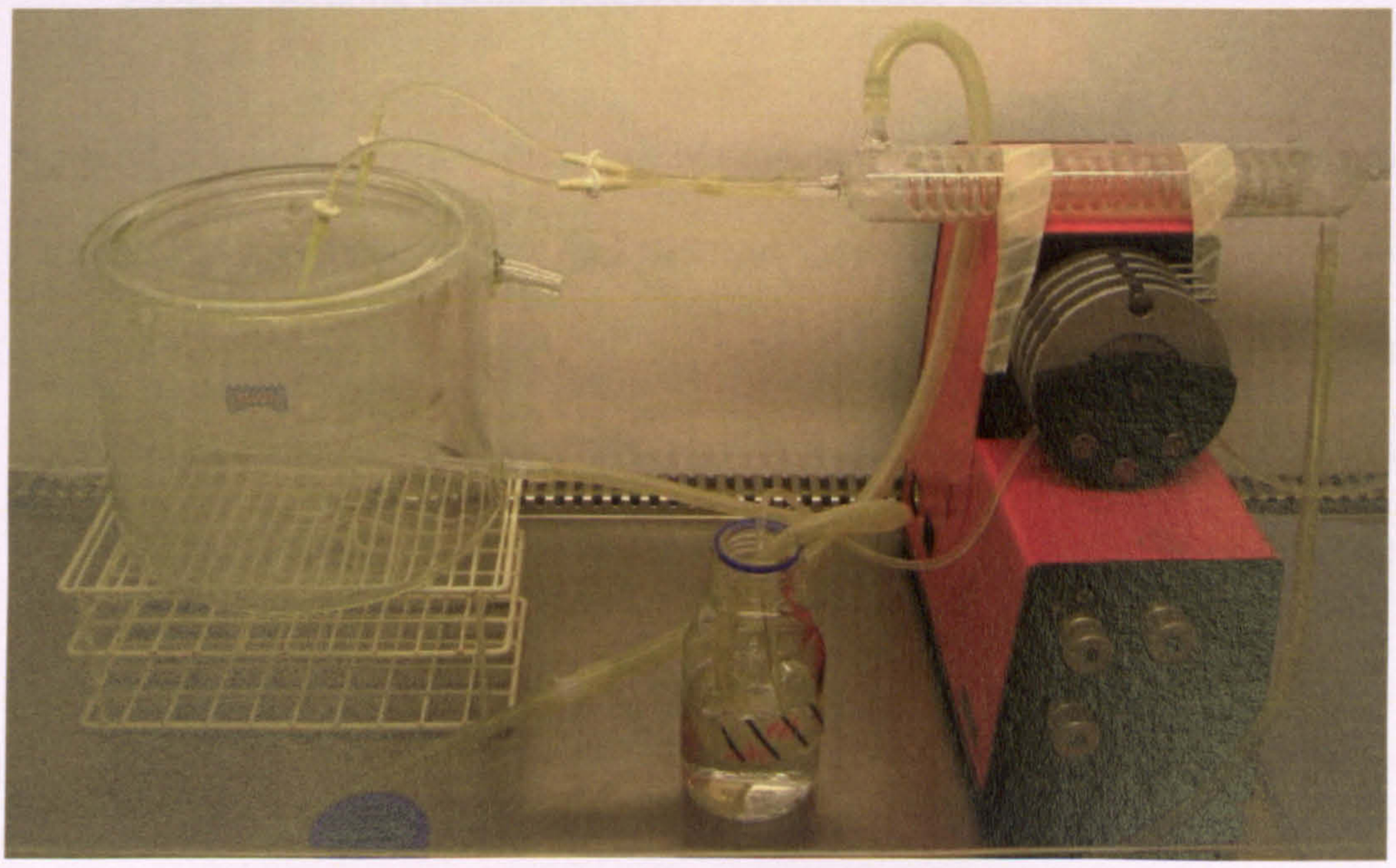

Figure 3.3: Photograph showing how the buffer circuit was flushed with phosphate buffer saline before use, in order to remove any detergent residue.

3.3.4. Preparing and perfusing the liver tissue in the laboratory

On arrival at the laboratory the pot containing the tissue specimen was weighed, so that the pre-perfusion tissue weight could be calculated. Working in a class 2 safety cabinet, the liver tissue specimen was then removed from the pot, which was also weighed, and processed as described below.

3.3.4.1. Securing vascular access to the liver tissue specimen

In a total of 8 initial experiments, plastic IV cannulae (Venflon 18G-22G) were used to introduce the perfusion buffers into the liver tissue. These soft plastic cannulae could be cut to length depending on the vasculature of each tissue specimen and were glued in place using quick-drying hardware glue ("superglue"). The glue was also used to seal any unused vessels from which perfusion buffer leaked during processing.

Experience showed that this approach presented a number of disadvantages. Firstly, an exact match of cannula diameter and blood vessel width was very difficult to achieve, and often the vessels were too large for even the largest cannula grades. Secondly, use of the glue to seal vessel gaps (in both cannulated and uncannulated vessels), although advocated by a number of workers in this field[91,98], was of unpredictable efficacy and also created substantial delay before and during the initial stages of tissue perfusion, thereby increasing the warm ischaemic time.

For subsequent experiments the IV cannulas were replaced with sterile plastic pipette tips (20-200 µl capacity), as shown in figure 3.4.

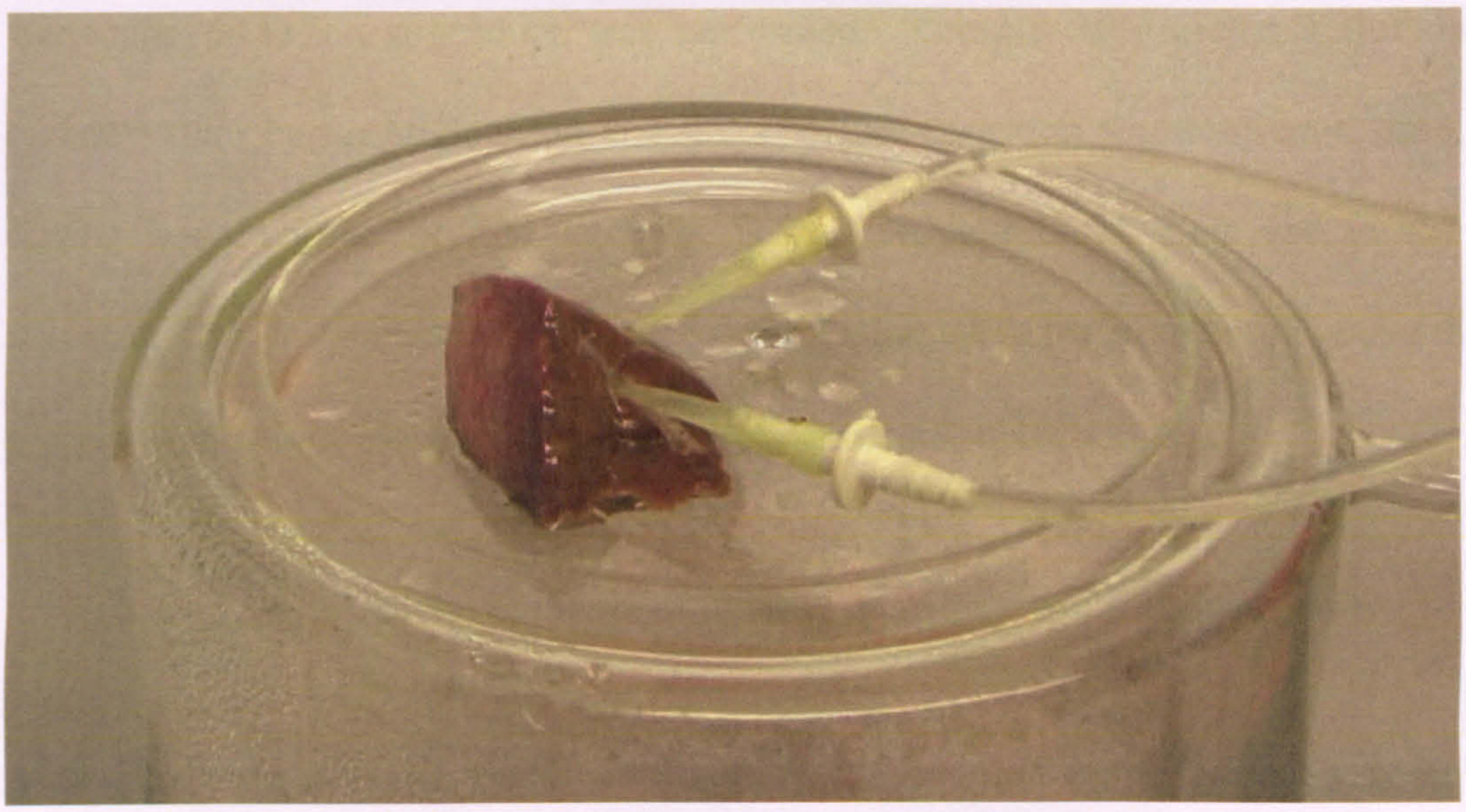

Figure 3.4: Photograph showing the cannulation of blood vessels on the cut sides of a liver tissue specimen, using sterile plastic pipette tips. Glisson's capsule is visible on the left hand surface of the specimen.

These pipette tips had the advantage of being rigid and therefore were not prone to kinking in the tissue vasculature. Most importantly, however, their tapering form allowed them to be cut to fit the tissue's blood vessels exactly. This improved fit had the added advantage of removing the need to glue the cannula in place and therefore reducing warm ischaemic time and increasing hepatocyte viability.

Once reliable access to the vasculature had been secured (using either method), a two-stage perfusion procedure was performed. Commercially available buffers (Gibco™ Liver Perfusion Solution and Gibco™ Liver Digestion Solution) were used, having been chosen on the basis of favourable reports of their use in the literature[86,91,99]. Appropriate values for several other variables were not recorded in the published literature and had to be determined, as described below.

3.3.4.2. First (chelating) stage of the two-stage liver perfusion procedure

A review of the available published literature showed that the reported optimum duration of the first stage, during which blood is flushed from the capillary beds and the cell junctions begin to dissociate as a result of calcium being removed by the chelating agent, ranged from 10 – 30 minutes, depending on the tissue size and buffer composition[86,89,91]. The procedure adopted for the first stage in the present work was essentially adapted from the rodent liver perfusion protocol (described in chapter 2), following the review of available literature. Bottles of Liver Perfusion Medium were pre-warmed to 40°C and connected to the perfusion circuit, as shown in figure 3.2. The peristaltic pump was activated and medium allowed to flow through the circuit (before cannulation of the liver tissue) until no visible air bubbles remained, whereupon the pump was halted. After cannulation of the tissue, as described above, the pump was restarted and flow of medium was allowed to

proceed for 20 minutes (using a maximum of 1 l of Liver Perfusion Medium, irrespective of sample size and the number of cannulae).

3.3.4.3. Second (digesting) stage of the two-stage liver perfusion procedure

For liver tissue to be optimally digested, tissue throughout the sample must be exposed to sufficient digestive enzyme at optimum temperature and for sufficient time. The activities of collagenases and other digestive enzymes in the Liver Digest Medium were not disclosed by the manufacturer but were stated to have been batch-adjusted for consistency. The optimum working temperature for all batches was stated to be 37°C.

Both stages of the two-stage liver perfusion technique expose the liver tissue to a further period of warm ischaemia. Furthermore, exposure to collagenases and other digestive enzymes, together with concurrent loss of the supportive extra-cellular matrix, is damaging to hepatocytes. The time allowed for tissue digestion is therefore critical, not only for the release of optimum numbers of cells, but also for the viability of those cells. Since the rate of flow and concentration of perfusion solution was pre-determined in this experiment (as described above), the mass of tissue to be treated was the main measurable variable affecting the time required for digestion. More specifically, the optimum time for perfusion was expected to be proportional to the perfused vascular area of the tissue wedge. For all practical purposes however (i.e. while retaining maximum numbers of viable hepatocytes), the vascular area and volume of tissue being satisfactorily perfused cannot be quantified prior to digestion.

Published reports of the time allowed for digestion of human liver range from 10-30 minutes, where stated[91,95,99], although many workers either used in-house preparations of digestion medium or whole lobes of liver tissue. In the present work, it was noted in a total of 13 preliminary experiments that a digestion time of 20-30 minutes sometimes resulted in very little tissue digestion and low hepatocyte yield (see Table 3.1). It was, however, hypothesised that the damaging effects of a longer digestion period would be mitigated if cold ischaemia was induced immediately after the second stage of perfusion (as in section 3.3.5.2), and consequently should produce higher total yields of intact hepatocytes. Subsequently, therefore, the digestion stage was allowed to proceed for 40-50 minutes, depending on total tissue weight and a visual estimate of the perfused volume, in order to test the following hypothesis:

Hypothesis 1: that prolonging circulation of the Liver Digest Medium, and reducing the duration of post-digestion warm ischaemia, could increase hepatocyte yield without causing an overall reduction in hepatocyte viability.

Following advice from other workers in the field (see Acknowledgements), 1 l of Liver Digest Medium was perfused, with recirculation of the second 500 ml, over this period. Recirculation in this way avoided consumption of up to an additional 1 l of this reagent, helping to limit the costs involved and apparently without compromising the yield and activity of the hepatocytes obtained.

3.3.5. Post-perfusion tissue disaggregation and hepatocyte isolation

The intra- and post-operative warm ischaemic time is inversely proportional to the viability of hepatocytes and the advantages of inducing cold ischaemia in liver tissue before and during transport to the laboratory are well documented[86,88,91]. Unfortunately, warm ischaemia is unavoidable during tissue perfusion, due to the temperatures required for enzyme activity. However, it was hypothesized that the re-induction of cold ischaemia during post-digestion processing (i.e. while harvesting the cell suspension and isolating the hepatocyte-rich fraction) would reduce the rate of hepatocyte deterioration and death during this part of the isolation procedure. A subset of experiments was therefore performed to assess whether hepatocyte viability could be improved by re-inducing cold ischaemia directly after tissue perfusion was completed and maintaining the cells at, or below, 4°C until placed into culture conditions.

In a total of 13 initial experiments the hepatocyte fraction was isolated as described

3.3.5.1. Harvesting the liver suspension and isolating the hepatocyte-rich fraction

Once the tissue matrix was judged, by appearance and feel, to have been adequately digested, the flow of buffer was stopped. Working quickly, the cannulae were removed and the liver tissue was carefully lifted from the perfusion platform and placed into a fresh sterile 1 l beaker containing 150 ml of medium 5, pre-warmed to 37°C. The capsule, and any superficial undigested liver tissue, was incised with sterile scissors. The tissue was gently agitated by hand to encourage the cell suspension to flow into the surrounding medium. Undigested lumps of material were then removed from the beaker using sterile forceps and weighed to assess the amount of

tissue digested. The remaining mixture was filtered into a sterile, 750 ml measuring cylinder via a polyester gauze mesh with 64 µm square apertures (as described in section 2.2.1.1) to produce a suspension of single cells.

Working quickly, the liver cell suspension was made up to approximately 200 ml with further pre-warmed medium 5 and then decanted into 4 x 50 ml Falcon tubes. After centrifugation at room temperature (5 minutes at 50 *g* with slow acceleration and braking), the supernatant (containing the non-parenchymal cell fraction) was removed and the cell pellets were carefully re-suspended in further medium 1. Two further, identical centrifugation-resuspension cycles were performed to wash the parenchymal cell fraction. After the final centrifugation, the hepatocyte-rich cell pellets were resuspended and combined in medium 5, giving a total volume of 25 ml. This hepatocyte-rich suspension was gently added to 25 ml of Percoll working solution and mixed by slow inversion and rotation of the tube. Finally, the cell suspension was centrifuged for 5 minutes at 100 *g* and room temperature to obtain a cell pellet enriched in intact hepatocytes. The pellet was then resuspended in cell culture medium as determined by subsequent experimental plans. The total number of hepatocytes obtained was calculated from the cell numbers counted, manually, by light microscopy using an improved Neubauer haemocytometric chamber. The percentage of viable hepatocytes present was determined by the Trypan Blue exclusion method (as described in Chapter 2) and viable cell numbers isolated per gram of sample tissue were calculated.

In a further 14 experiments this protocol was adjusted, as described in section 3.3.5.2, in order to test the following hypothesis:

Hypothesis 2: that post-digestion cooling of the liver cell suspension from 37°C to between 0 and 4°C would increase hepatocyte viability.

3.3.5.2. Induction and maintenance of cold ischaemia in the isolated liver cells

In a total of 14 experiments, the final two steps of the liver cell isolation method were adjusted as follows:

Following removal of the cannulae (or pipette tips) at the end of the two-stage perfusion procedure, the tissue was placed into a fresh sterile beaker containing 200 ml of ice-cold medium 5. The cell suspension was liberated and undigested material was removed, as in 3.3.5.1. The remaining mixture was diluted with further ice-cold medium 5 and, using a procedure that was otherwise similar to that in 3.3.5.1, it was filtered into a pre-cooled sterile, 750 ml measuring cylinder.

The cell suspension thus obtained was equally divided between 4 pre-cooled 50 ml Falcon tubes which were placed into a beaker of melting ice between subsequent centrifugation cycles. Centrifugation purification steps were carried out as in 3.3.5.1 but the centrifuge chamber was refrigerated to 4°C before and during use.

The final number and viability of hepatocytes was, again, assessed by counting and the Trypan Blue dye exclusion test.

3.4. Results

The effects of two variables in the human hepatocyte isolation method, digestion time and post-isolation temperature (as outlined in sections 3.3.4.3 and 3.3.5.2, respectively), were the main subjects of experiments to optimise the numbers and viability of hepatocytes obtained. Results from 13 experiments involving digestion periods of 20 or 30 minutes coupled with post-digestion procedures conducted at room temperature are presented in table 3.1. Results from 14 subsequent experiments, which combined longer periods of tissue digestion with re-induction of cold ischaemia after digestion, are shown in table 3.2.

Table 3.1: Hepatocyte yields from human liver samples, using digestion periods of 20 or 30 minutes at 37°C and performing post-digestion procedures at room temperature (~22°C) (i.e. without re-induction of cold ischaemia)

Liver sample	Sample weight (g)	Digestion time (min)[1]	Undigested material remaining (g)	Digested tissue weight (g)	Proportion of sample digested (%)	Hepatocyte viability[2] (%)	Total viable hepatocytes from whole sample ($x10^6$)	Viable hepatocytes per gram of whole sample ($x10^6$)	Viable hepatocytes per gram of digested sample ($x10^6$)
1	45	20[3]	34	11	24.4	NM[4]	NM[4]	-	-
2	55	30[3]	35	20	36.3	90	341.4	6.20	17.07
3	251	30[3]	246	5	2.0	85	10.3	0.04	2.06
4	220	30[3]	211	9	4.1	91	120.0	0.55	13.33
5	100	30	87	13	13.0	25	24.1	0.24	1.85
6	NR	20	NR	-	-	NM[5]	NM[5]	-	-
7	192	20	165	27	14.1	25	600.0	3.13	22.22
8	74	20	66	8	10.8	44	97.6	1.32	12.20
9	67	20	54	13	19.4	68	86.1	1.29	6.62
10	41	20	35	6	14.6	33	30.0	0.73	5.00
11	NR	20	NR	-	-	70	1.5	-	-
12	NR	20	NR	-	-	31	240.0	-	-
13	NR	20	NR	-	-	40	25.7	-	-

NR = not recorded. NM = not measurable.

[1] After Sample 8, tissue samples were cannulated with plastic pipette tips, reducing warm ischaemic time and improving perfusion efficiency.

[2] Post-Percoll centrifugation.

[3] Digestion temperature was ~35°C in these experiments, as the perfusion fluid temperature (37°C) did not take into account cooling during circulation.

[4] Gross contamination of the final cell suspension with red blood cells prevented accurate counting of hepatocytes and arose from the extreme difficulty in achieving satisfactory initial clearance of blood from the sample, using method (a) (section 3.3.2.4, above), in the operating theatre.

[5] The hepatocyte fraction was accidentally discarded following a failure of the cells to sediment during centrifugation, due to steatosis.

Table 3.2: Hepatocyte yields from human liver samples, using digestion periods of 30, 40 or 50 minutes and performing post-digestion procedures at 0-4°C (i.e. with re-induction of cold ischaemia)

Liver sample	Sample weight (g)	Digestion time (min)	Undigested material remaining (g)	Digested tissue weight (g)	Proportion of sample digested (%)	Hepatocyte viability[1] (%)	Total viable hepatocytes isolated from whole sample ($x10^6$)	Viable hepatocytes per gram of whole sample ($x10^6$)	Viable hepatocytes per gram of digested sample ($x10^6$)
14	NR	30	NR	-	-	65	4.2	-	-
15	NR	30	NR	-	-	70	37.0	-	-
16	28	40	22	6	21.4	84	51.4	1.84	8.57
17	NR	40	NR	-	-	97	27.0	-	-
18	NR	40	NR	-	-	87	45.3	-	
19	23	40	7	7	30.4	92	53.0	2.30	7.57
20	57	50	45	12	21.1	94	62.6	1.10	5.22
21	8	40	5	3	37.5	92	37.6	4.70	12.53
22	140	50	127	13	9.3	91	7.7	0.06	0.59
23	72	50	61	11	15.3	95	312.0	4.33	28.36
24	62	50	51	11	17.7	94	412.0	6.65	37.45
25	43	50	26	17	39.5	95	152.0	3.53	8.94
26	25	40	19	6	24.0	88	94.8	3.79	15.80
27	79	50	68	11	13.9	91	254.0	3.22	23.09

NR = not recorded.
[1] Post-Percoll centrifugation.

3.4.1. Effect of digestion time on the proportion of tissue digested

Figure 3.5 shows the effect of digestion time on the proportions of tissue digested, for samples where pre- and post-digestion sample weights were recorded. The proportion of tissue digested cannot have been influenced by differences in the post-digestion experimental conditions and therefore the data are not differentiated on the basis of this criterion.

3.4.2. Viability of the harvested hepatocyte populations

Figure 3.6 shows the effect of post-digestion processing temperature on hepatocyte viability.

Hepatocyte cell populations isolated (post-perfusion/digestion) at, or below, 4°C had a mean viability of 88.2% (95% confidence interval 83.2-93.2 %), compared with 54.7% (95% confidence interval = 39.7-69.7%) for cells isolated at room temperature. Failure to maintain the isolated hepatocytes at or below 4°C was therefore associated with a very significantly lower heptocyte viability ($P = 0.0002$ by unpaired t-test) as shown in Figure 3.6.

3.4.3. Total and relative yields of viable hepatocytes

The weight of original tissue sample, and remaining undigested tissue weight following perfusion, was measured and recorded in a total of 19 experiments. The recording of these parameters allowed calculation of the digested tissue weight and, thence, the yield of viable hepatocytes per gram of digested tissue, as shown in tables 3.1 and 3.2.

Somewhat surprisingly, little or no correlation was observed between sample weight and the yield of viable hepatocytes, whether the yield was expressed as numbers per whole sample weight (Figure 3.7), as numbers per gram of sample tissue (Figure 3.8), or as numbers per gram of digested tissue (Figure 3.9). The lack of correlation remained if the data for room temperature and chilled post-digestion processing were plotted separately.

However, some correlation was evident between digestion time and hepatocyte yield and, in particular, the correlation differed according to the post-digestion isolation protocol used. When post-digestion processing of hepatocytes was carried out at room temperature, there was a trend towards lower total yields of viable hepatocytes as second perfusion stage (digestion) time was increased from 20 to 30 minutes. In contrast, when post-digestion processing was carried out at 0-4°C, the viable cell yield tended to increase as digestion time from 30 to 40 or 50 minutes, as shown in figure 3.10.

The mean tissue weight for samples processed at room temperature, post-digestion, was 125 g (range 41-251 g), with a mean viable hepatocyte yield of 1.69×10^6/g of whole sample weight or 10.04×10^6/g of digested tissue weight. The mean tissue weight for samples processed at 0-4°C, post-digestion, was 54 g (range 8 – 140 g), with a mean viable hepatocyte yield of 3.15×10^6/g of whole sample weight or 14.81×10^6/g of digested tissue weight. The increases in yield produced by isolation under chilled conditions (whole sample average ~100% higher and digested tissue average ~50% higher than was achieved by isolation at room temperature) (figure 3.11) were not statistically significant however ($P = 0.629$ and 0.320 respectively).

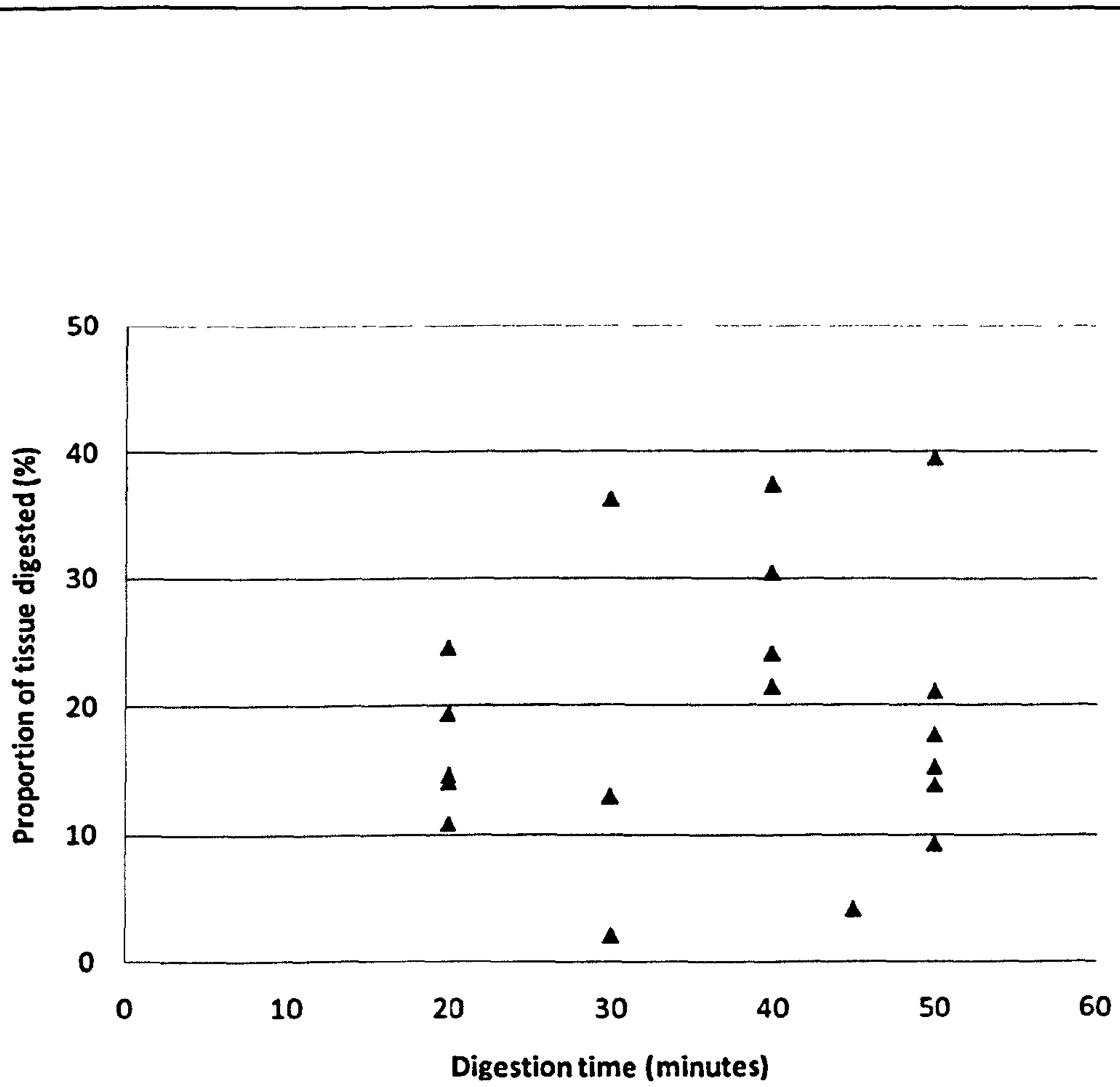

Figure 3.5: *No correlation was observed between the digestion time and the proportion of tissue sample digested.*

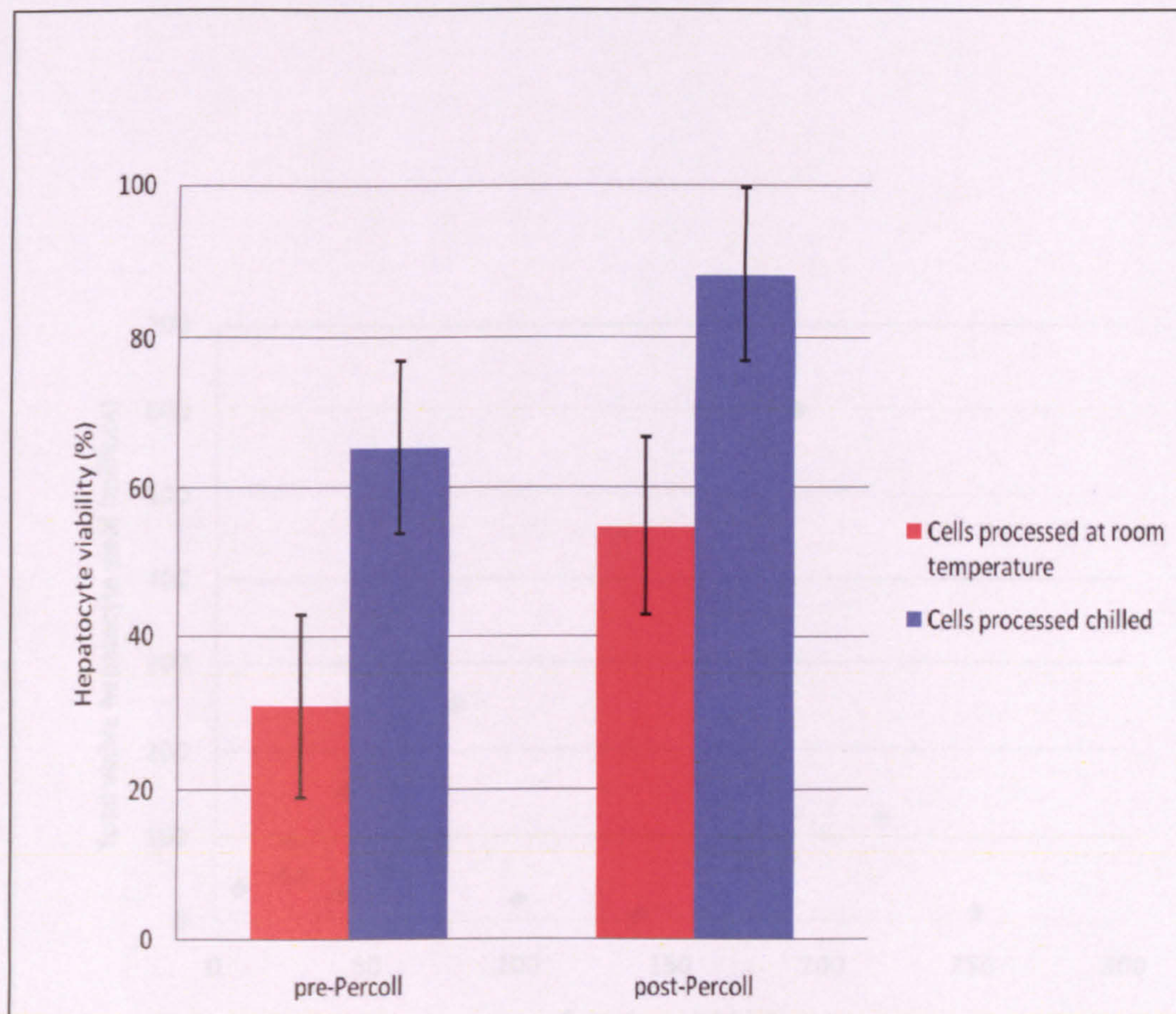

Figure 3.6: *Viability of isolated hepatocytes is significantly increased by re-induction of cold ischaemia during post-digestion processing, both before and after enrichment of the viable hepatocytes fraction by centrifugation through Percoll (showing standard error bars; P = 0.004 and 0.0002 respectively)*

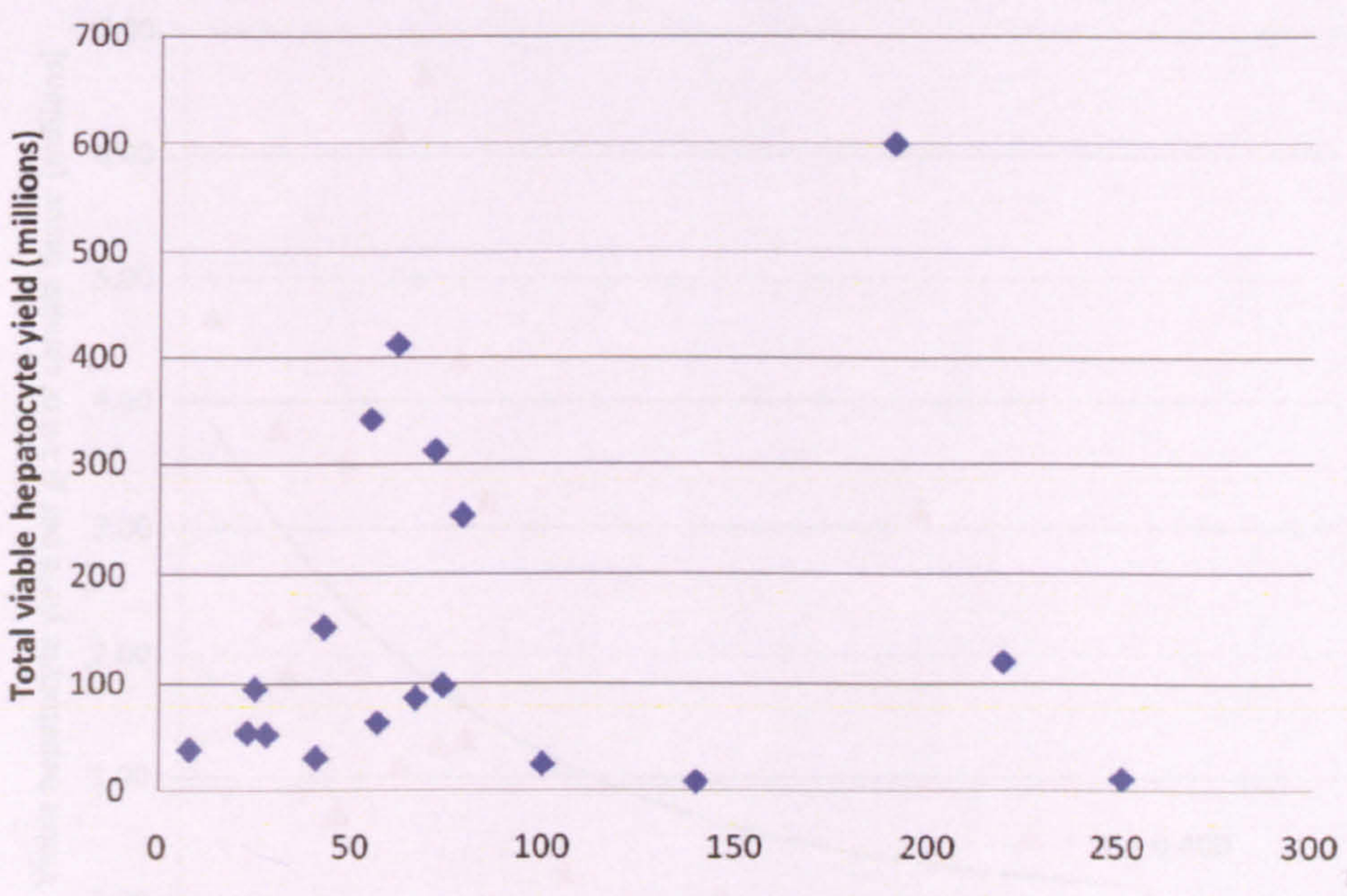

Figure 3.7: Relationship between human liver sample weight and the total yield of viable hepatocytes obtained, showing essentially no correlation.

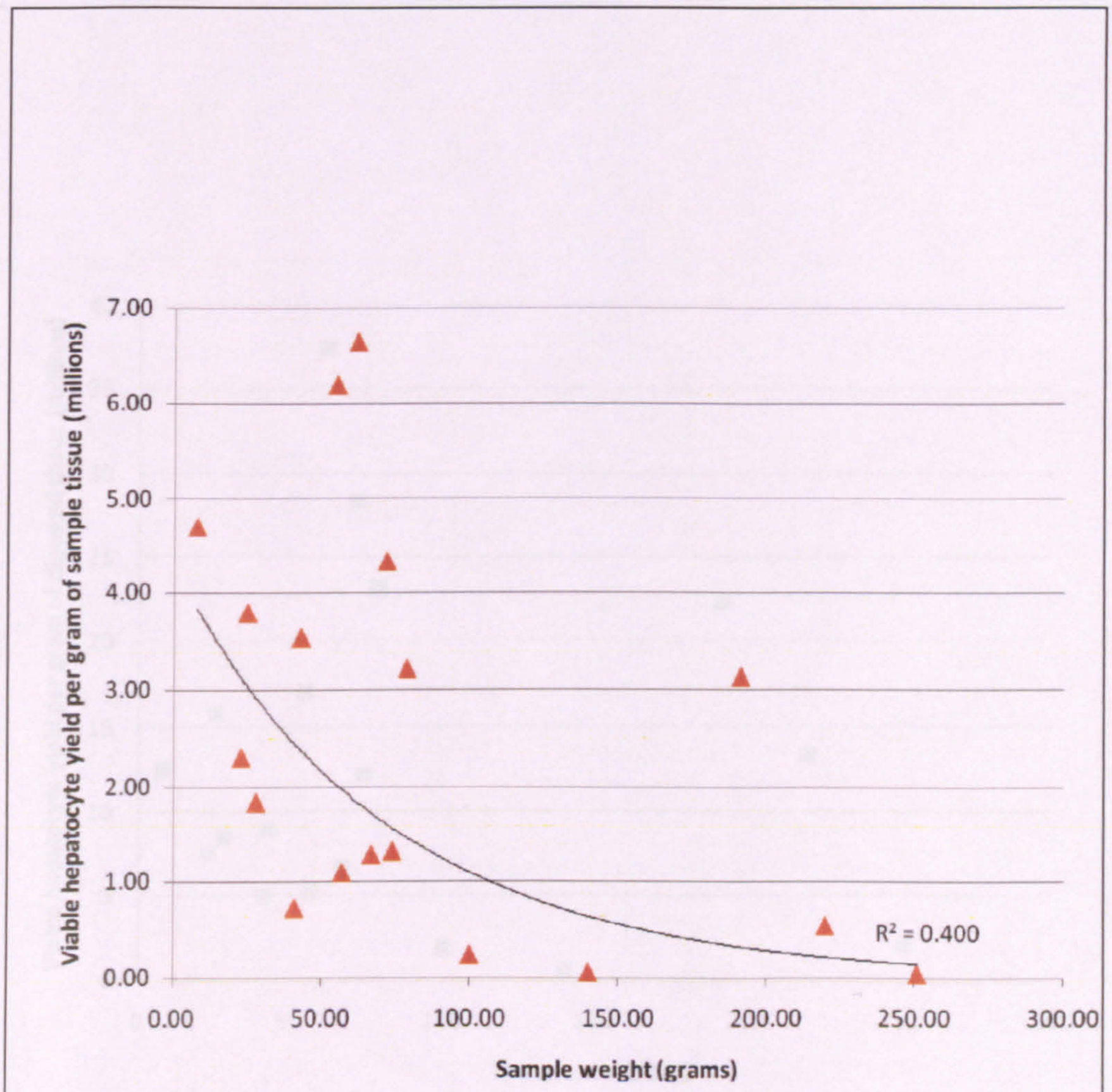

Figure 3.8: Relationship between human liver sample weight and the yield of viable hepatocytes per gram of sample tissue, showing poor correlation ($R^2 = 0.400$).

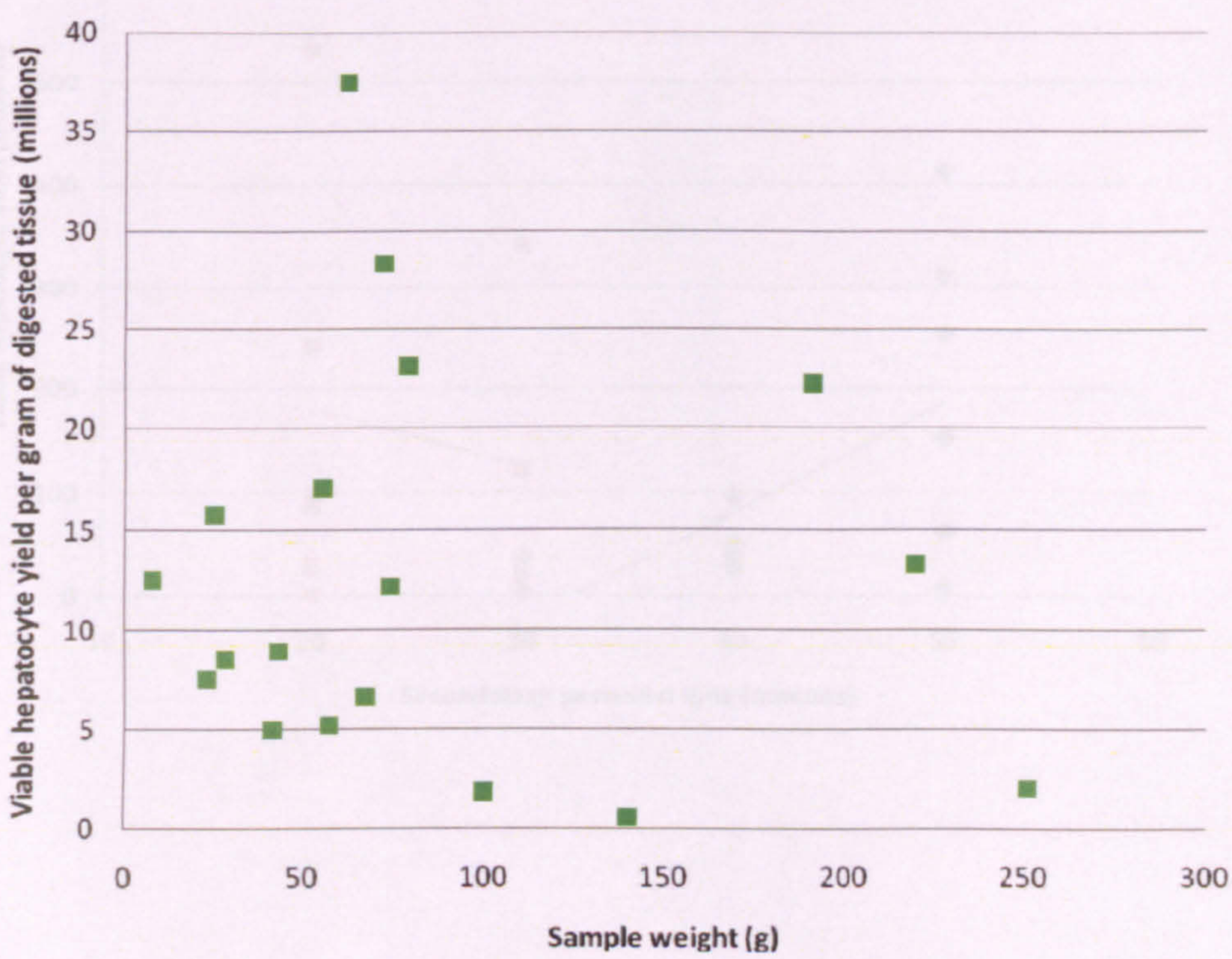

Figure 3.9: Relationship between human liver sample weight and the yield of viable hepatocytes per gram of digested tissue, showing essentially no correlation.

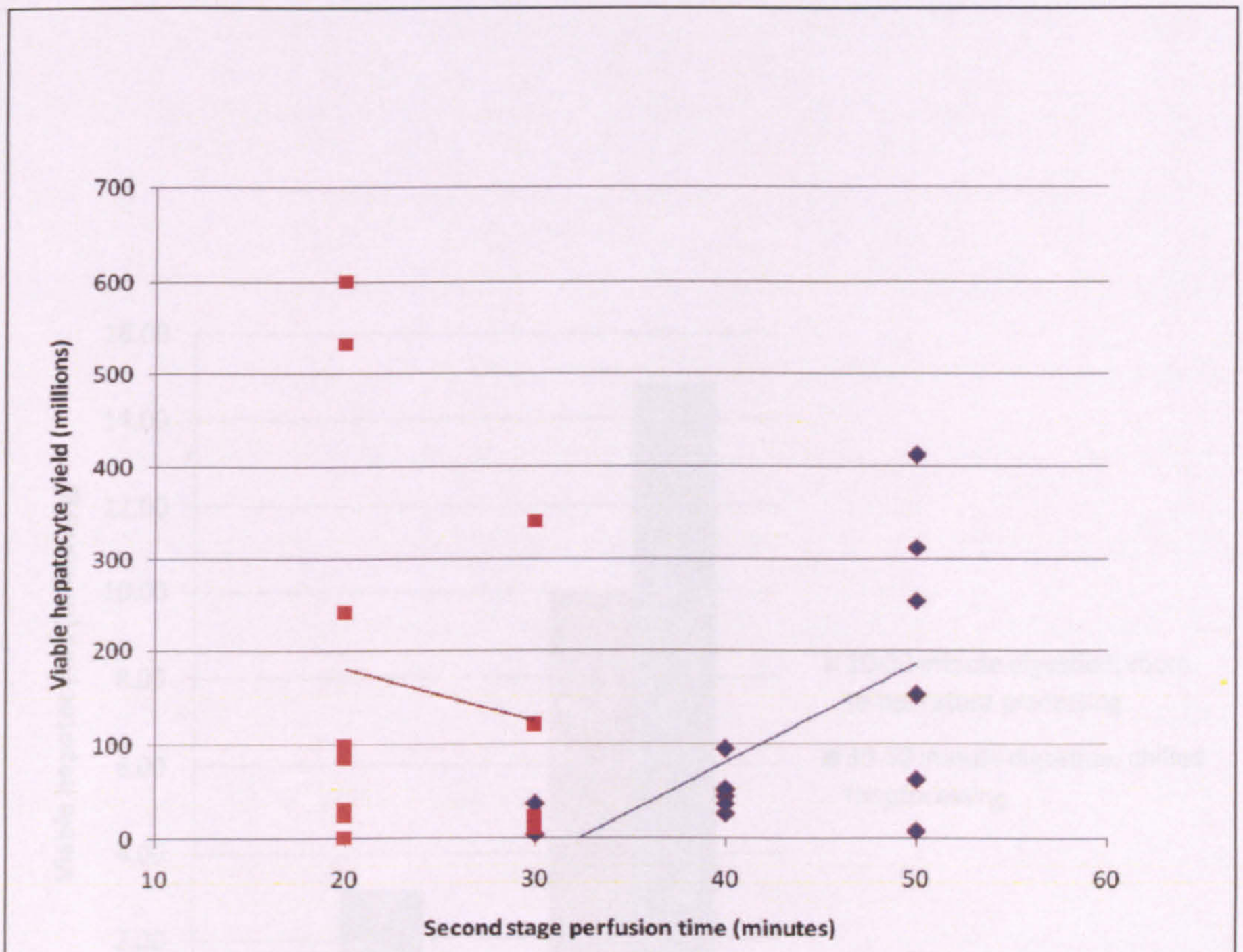

Figure 3.10: *Samples which were processed at room temperature post-digestion showed a trend to yield fewer viable hepatocytes, in total, if the digestion period was allowed to proceed for more than 20 minutes (■; trend-line shown in red). Chilling the cell suspension to 0-4°C during post-digestion processing tended to allow a higher proportion of viable hepatocytes to be obtained without compromising overall viable hepatocyte numbers (◆; trend line shown in blue).*

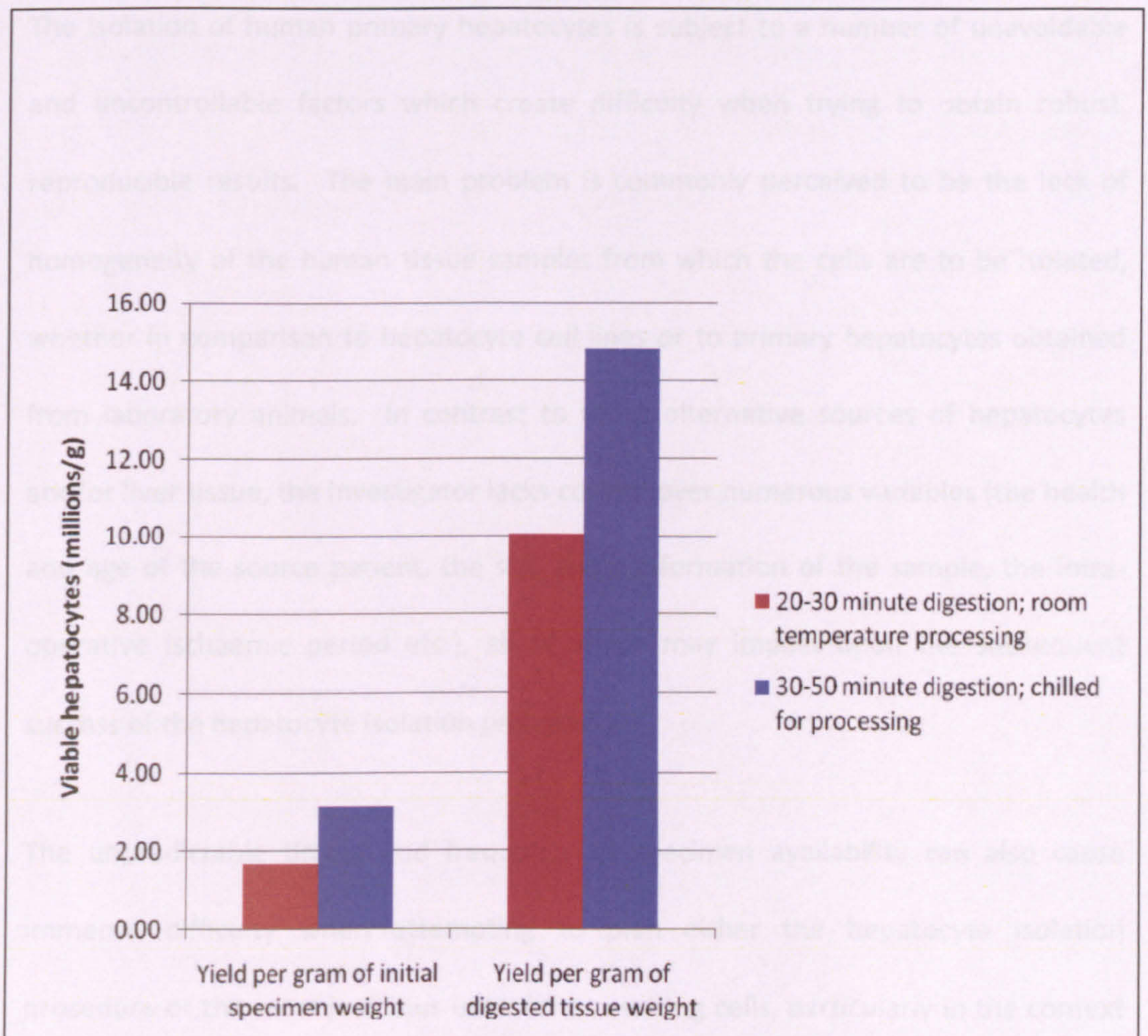

Figure 3.11: Use of a prolonged digestion time with post-digestion chilling to between 0-4°C showed a trend to increase the yield of viable cells as a proportion of the digested tissue weight. However, this difference did not reach significance (p = 0.320).

3.5. Discussion

The isolation of human primary hepatocytes is subject to a number of unavoidable and uncontrollable factors which create difficulty when trying to obtain robust, reproducible results. The main problem is commonly perceived to be the lack of homogeneity of the human tissue samples from which the cells are to be isolated, whether in comparison to hepatocyte cell lines or to primary hepatocytes obtained from laboratory animals. In contrast to these alternative sources of hepatocytes and/or liver tissue, the investigator lacks control over numerous variables (the health and age of the source patient, the size and conformation of the sample, the intra-operative ischaemic period etc.), all of which may impact upon the subsequent success of the hepatocyte isolation process.

The unpredictable timing and frequency of specimen availability can also cause immense difficulty when attempting to plan either the hepatocyte isolation procedure or the most judicious use of the resulting cells, particularly in the context of shared laboratory resources and facilities. In ideal circumstances, a dedicated tissue perfusion and cell isolation facility would be maintained, in order to make maximum and optimal use of whatever tissue specimens become available, even when liver resection operations are planned and executed at relatively short notice. A number of such facilities now exist in the UK. The potential advantages of such laboratories (and the skilled staff who must use them), are tempered by the geographical, temporal, and financial factors encountered when distributing the resulting hepatocytes, as there is still no published protocol for cryostorage of these cells which does not result in significant loss of viability. However, as the NHS becomes increasingly structured to form fewer, but larger, Centres of Excellence for

complex procedures such as partial hepatectomy, it may become increasingly unusual for individual researcher in the UK to have either the need or the opportunity to isolate their own primary cells from human liver tissue. In the meantime, several further challenges confront those researchers who are able to source human liver tissue for hepatocyte isolation.

In the experiments described above, sample size and anatomy were found to be highly variable. In particular, the numbers, and distribution, of intact and sufficiently sized blood vessels were frequently sub-optimal. The extent to which each specimen remained encapsulated by Glisson's capsule was also extremely variable. At least half of the tissue specimens obtained during these experiments had more than one cut surface. This commonly gave rise to two problems affecting tissue perfusion. Firstly, many of the blood vessels on one surface might pass almost directly through to the second surface without any significant supply to the capillary beds in between, and therefore it could be difficult to find sufficient vascular access for perfusion. Secondly, the increase in unencapsulated surface area probably allowed more perfusion fluid to leak out of the tissue during perfusion, thereby reducing the internal vascular pressure (and hence the extent of distribution of the perfusion fluid through the sinusoidal beds).

The above factors frequently made it impossible to achieve extensive parenchymal distribution of the perfusion fluids, thereby significantly limiting the potential yield of hepatocytes. Due to the relative infrequency of sample availability, hepatocyte isolation was attempted from all samples of relatively healthy tissue that were obtained. However, particular challenges were encountered with the samples which were under 30 g in weight, as most were thin strips of tissue which had a

proportionately larger cut surface from which leakage could occur. Despite these difficulties, the lack of an obvious correlation between sample weight and hepatocyte yields (as described and shown in section 3.4.1.2) indicates that a flexible approach to cannulation and perfusion of low-weight specimens could produce acceptable (if perhaps sub-maximal) yields of viable hepatocytes.

Securing good vascular access to the tissue, in order to achieve widespread perfusion with minimal unwanted leakage of perfusion fluid, presented an unpredictable challenge for each individual specimen. The precise methods and materials used for tissue cannulation were determined, during preliminary experiments, to be highly influential on the ultimate success of the hepatocyte isolation procedure. The optimal technique derived from the experiments described above had a number of advantages. Firstly, matching pipette tip diameter to the vasculature aperture, for successful cannulation, was simply and rapidly achieved by visual inspection and cutting the tip with a pair of sterile scissors. Furthermore, the rigid nature of the pipette tips was useful both to stabilise the tissue specimen on the perfusion platform and to ensure un-occluded flow of the perfusion fluids. Lastly, as the pipette tips were cut to fit each blood vessel in a multiply-cannulated piece of tissue, the perfusion volume passing through each would have been approximately in proportion to the size of the blood vessel in question. As the blood vessel diameter is itself likely to be proportionate to the volume of tissue it perfuses (assuming that the tissue is homogeneous, healthy and uninterrupted, which was unfortunately not always the case), the sample might therefore have been more uniformly perfused than if the tip outlet size was the same (i.e. uncut) in each blood vessel.

Gluing of large, obviously leaking, unused blood vessels was found to be helpful on occasion and was performed when necessary. However, gluing of the whole cut surface, in order to contain the perfusion fluids within the tissue sample, was found to be difficult and time-consuming. An over-the-counter coagulation spray, sold for use on superficial wounds, was more simple to apply, but did not result in a good seal to the surface and appeared to cause excessive hepatocyte mortality during subsequent tissue disaggregation and post-digestion processing of the cell suspension.

Perhaps the most significant challenge, present during the above experiments, was the maintenance of hepatocyte viability. If viability was not a concern of the researcher, it would have been possible to collect the sample from the operating theatre without particular urgency and with no special transport conditions, store it until a convenient time, and then perhaps apply a combination of mechanical mincing and crude collagenase digestion until 100% of the parenchymal matter had been liberated into suspension, thus obtaining a very high yield indeed. However, this is a physiologically unrealistic scenario for working with hepatocytes and many precautions must be followed to minimise the proportion cells dying before, during, and after isolation of the hepatocytes from the tissue matrix. In other words, increasing the number of hepatocytes liberated by digestion for longer periods is only worthwhile if their viability is maintained, otherwise the increase in liberated cell numbers is outweighed by their increased loss of viability.

As described in section 3.1.5, the advantage of chilling the ischaemic tissue as soon as practicable following its removal from the patient's body cavity (i.e. pre-digestion) has been widely reported. Swift induction, and strict maintenance, of a temperature

between 0 and 4°C is required, and the materials utilised for this procedure may need to be maintained chilled for several hours in the (usually very warm) operating theatre prior to use. Here, again, good communication with the more experienced members of theatre staff is required, in order to avoid prematurely setting up (and increasing the warming of) the materials to receive the tissue specimen. Rapid transport of the sample to the laboratory from the hospital was, fortunately, aided by the adjacent locations of these facilities in the present study.

The re-induction of cold ischaemia, immediately after digestion of the liver tissue samples, was associated with a significant increase in the proportion of viable hepatocytes obtained following processing of the liver cell suspension. Maintenance of the low temperature required strict adherence to the regime at all steps of the post-digestion procedure in order to achieve success. All plastic-ware and solutions had to be pre-cooled at -20°C and 4°C, respectively, and then contained in or on melting ice during use to maintain a temperature of 0-4°C. However, it was important not to allow the hepatocytes to settle into contact with the cooled sides of their containers for any prolonged period of time, in order to avoid direct thermal damage to the cells.

In the above experiments, the mean hepatocyte viability of 88%, achieved when using the adjusted isolation method with prolonged digestion time and induction of cold ischaemia after digestion, was high in comparison with the results published by other workers. In contrast, the low percentage viability (mean 55%) which was obtained prior to alteration of the main method precluded the use of the resulting hepatocytes in downstream experiments due to the high, and detrimental, percentage of dead and/or dying cells. Such cells cause unwanted clumping due to

the release of DNA and may induce damage in co-existing viable cells, due to apoptotic signalling and cell breakdown. Due to the relative lack of proliferative capacity of primary hepatocytes, they are commonly only used in cell culture applications if the population viability is at least 75%. Thus the ability, using a modified isolation protocol, to reduce the dead hepatocyte fraction to only 5-15% of the total was not only statistically significant but also presented an ongoing advantage when using the cells in subsequent experiments.

Perhaps even more importantly, processing at room temperature produced much more variable results (viability RSD = 49%) in contrast to the more predictable results obtained when using chilled processing (viability RSD = 11%). This conferred another extremely important advantage to the modified protocol, particularly in view of the infrequent nature with which human liver tissue samples could be obtained.

The mean yields of viable hepatocytes, per gram of digested tissue, were within the ranges published by other researchers, as detailed in section 3.1.5. As the absolute hepatocyte numbers obtained from these human liver specimens were relatively high with respect to the quantities that are typically required for many *in vitro* applications, the yield was usually sufficient for any subsequent experiments. However, unlike the percentage viability of the cell population, which was rendered much more predictable through use of the modified protocol, the overall yield of viable cells remained extremely variable, even after accounting for differences in tissue sample sizes.

Therefore, there was a statistically significant increase in the proportion, but not the yield (however yield is expressed), of viable hepatocytes when cold ischaemia was re-

induced post-digestion. It thus appears that using a longer digestion period followed by re-induction of cold ischaemia enabled the number of (contaminating) non-viable hepatocytes to be decreased significantly, though it did not increase the overall number of viable hepatocytes sufficiently to be statistically significant. This is a surprising and somewhat counter-intuitive result.

It seems likely that prolonging the perfusion process essentially prolongs warm ischaemia (in both digested and undigested areas of the tissue), thereby reducing the overall proportion of viable hepatocytes in the harvested cell suspension, but increasing the overall numbers of liberated cells. The proportion of the tissue sample which is digested might be expected to increase. However, the rate of perfusion, and total volume, of digestion fluids remained unchanged and, thus, a proportionate increase in the volume of tissue digested cannot reasonably be expected. Upon further consideration, it is also logical that the area (or volume) of distribution of perfusion fluids is influenced mainly by the anatomy of the vasculature and that a longer period of digestion merely increases the extent of physical dissociation of the parenchyma within that same area or volume. This would result in fewer or weaker remaining associations with the ECM, in regions exposed to the perfusion fluid, on completion of the digestion period and thereby easier physical separation of hepatocytes within those regions when the tissue was incised and manipulated at the end of the perfusion procedure. So it might be hypothesised that the proportion of cells, within the liberated population, which had suffered fatal mechanical trauma was decreased. Whatever the correct explanation, it is possible that chilled processing was the main beneficial factor and acted by preserving the viability of cells immediately following digestion, whereas processing at room temperature led to the continued degradation and death of cells during and after the centrifugation stages.

Centrifugation through an iso-dense solution of Percoll and cell culture medium proved to be an effective means of enriching the intact hepatocyte fraction. Percoll is a 23% w/w suspension of colloidal silica particles coated with polyvinylpyrrolidone. The suspension is non-toxic to cells when used in an iso-tonic solution, and has a low viscosity, thus reducing shear forces during centrifugation. Cell fragments or damaged cells which have lost intra-cellular contents are less dense than viable cells and do not sediment through the Percoll column. This procedure is not affected by differences in the antecedent parts of the protocol and can be performed either at room temperature or at 4°C. The density of the liquid phase the Percoll suspension would have been higher at 4°C than at room temperature and there would have been a higher rate of interaction with the particulate matter. Both factors might contribute to a reduction in the proportion of damaged cells precipitated during centrifugation, but these effects are likely to have been minimal, since the hepatocytes themselves would also have increased in density. Regardless of temperature of processing, it was found that hepatocyte populations with a very low initial proportion of viability did not achieve a sufficiently high viability for subsequent experimental use, even after Percoll centrifugation. This may reflect either an overloading effect of the column by high numbers of dead cells or higher numbers of apoptotic cells, which would be expected to possess a higher density than viable cells (following cell shrinkage) and therefore precipitate through the column without separation.

Some outlying results of hepatocyte yield were amenable to rational explanation. An early experiment (1) yielded a cell suspension containing extremely high numbers of contaminating red blood cells, which obscured much of the field in the haemocytometer and therefore prevented accurate quantification of hepatocyte

yield. This is thought to have resulted from poor initial flushing of blood from the tissue sample using the IV giving-set method. In three experiments (6, 11 and 22) which resulted in relatively poor yields of viable hepatocytes, the cells obtained following digestion of the tissue samples were observed, by light microscopy, to contain large, clear intra-cellular inclusions and the cell suspensions exhibited a thick, pale, creamy froth above the media layer following centrifugation. It was inferred that the hepatocytes of these specimens were steatotic and thus more than usually susceptible to fragmentation, resulting in a high level of attrition during processing. Due to their high fat content, remaining cells were also likely to be of lower than usual density, and therefore precipitated with less efficiency by the Percoll centrifugation step. Both factors help to account for the very low hepatocyte yields from these experiments.

Undoubtedly, one of the main variables affecting post-digestion processing was the innate heterogeneity of the human primary hepatocyte population, with relation to both cell size and cell density. This heterogeneity results from differences in anatomical distribution, functional differentiation, and the numbers (and types) of storage granules and organelles contained in these large, complex cells. The effect of this heterogeneity was most obvious when processing visibly steatotic cells, as described above. However, such factors are applicable to all primary hepatocyte populations and their variability makes it difficult, if not impossible, for the researcher to counteract their effects.

To conclude, human primary hepatocytes with high percentages of viability were obtained from surgically resected human liver tissue. A protocol for two-stage perfusion was developed from previously published methods and from locally-

established methods for rodent hepatocyte isolation. Several physical issues relating to tissue perfusion were explored and optimised. A modified protocol, utilising a longer period of liver tissue digestion in combination with re-induction of cold ischaemia to preserve the cells after digestion, was found to be associated with significantly higher levels of hepatocyte viability. Unavoidable delays, enormous inter-sample variation and lengthy experimental procedures created complex conditions in which it was not possible to obtain reliable and predictable results or conventional standardisation of experimental parameters. Despite the challenges, the aim of the experiment series – to optimise experimental condition for isolating highly viable suspensions of hepatocytes from human liver tissue – was achieved. The cells thus obtained facilitated many of the subsequent experiments described in this thesis.

4. TRANSFECTION STRATEGIES FOR PRIMARY HEPATOCYTES

4.1. Introduction

4.1.1. General overview of transfection

Transfection is a process by which non-native genetic material is inserted into target prokaryotic or eukaryotic cells to enable them to express foreign proteins. This is an essential technique for investigating the roles of individual genes and their products in the pathogenesis of disease or as targets for its treatment. There are a number of different methods existing for transfection: the best choice in each instance largely depends upon the cell type to be targeted and the effect or product to be generated and/or measured. A number of different virus types are commonly used for transfection; making use of their natural ability to enter cells and use the host cell machinery to generate the corresponding proteins/polypeptides from the genes they are carrying, including any incorporated foreign genes. There are also various non-viral methods for transfection.

There are two main types of transfection: transient transfection and stable transfection. In transient transfection, the foreign DNA or RNA carries its own promoter sequence(s) and therefore may be rapidly translated. The gene, or genes, of interest is/are commonly encoded in a carrier plasmid or recombinant virus, referred to as a vector. Viruses, such as adeno-associated viruses and Semliki Forest virus, are commonly used to achieve high-level transient transfection of host cells[100-102]. Many copies of the vector may enter each cell so that a high level of translation (the process by which mRNA is processed by host cell ribosomes to produce the protein it encodes) may be achieved. However, expression of the transfected vector

sequence generally declines after a few days, as the foreign DNA or RNA is lost from the host cell population, due to active elimination or cell turnover[103].

During stable transfection, which can be performed only using DNA, the vector sequence is permanently incorporated into the host cell's genetic material. The transfected gene or genes may be integrated into the host's genome by splicing, or may persist as a nuclear episome. Retroviral vectors are useful for stable transfection, as they naturally integrate genetic material into the host chromosome(s)[102]. As only one or two copies of the sequence are likely to be integrated per cell, amounts of the foreign protein expressed by each cell are usually lower than with transient transfection (at least initially). However, depending on the vector and cell line used, the expression may be maintained indefinitely and the incorporation of additional genes which conferring a drug resistance can enable a homogenous cell population to be selected in culture, i.e. where, finally, all the cells in the *in vitro* culture carry the inserted genes. Unfortunately, because cell proliferation is required, both for the DNA integration and subsequent selection phases, stable transfection has little utility in non-proliferating cells (including primary hepatocytes)[104].

Optimal transfection methods for any given cell type result in a high level of gene expression (efficacy) and confer a low level of host cell toxicity. A number of different methods have been developed for the transfection of eukaryotic cells. These methods are commonly categorised as viral transfection (also known as transduction) and non-viral transfection.

Viruses possess natural mechanisms by which they can bind to, then release genetic material into, target cells (see figure 4.1). In many cases, proteins on the surface of the virion act as ligands for naturally occurring host cell receptors and then trigger endocytosis. If a virus has been genetically modified, through recombination, to carry additional genes (as shown in figure 4.1), the foreign genes will also be carried into the infected cells. The virus's own (and usually highly efficient) mechanisms for directing transcription and/or translation of its genome usually results in a high level of expression of the foreign gene of interest.

Generally, non-viral transfection relies either on causing nucleic acid-containing particles to associate with, or pass through, cell membranes prior to releasing their contents into the cell or on the creation of pores in the membranes of target cells (generally using an electric current by a process known as electroporation). In the same way as viral transfection, non-viral transfection can produce either transient or stable transfection, depending on the cell type, transfected genes and selection pressures applied (if any).

Positively-charged substances, such as diethylaminoethyl (DEAE)-dextran, were first used for transfection in 1965[105]. DEAE-dextran is cationic under acidic conditions, in common with many other non-viral transfectants, and therefore binds to the negatively-charged (acidic) phosphate groups on DNA. The resulting complex salts, which may be extremely large molecules, can interact with the hydrophilic moieties of lipid membranes of target cell and subsequently can be endocytosed.

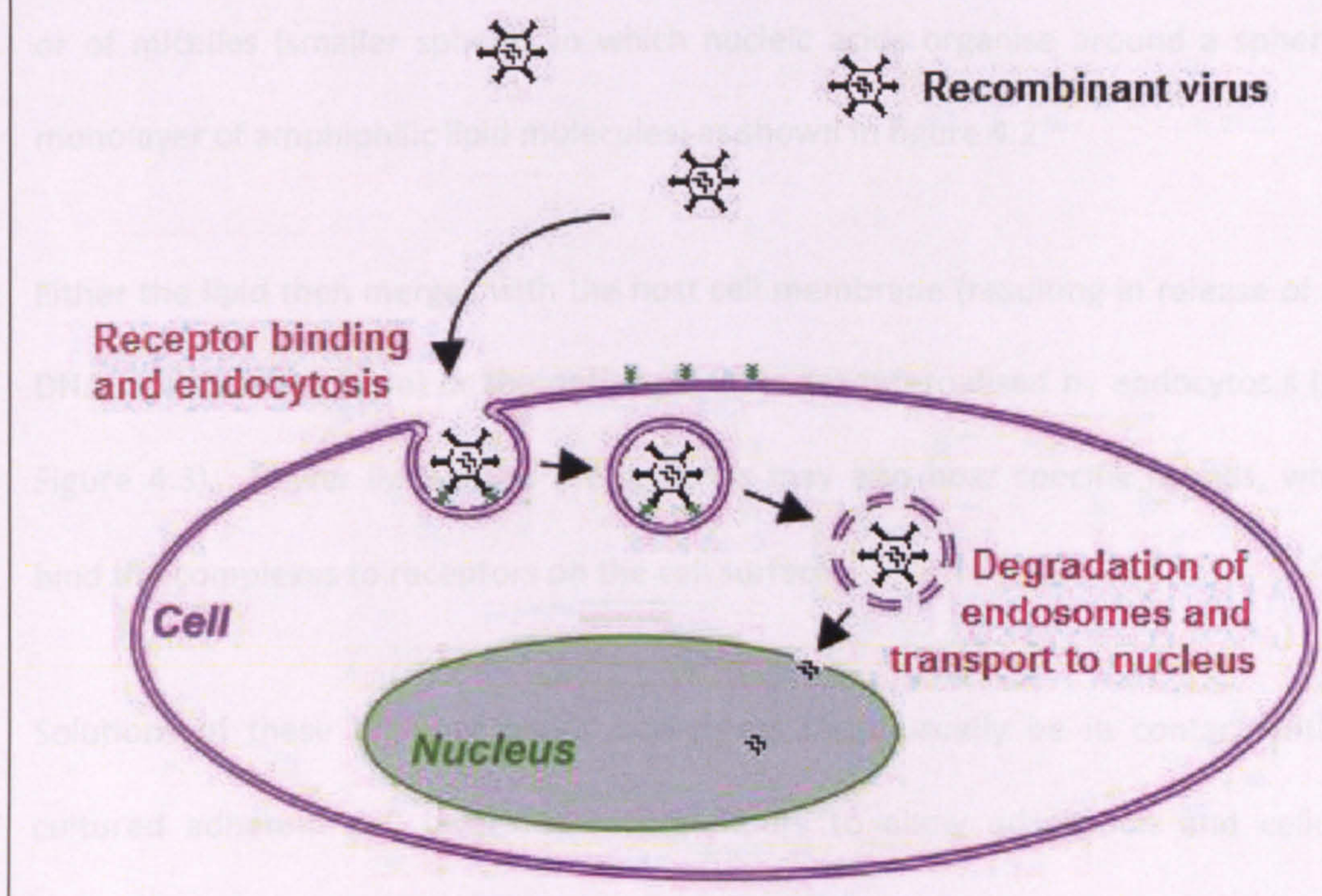

Figure 4.1: Introduction of DNA into eukaryotic cells *by viral transfection. Positive-sense RNA viruses are directly translated in the endoplasmic reticulum.*

Cationic lipids are now among the most commonly used reagents for non-viral transfection. These lipid molecules are comprised of two (hydrophobic) fatty acid chains, a linker sequence, and a hydrophilic amino group[106] and they can bind to DNA or RNA to form transfection complexes. These complexes may take the form of liposomes (spherical structures in which a lipid bilayer encapsulates the nucleic acids) or of micelles (smaller spheres in which nucleic acids organise around a spherical monolayer of amphiphilic lipid molecules) as shown in figure 4.2.

Either the lipid then merges with the host cell membrane (resulting in release of the DNA into the cytoplasm) or the entire particles are internalised by endocytosis (see Figure 4.3). Newer lipid-based preparations may also bear specific ligands, which bind the complexes to receptors on the cell surface.

Solutions of these DNA-containing complexes must usually be in contact with a cultured adherent cell layer for several hours to allow adsorption and cellular endocytosis to occur. Transfection then requires the host cell's normal intra-cellular trafficking processes to transport the DNA to the nucleus[107]. The efficiency of non-viral transfection tends to vary between different cell types and species and, in addition, the formulation of the complexes, incubation time, cell density and medium composition all need to be optimised accordingly.

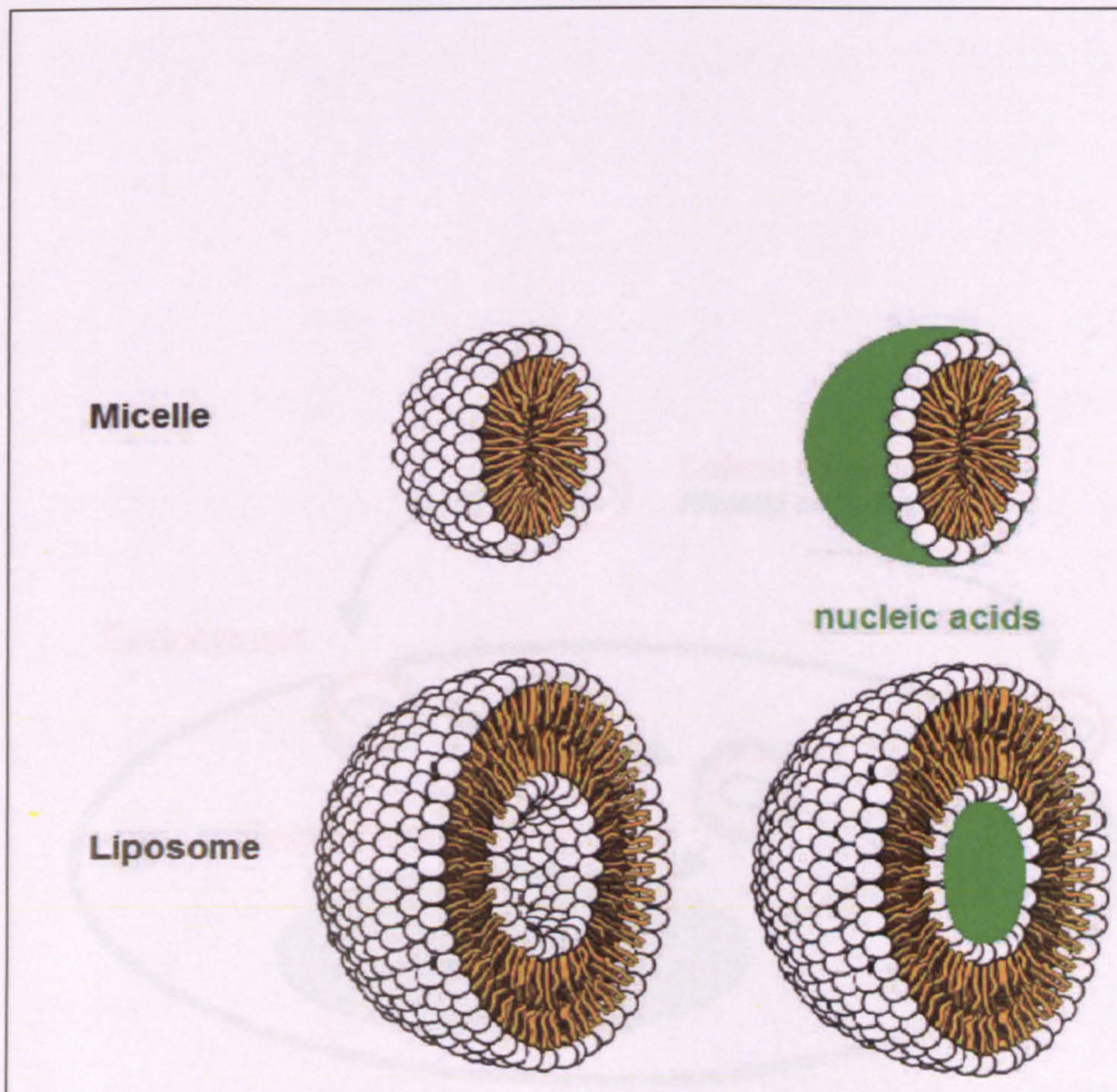

Figure 4.2: Schematic diagram showing the monolayer nature of a micelle, in contrast to the bilayer structure of a liposome, when formed in the presence (right) or absence (left) of DNA or RNA. Hydrophilic 'heads' of the molecules organise to the exterior when formed in aqueous solutions and associate with the nucleic acids (shown in green). (Adapted from Villarreal[108])

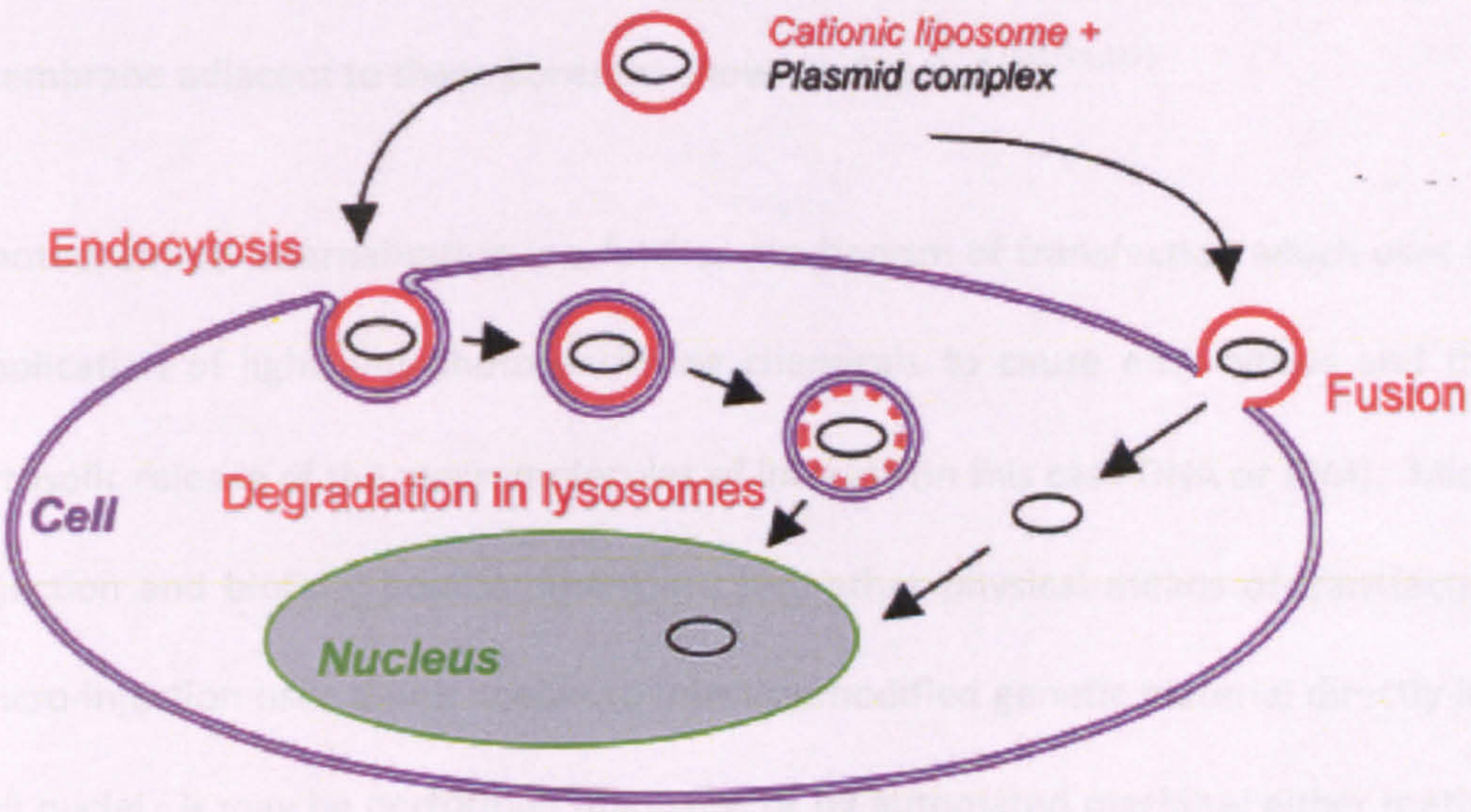

Figure 4.3: Introduction of DNA or RNA into eukaryotic cells by cationic lipid transfection (adapted from Lee et al. [109]).

Electroporation is a procedure in which one or more short, high-voltage pulses of electricity is passed through a specially designed cuvette containing a carefully selected electrolyte solution, in which the target cells have been suspended, and in which the nucleic acid to be transfected is also present[110]. This disrupts the normally stable potential difference across the cell membranes and small, temporary pores develop in them. Naked DNA or RNA, present in the buffer solution, is thought to enter cells mainly by electrophoretic transfer* following association with the cell membrane adjacent to these pores (as shown in figure 4.4)[111,112].

Photochemical internalisation is a further mechanism of transfection which uses the application of light and photosensitising chemicals to cause endocytosis and then cytosolic release of the macromolecules of interest (in this case DNA or RNA). Micro-injection and biolistic bombardment are two other physical means of transfection. Micro-injection uses a fine needle to inject unmodified genetic material directly into cell nuclei. It may be performed manually, or by automated machine: either method requires highly specialised equipment. Biolistics refers to the technique of inserting particles into target cells using compressed air. This technology is also described as a "gene gun". The "bullets" comprise microscopic particles of metal coated with plasmid DNA[103].

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* A non-active transfer process, driven by the potential difference existing between the inside and outside of the cell (as the result of active ion-transfer processes). For simplicity, this "unintentionally-aided" process is referred to as passive transfer in this thesis.

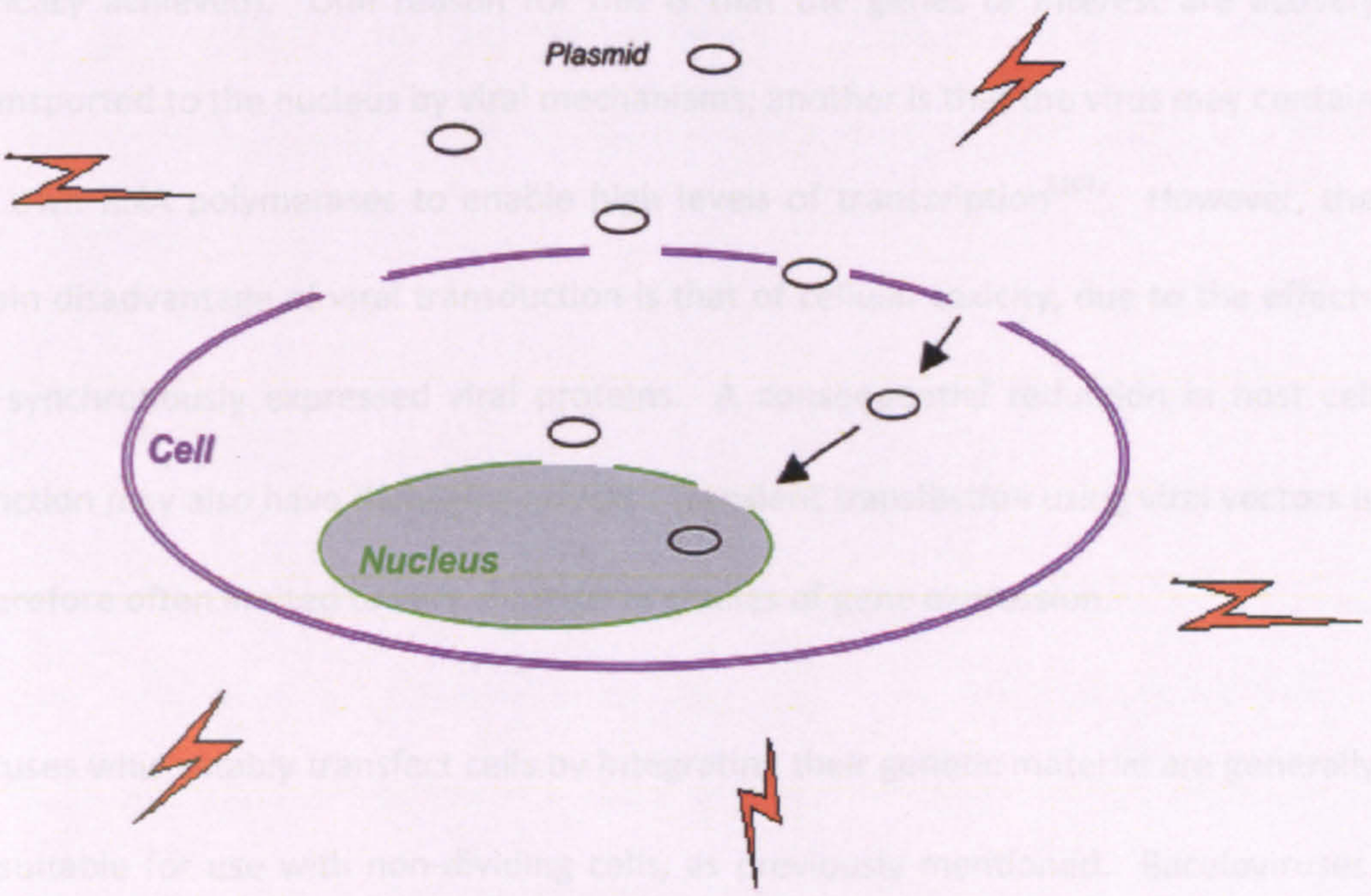


Figure 4.4: Introduction of genetic material into eukaryotic cells by electroporation. DNA sequences enter the nucleus by non-active transfer mechanisms to undergo transcription, whereas single stranded RNA sequences which contain a suitable ribosome entry site can be directly translated in the endoplasmic reticulum.

4.1.2. A comparison of methods for viral transduction with those for non-viral transfection, with reference to the advantages and disadvantages of each.

Viral transduction commonly has high levels of efficacy (i.e. a high proportion of target cells express the protein(s) of interest) and overall efficiency (i.e. the amount of genetic material required for transfection is low, relative to the transfection efficacy achieved). One reason for this is that the genes of interest are actively transported to the nucleus by viral mechanisms; another is that the virus may contain its own RNA polymerases to enable high levels of transcription[102]. However, the main disadvantage of viral transduction is that of cellular toxicity, due to the effects of synchronously expressed viral proteins. A consequential reduction in host cell function may also have damaging effects. Transient transfection using viral vectors is therefore often limited to very short-term studies of gene expression.

Viruses which stably transfect cells by integrating their genetic material are generally unsuitable for use with non-dividing cells, as previously mentioned. Baculoviruses, which offer good efficiency in producing correctly folded and functional target proteins, are generally unsuitable for the transfection of mammalian cells as they can only express protein in insect cells, although recent advances have permitted the transfection of some mammalian cell lines[113,114]. Recombinant adeno-associated viruses or vaccinia viruses can achieve tremendously high levels of target gene expression and are useful for over-expressing proteins within a cell[101,102]. However, the translation level of both viral and foreign genes may be so high that it has a detrimental effect on the host cells, which usually die after 1-2 days. Furthermore, the expression and presence of vector virus proteins may be a confounding factor in interpreting the results of experiments which examine cell function and interactions.

Non-viral methods of transfection are least damaging to the target cells but also tend to be the least effective. Most such methods lack any means of active transfer to the nucleus and this can substantially limit transcription (when using DNA) and therefore the effectiveness of transfection. However, if the target cells are to be subsequently examined individually, or if the successfully transfected cells can be selected in some way, these may still be suitable techniques to use for achieving a sufficient level of foreign protein expression. Non-viral carriers can be usually be used to introduce larger genetic fragments than viral vectors, which may be advantageous in some circumstances.

The oldest method of non-viral transfection, diethylaminoethyl (DEAE)-dextran, is simple to use and gives reliable results, but is frequently disadvantaged by consequential cytotoxicity. The cytotoxicity may be exacerbated by the need to remove, or reduce the concentration of, any serum component of the cell culture medium, to avoid interference with the transfection mixture during incubation. A number of cell types are also non-permissive to (DEAE)-dextran[103]. As one of the other non-viral transfection procedures, calcium phosphate-DNA complexes are relatively cheap to produce and easy to apply. A solution of DNA in a phosphate buffer is mixed with a solution of calcium chloride to form the complexes. However, the efficiencies achieved are often unpredictable because it can be difficult, reproducibly, to obtain optimally- and homogenously-sized complexes[115]. Many primary cell types are also resistant to calcium-phosphate transfection[105]. Synthetic cationic polymers, such as polyethyleneimine (commonly referred to as PEI), can also be complexed with the anionic phosphate groups of nucleic acids. This technique offers a higher efficiency than older methods in some cell types and, because these chemicals lack strongly hydrophobic moieties, they are not sequestered by

interaction with the serum in the cell culture medium. However, the macromolecular complexes formed are likely to present many uncomplexed ionic sites and may cause toxicity through unintended interactions with cellular membranes and other components[116].

There are also several lipid-based transfection reagents commercially available, which may offer improved efficiency and reproducibility of transfection as compared with the DEAE-dextran or calcium-phosphate methods. However, the amphiphilic character of any excess (uncomplexed) reagent may cause direct cytotoxicity by disruption of the cells' membranes[116]. A further disadvantage of lipid-mediated endocytosis is that phospholipids and other fats within serum, if present within the cell culture medium, may associate with the hydrophobic sites of the complexes formed and inhibit their interaction with plasma cell membranes, thereby reducing transfection efficiency. On the other hand, reducing the level of serum in the cell culture medium, as when using DEAE-dextran, may increase the risk of cytotoxicity from the transfection complexes or the genes carried in them[105].

Electroporation techniques are fast, simple and among the most efficacious methods of non-viral transfection but these advantages generally come at the expense of irreversible damage to a proportion of the target cells. The electric pulse (or pulses) used causes disruption to the cells' membranes so that, in addition to molecules within the buffer solution being able to diffuse passively into the cell cytoplasm and nucleus (see figure 4.4), intra-cellular contents can also diffuse out, adversely affecting viability of the cells[112].

Recovery of the cells' membranes from this trauma is highly dependent upon the cell type and robustness and also on the buffer, cell handling and duration and amplitude of the pulse. A high proportion of cell death (50-70%[105]) may occur, although this drawback may be of less concern if using a rapidly proliferating cell line. However, there is no restriction on the use of serum in the culture medium and its presence may help to minimise some of the damaging effects.

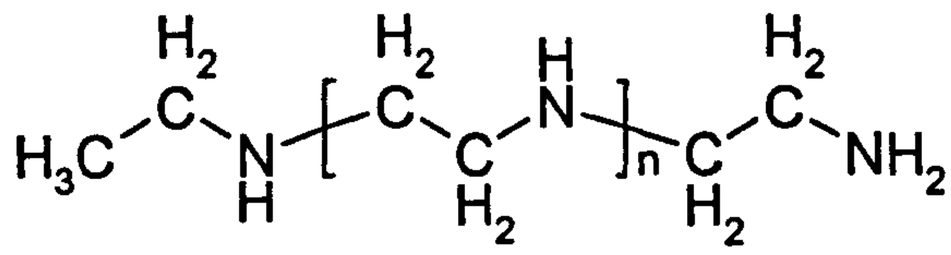
Photochemical internalisation has the potential for very broad applicability, and enables multiple samples to be processed at once, but the potentially many photochemical reactions induced can result in significant and permanent cell toxicity. Micro-injection and biolistic bombardment both have limited applicability due to issues of complexity, high cost and very restricted scale.

4.1.3. Transfection using cationic polymeric polyethylenimines

4.1.3.1. Principle of cationic polymer-mediated transfection

Polyethylenimines (PEIs) were first developed for use by soap manufacturing and water purification industries[117]. Their suitability as transfection reagents was first described in 1996[116]. PEIs have been found to condense DNA extremely efficiently, to form small polyplexes which may be more readily endocytosed than lipoplexes[107]. They are very stable, water-soluble polymers that can be synthesised in two forms, branched chain and linear (as shown in figure 4.5). Branched PEIs are made by polymerising aziridine monomers, whereas the linear forms of PEI are made by polymerising 2-substituted 2-oxazoline monomers.

(a)



(b)

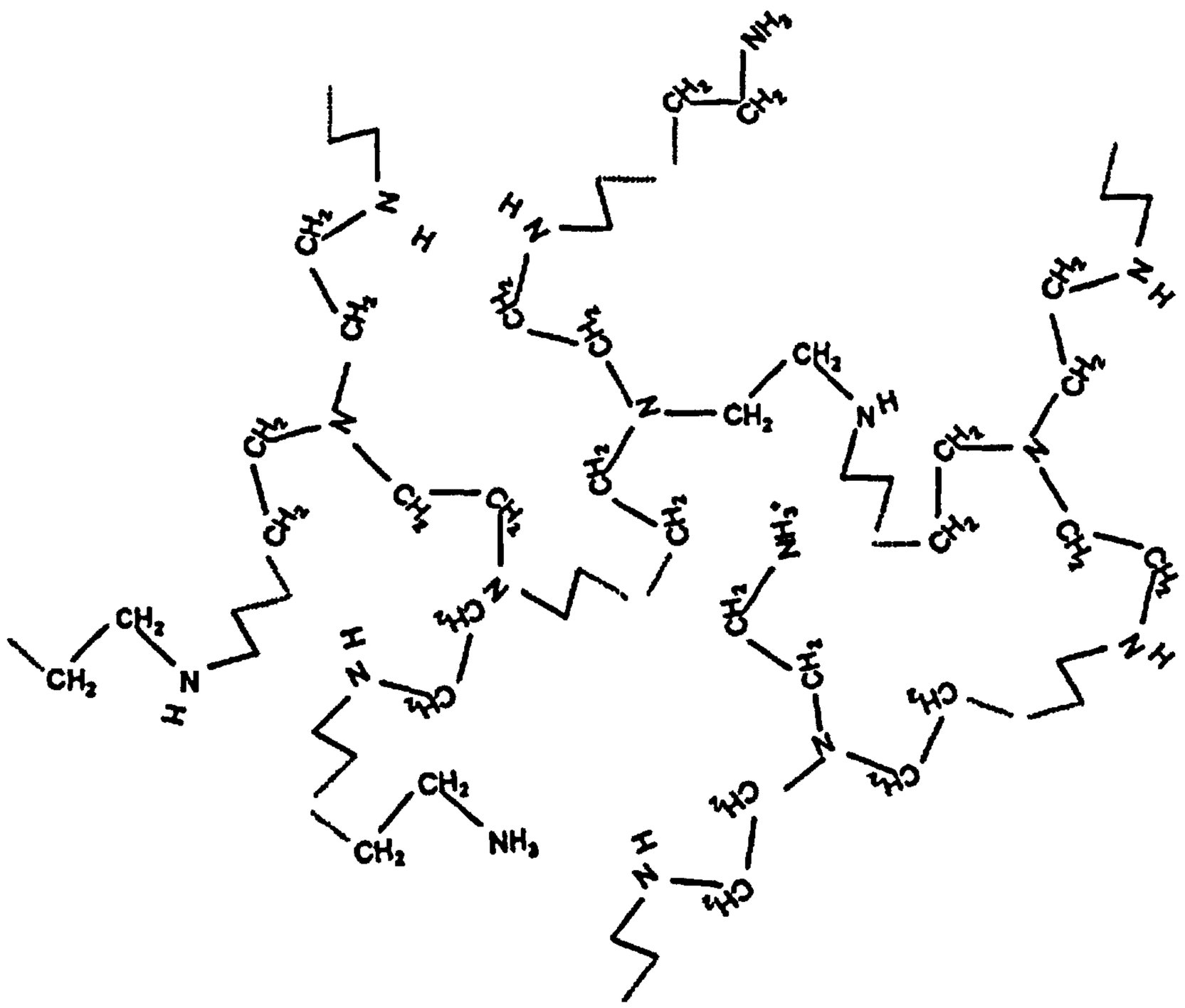


Figure 4.5: Diagram showing the chemical structure of the (a) linear and (b) branched forms of polyethylenimine. Multiple amine groups accept protons at physiological pH. (Adapted from Godbey et al.[117]).

As shown in figure 4.5, both branched and linear PEI possess numerous proton-accepting amino groups (potentially more than 1000, depending on the polymer's molecular weight[117]), conferring excellent buffering capacity in relation to the polymers' size. Branched chain PEI possesses primary, secondary and tertiary amine groups, with their pKa ranging from 5.5 to 9.0. The high residual buffering capacity of the PEI component allows the polyplexes to resist lysosomal degradation following endocytosis, by reducing the acidification of the endolysosome that would otherwise normally occur following endocytosis[118]. Acidification causes destabilisation, swelling and degradation of the endolysosome and permits escape of the intact polyplexes once the endolysosome breaks down[119].

When mixed with an aqueous solution of plasmid DNA, PEI surrounds, and causes condensation of, the DNA. In this context, condensation describes the process by which double-stranded DNA collapses into a compact toroid-shaped particle. This process occurs if more than 90% of the DNA's charge is neutralised by the presence of cations and the electrostatic charge which usually maintains its open coil structure is therefore removed. The small, polyplexed particles of PEI and DNA may each contain several plasmids. The diameter of these particles can range between 20-130 nm[117,120], and is influenced by the ionic strength of the solution in which the reaction is performed, the ratio of nitrogen atoms in the polymer to phosphorus atoms in the DNA (N:P ratio) in the whole mixture, and the type and molecular weight of the PEI used[120]. Different configurations of complexes are inevitably created in the same mixture, and are probably influenced by physical mixing techniques[117]. When PEI:DNA polyplexes are overlaid onto adherent cell cultures, they associate with the plasma membranes of cells[120]. The main interaction is

thought to occur with negatively-charged sulfated proteoglycan molecules[117]. A proportion of the complexes are subsequently endocytosed.

Generally, smaller particles are more easily endocytosed by cells and denser complexes of PEI may protect the DNA from degradation[117]. The influence of particle size and surface charge on uptake into cells, intra-cellular trafficking, and DNA expression appears to be complex[116].

4.1.3.2. Considerations when using cationic polymer-mediated transfection

The choice between branched chain and linear PEI is not straightforward. Branched chain PEI (around 25 kDa) was initially found to give better general transfection success[117]. However, it also seems that very low molecular weight linear PEI (around 5 kDa) can result in good transfection efficiencies and may also result in lower toxicity to the transfected cells. The relationships between PEI form or molecular weight and transfection efficiency are likely to be influenced by the size and density of polyplexes formed which, as described above, may vary between (and even within) batches of polyplex made with the same polymer[119]. Whichever form of PEI is chosen, it is therefore necessary to spend some time optimising the N:P ratio as well as carefully regulating the chemical, physical and mechanical conditions under which the complexes are formed.

Proteins can bind to, and effectively inactivate, PEI:DNA complexes[117], although this adverse effect may be minimised by altering the N:P ratio of the complexes and/or washing serum from the target cell layer prior to overlaying the PEI:DNA mixture. Lastly, uncomplexed PEI increases the permeability of cell membranes and is therefore toxic if present at too high a concentration.

4.1.4. Transfection by electroporation

4.1.4.1. Principle of electroporation

Electroporation, also known as electropermeabilisation or electrotransfer, is a technique which uses voltage pulses to disrupt the plasma membranes of target cells, thereby allowing naked DNA or RNA to diffuse into the cell and its nucleus. Target cells, either in suspension or in monolayer culture in specially adapted culture vessels, are placed between two conductive metal plates and a very short pulse of electricity is applied (typically, for mammalian cells, around 260 V[110]). Genetic material, in solution surrounding the cells, diffuses passively into the cells through the pores transiently created in their plasma and nuclear membranes by the electrical pulse, as shown in figure 4.4. These pores are naturally resolved within a short time and the transfected sequences are usually rapidly transcribed and/or translated, following recovery of the host cell.

4.1.4.2. Considerations when using electroporation

As there is no targeted or active transport into the cell, a relatively high external concentration of DNA or RNA is required for effective transfection. However, overall efficiency tends to be high due to the high proportion of cells that are likely to be permeabilized by this technique. Rapid translocation of the transfected genes usually allows production of the protein of interest to begin very quickly, in contrast to many other transfection techniques. This advantage may however be lost if the unprotected DNA or RNA is ejected from, or destroyed by, the host cell. The use of super-coiled DNA, or capped RNA, may be necessary to mitigate this degradation[121-123].

The number and size of the membrane pores created depends upon the voltage, capacitance, the duration of the pulse, the composition of the cell suspension medium, and the cell type. The same factors will also influence the directly damaging effects of the method: creating too much permeability will kill the target cell. Most small membrane pores will resolve quickly but a proportion of cells will be more liable than the average to irreversible membrane damage and will die for this reason. There is therefore a need for careful optimisation of the cell concentration, ionic composition of buffers and electrical parameters used for each cell type.

In principle, cells might be electroporated in any standard physiological buffer or medium. However, the ionic composition of the culture medium or cell buffer will affect both the transmission of the pulse and its effect on the cells' membranes, so in some methods a specialised (sometimes proprietary) solution may be used to create the cell suspension. This is the case for nucleofection, a method of electroporation using a commercial electroporation machine (Nucleofector®; Amaxa® AG, Germany) with proprietary buffers. Nucleofection has been widely reported to confer the high efficiency of electroporation with lower levels of cell damage and death[124,125]. The contents of its cell suspension buffers and the parameters of its pre-coded programmes of electrophoretic pulsing are not disclosed by Amaxa® and therefore unable to be openly assessed.

During the initial phases of the present experimental work, the ultimate intention had been to transfect primary hepatocytes with single gene constructs from the hepatitis C virus and to examine the effects on lipid metabolism. Immuno-histochemical techniques would have enabled the examination of individual transfected cells, thereby obviating the need to successfully transfect a high

proportion of the hepatocyte population. The development and availability of the HCV JFH1 clone and subgenomic replicon (described further in Chapter 5) offered numerous additional opportunities and potential advantages in a primary hepatocyte culture model and therefore investigation of the replication of JFH1 in primary cells became the main aim of the project. However, investigation of its capacity for replication would be problematic unless a high proportion of transfected cells could be achieved, as the chief methods to measure replication involve the (usually indirect) quantification of marker protein production from an entire population of cells, rather than a cell-by cell immunocytochemical analysis. As has already been mentioned, differentiated primary hepatocytes do not proliferate freely in culture, thus the selection and expansion of successfully transfected cell populations was not feasible. In hepatocytes, expression of a potentially cytotoxic set of genes from the hepatitis C virus would offer temporal limitation to the utility of this approach, in any case. Re-suspension and selection of cells expressing the replicon after an initial culture period, using fluorescence-assisted cell sorting (FACS), would be physically intolerable to live hepatocytes* and thus similarly unfeasible.

There are few publications containing details of techniques for non-viral transfection of primary hepatocytes and fewer still which consider transfection methods for human primary hepatocytes. Calcium phosphate transfection has been described in a number of studies of rat primary hepatocytes, achieving efficacies of 20-25%[126,127]. However, a later study suggested increased toxicity and only low levels of efficiency

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* Preliminary studies (not presented in this thesis), using rat hepatocytes, found that chemical and / or mechanical removal of these fragile cells from the culture surface, and the shear forces of the sheath fluid during subsequent FACS analysis, caused almost universal cell fragmentation and death.

in comparison to newer methodologies[128]. Cationic lipid-mediated transfection of rat hepatocytes has also been described, using a number of commercially available reagents. Efficacies of 5-22% were described in one study, which used GFP as a reporter gene in rat hepatocytes[129]. In a similar study which used a luciferase reporter, the level of transgene expression was found to decline after only 24-31 hours[130]. Cationic lipid transfection methods have been reported to produce varying levels of cell toxicity and reduced duration of viability in subsequent culture, and may be inhibited by serum in the culture medium[131-133]. There is some evidence that optimum transfection levels are achieved by prolonging the post-isolation/pre-transfection period[134,135]; unfortunately, this may be disadvantageous for the examination of any downstream effects of transgene expression, as there may be greater relative de-differentiation of the hepatocyte phenotype prior to transfection.

Chemin *et al.*[136] described the use of both linear and branched chain PEI to successfully transfect primary hepatocytes from ducks and tree shrews. They were able to achieve efficacies of up to 10% and 50%, respectively. The optimum N:P ratio range was found to be 10:1. In some contrast to these high levels, another study comparing various types of PEI to transfect reporter genes into rat hepatocytes reported maximum average efficacies of 0.56% to 14.7%, depending on the plasmid used (but not the amount of DNA, type of PEI, or incubation time)[137]. Efficacy did not increase beyond 24 hours. A further study demonstrated transfection of both human and rat primary hepatocytes using a PEI-mellitin conjugate, but did not provide data for efficacy or efficiency[138].

A limited number of studies have now reported the successful use of nucleofection to transfect primary hepatocytes. Rat hepatocytes were reported to be successfully

transfected by Ishihara *et al.*[124], although efficacy was not reported, and porcine hepatocytes were transfected with 40-50% efficacy by Chen *et al.*[139].

In the remainder of this chapter, work to optimise two methods for the transient transfection of primary hepatocytes is described. The most effective DNA concentration and N:P ratio for PEI:DNA transfection was determined, using adherent cultures of rat hepatocytes approximately 18 hours after isolation. The optimal DNA concentration and Nucleofector® programme for nucleofection was determined using freshly isolated rat and human hepatocytes in suspension. The results obtained from both methods are presented and the efficacy, advantages and disadvantages of the two methods are then compared and summarised.

4.2. Materials and methods

4.2.1. Cationic polymer-mediated transfection

4.2.1.1. Materials for cationic polymer-mediated transfection

The gWizGFP plasmid, (5757bp; MW 3800 kD), was obtained from Aldevron [see appendix 8.2 for vector gene map]. Linear PEI (25kDa, Polysciences) was obtained from Park Scientific Ltd.

Standard hepatocyte culture media were prepared as described in chapter 2; additional media were prepared as follows.

Medium 7 comprised William's Medium E (Gibco©) (500 ml), 2 mM L-glutamine (Gibco©), 5 mM nicotinamide and 10 µg/ml bovine pancreas insulin (final concentrations of supplements indicated in each case).

Medium 8 comprised William's Medium E (Gibco©) (500 ml), 2 mM L-glutamine (Gibco©), 5 mM nicotinamide, 10 µg/ml bovine pancreas insulin and 1.5% v/v dimethylsulfoxide (DMSO) (final concentrations of supplements indicated in each case).

Medium 9 comprised William's Medium E (Gibco©) (500 ml), supplemented by the addition of 50 ml fetal calf serum (PAA Laboratories©), 2 mM L-glutamine (Gibco©), 5 mM nicotinamide and 10 µg/ml bovine pancreas insulin (final concentrations of supplements indicated in each case).

4.2.1.2. Methods for cationic polymer-mediated transfection

4.2.1.2.1. Preparation of cell cultures

Freshly isolated primary rat hepatocytes (prepared as described in Chapter 2), were cultured in a monolayer applied at 10^5 cm^{-2} to 6-well collagen-coated plates in medium 1. The hepatocyte cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. After an initial 2-hour period of incubation, non-adherent hepatocytes were removed from the monolayer culture by aspiration of the culture medium and by washing the monolayer with PBS (pre-warmed to 37°C), after which the cultures were re-incubated overnight with medium 2. Following overnight incubation, and just prior to addition of the transfection complexes, the medium was aspirated and 400 µl fresh medium 2 was applied to each well.

Some disruption to the hepatocyte layers was visible following PEI transfection during preliminary experiments. Subsequent to this observation, medium 7 was used during overnight culture of the monolayers prior to transfection, as many manufacturers of transfection agents suggest that certain antibiotic solutions may cause cellular toxicity if present during transfection, due to increased cellular permeabilization. Also, medium 7 was replaced with medium 8, containing DMSO as a cell protectant, immediately before addition of the transfection complexes (instead of medium 2)[136]. These two modifications resulted in improved retention of the cell monolayers and were then adopted for all subsequent experiments.

4.2.1.2.2. Preparation of DNA

Working stocks of the gWiz plasmid were obtained by transformation of competent *E. coli*. For these experiments the unmodified plasmid was used. After lysis of the bacterial cultures and ultra-centrifugation to obtain the DNA, the plasmid DNA was purified by ultra-centrifugation, using a caesium chloride gradient, and then extracted by needle aspiration. The plasmid was precipitated using ethanol and re-dissolved in RNase- and DNase-free water. The DNA concentration was then measured, and adjusted to 5 µg/µl, using a Nanodrop™ ND1000 UV spectrophotometer. Plasmid DNA stocks were stored at -20°C prior to use.

4.2.1.2.3. Preparation and application of PEI:DNA transfection complexes

A 10 mM stock solution of PEI was prepared in de-ionised and autoclaved water. The pH was adjusted to 7.0 with HCl and the final solution was filter-sterilised and stored at 4°C. The stock PEI and DNA solutions were diluted to various working concentrations (as shown in table 4.1), being made up to 200 µl, using PBS and vortexed briefly to mix. The working-strength PEI solution was added drop-wise to the working-strength DNA solution, with intermittent manual shaking. The resultant PEI:DNA mixture was then vortexed briefly and incubated at room temperature for 15 minutes to allow the complexes to form.

Initial experiments showed some toxicity to the monolayered hepatocytes at higher concentrations of the complexes and thereafter PBS containing 5% sucrose w/v was used as a diluent (as shown in table 4.1)[136].

Table 4.1: Amounts of DNA and PEI used to prepare PEI:DNA complexes for the transfection of rat primary hepatocytes. Each of the calculated volumes was made up to 200 µl using 5% sucrose PBS diluent (except where otherwise indicated) prior to mixing.

N:P ratio	DNA			PEI	
	Mass (µg)	Volume (µl)	Phosphate (nmol)	Nitrogen (nmol)	Volume (µl)
4	0.5	0.1*	1.5	6	0.6
	1.0	0.2	3.0	12	1.2
	2.0	0.4	6.0	24	2.4
	4.0	0.8	12.0	48	4.8
10	0.5	0.1	1.5	15	1.5
	1.0	0.2	3.0	30	3.0
	2.0	0.4	6.0	60	6.0
	4.0	0.8	12.0	120	12.0
	6.0	1.2	18.0	180	18.0
	8.0	1.6	24.0	240	24.0
	10.0	2.0	30.0	300	30.0
	12.0	2.4	36.0	360	36.0
12.5	6.0	1.2	18.0	225	22.5
	8.0	1.6	24.0	300	30.0
	10.0	2.0	30.0	375	37.5
	12.0	2.4	36.0	450	45.0
15	6.0	1.2	18.0	270	27.0
	8.0	1.6	24.0	360	36.0
	10.0	2.0	30.0	450	45.0
	12.0	2.4	36.0	540	54.0
20	6.0	1.2	18.0	360	36.0
	8.0	1.6	24.0	480	48.0
	10.0	2.0	30.0	600	60.0
	12.0	2.4	36.0	720	72.0

* The experiments using these parameters were carried out using PBS as diluent for the PEI:DNA complexes.

The first three experiments, which examined N:P ratios of 4:1 and 10:1 as recommended in previously published studies, failed to achieve any observable transgene expression using a ratio of 4:1. Therefore, only ratios of around 10:1 were tested during the remaining experiments.

The PEI:DNA complexes (400 µl per culture well) were then added by pipette to the culture wells (prepared as in 4.2.1.2.1 and containing the medium already overlaying the hepatocyte monolayer) and the cultures were re-incubated. Control cell cultures were also prepared for each batch and comprised:

a. *hepatocyte preparation control,* one culture well prepared and maintained using standard media (as described in Chapter 2);

b. *untransfected media and buffer control,* one culture well prepared and maintained using modified transfection medium 7 and 8 (as described above) and with the addition of 400 µl of 5% sucrose PBS.

After 4 hours, the transfected hepatocytes cultures were examined by light microscopy, in order to observe visible signs of toxicity relative to untransfected control cultures, such as cell detachment, dysmorphology and increased granularity or vacuolation. Also at 4 hours post-transfection, 1 ml of medium 9 was added to each well, without prior aspiration of the existing contents. The transfection solution was allowed to remain in contact with the cell cultures for 24 hours in total. The hepatocyte cultures were examined for evidence of transgene expression at 20 hours after application of the transfection complexes.

4.2.2. Electroporation-mediated transfection using nucleofection

4.2.2.1. Materials for electroporation by nucleofection

Nucleofection kits for rat primary hepatocyte transfection were purchased from Amaxa®. A limited number of nucleofection kits for human primary hepatocyte transfection were a kind gift from Amaxa®, as they were under development and not commercially available at the time of these experiments. Both kit types contained sterile Nucleofector® cuvettes and pipettes, rat- or human-specific Nucleofector® solution, Nucleofector® solution supplement and plasmid pMaxGFP™ (MW 2303 kDa; 0.5 µg/µl in 10 mM tris pH 8.0). Before use, the entire volume of Nucleofector® solution supplement was added to the Nucleofector® solution and thoroughly mixed by inversion to produce the working solution. This solution was brought to room temperature before use.

4.2.2.2. Methods for electroporation of rat hepatocytes by nucleofection

4.2.2.2.1. Preparation of rat primary hepatocyte cell suspensions

The required number of freshly isolated rat hepatocytes in suspension (prepared as described in chapter 2) was placed into a 50 ml Falcon tube and sedimented by centrifugation (5 minutes; 50*g*; 4°C). Working quickly, the supernatant was discarded and the hepatocyte pellet was resuspended in Nucleofector® working solution, to produce a suspension with a measured concentration of 10^6 cells per 100 µl.

4.2.2.2.2. Nucleofection techniques for rat primary hepatocytes

The cell mixture (100 µl) and varying amounts of gWiz DNA (as shown in Table 4.2), diluted to 0.5 µg/µl, were added to each cuvette, avoiding air bubbles. The cuvette was immediately capped and placed into the machine for nucleofection, in order to avoid sedimentation or unnecessary warm ischaemia of the hepatocytes. Following nucleofection (whereby the machine was discharged using programme Q25 as recommended for rat primary hepatocytes by the manufacturer), the cuvette was removed.

Control cell cultures were also prepared for each batch as follows.

a. *Hepatocyte preparation control.* One well of non-nucleofected hepatocytes prepared and maintained using standard media (as described in Chapter 2).

b. *DNA control.* One well of non-nucleofected hepatocytes plus DNA, prepared as nucleofected cells, with 10 µg DNA added to the cuvette but NOT electroporated; rested for 15 minutes and cultured and maintained in the same way as nucleofected cells.

c. *Nucleofector® buffer control.* One well of non-nucleofected hepatocytes, prepared as nucleofected cells up to and including suspension in Nucleofector® buffer; added to the culture wells at 100 µl per well and maintained in the same way as nucleofected cells.

d. *untransfected electroporation control.* One well of DNA-free Nucleofected hepatocytes, prepared as nucleofected cells but with no gWiz DNA added to

the cuvette; nucleofected and maintained in the same way as standard nucleofected cells.

e. *untransfected electroporation control for viability.* One well of DNA-free nucleofected hepatocytes, prepared as nucleofected cells but with no DNA added to the cuvette; nucleofected and used immediately to assess post-nucleofection viability by Trypan Blue assay (as described in Chapter 2).

4.2.2.2.3. Post-nucleofection procedures for rat primary hepatocytes

Pre-warmed Medium 1 (500 µl) was added to the Nucleofector® cuvette, following a 15 minute "rest" period at room temperature. The entire cell suspension was gently aspirated by pipette and transferred to a collagen-coated cell culture well containing 1.5 ml Medium 1, which had been pre-warmed to 37°C. The culture plate was gently agitated by planar reciprocating motion, to distribute the hepatocytes evenly, and incubated at 37°C in a humidified 5% CO_2 atmosphere.

After 4 hours, non-adherent hepatocytes were removed from the monolayer culture by aspiration of the culture medium and washing the layer with PBS (pre-warmed to 37°C), after which the cultures were re-incubated with medium 2 (2 ml per well). The hepatocyte cultures were examined for evidence of transgene expression at 20 hours after nucleofection.

Table 4.2: Amounts of DNA used during nucleofection of primary rat or human hepatocyte suspensions, and the resulting, relative concentrations of cells and/or DNA. Note that, although the total reaction volumes are altered depending on the amount of DNA used, the relative concentrations of cells to DNA remain proportional to the mass of DNA added. [1]10 µg of DNA was used for the transfection of rat hepatocytes only.

DNA		Total suspension volume (µl)	Final concentrations		
mass (µg)	volume (µl)		hepatocytes (cells/µl)	DNA (ng/µl)	Hepatocytes (cells/ng DNA)
2	4	104	9615	19.2	501
4	8	108	9259	37.0	250
6	12	112	8929	53.6	167
8	16	116	8621	69.0	125
10[1]	20	120	8333	83.3	100

4.2.2.3. Methods for electroporation of human hepatocytes by nucleofection

4.2.2.3.1. Preparation of human primary hepatocyte cell suspensions

The required number of freshly isolated human hepatocytes in suspension (prepared as described in Chapter 3 and suspended in medium 5) was placed into a 50 ml Falcon tube and sedimented by centrifugation (5 minutes; 50*g*; 4°C). Working quickly, the supernatant was discarded and the hepatocyte pellet was resuspended in Nucleofector® working buffer solution to produce a suspension with a measured concentration of 10^6 cells per 100 µl.

4.2.2.3.2. Nucleofection techniques for human primary hepatocytes

An optimised protocol for nucleofection of human primary hepatocytes was not available. Following advice from the manufacturer, the effects of a number of different variables affecting the procedure were explored. For each reaction, 100 µl of the cell mixture (10^6 cells), and varying amounts of pMaxGFP™ DNA at 0.5 µg/ml (as shown in Table 4.2), were added to each cuvette, avoiding the creation of air bubbles. The cuvette was immediately capped and placed into the machine for nucleofection, in order to avoid sedimentation or unnecessary warm ischaemia of the hepatocytes. Following nucleofection (whereby the machine was discharged under various proprietary pre-programmed settings as suggested by the manufacturer and detailed in section 4.3.3), the cuvette was removed.

Control cell cultures were also prepared for each batch, although limited by availability of reagents, and comprised the following.

a. *Hepatocyte preparation control.* One well of non-nucleofected hepatocytes, prepared and maintained using standard media (as described in Chapter 2).

b. *untransfected electroporation control.* One well of DNA-free nucleofected hepatocytes, prepared as nucleofected cells but with no pMaxGFP™ DNA added to the cuvette; nucleofected and maintained in the same way as standard nucleofected cells.

4.2.2.3.3. Post-nucleofection procedures for human primary hepatocytes

The manufacturer reported that expression of the transfected gene may be increased, in certain cell types, by allowing the suspension of electroporated cells and DNA to remain undisturbed, and undiluted by the addition of cell culture media, for a period of 15 minutes following discharge of the machine. No data were available to indicate the effect of this "rest" period when nucleofecting human hepatocytes. Pre-warmed Medium 5 (500 µl) was therefore added to the Nucleofector® cuvette, either immediately or following a 15-minute "rest" period at room temperature. Working quickly, the entire cell suspension was then gently aspirated by pipette and transferred to a collagen-coated cell culture well containing 1.5 ml Medium 5 which had been pre-warmed to 37°C. The culture plate was gently agitated by planar reciprocating motion, to distribute the hepatocytes evenly, and incubated at 37°C in a humidified 5% CO_2 atmosphere.

After overnight incubation, non-adherent hepatocytes were removed from the monolayer culture by aspiration of the culture medium and washing the layer with PBS (pre-warmed to 37°C), after which the cultures were incubated with medium 6.

The hepatocyte cultures were examined for evidence of transgene expression at 20 hours after nucleofection.

4.2.4. Determination of transfection efficacies

The GFP protein, encoded by both plasmids, is fluorescent when exposed to ultra-violet light, therefore the cell cultures were examined using a Leica DM IRB microscope fitted with a mercury lamp UV light source and a standard fluorescein 530/25 filter cube set.

Total cell numbers, and the numbers of cells displaying visible green fluorescence, were counted at 100x magnification in three fields per cell culture well, to allow calculation of the mean proportion of cells expressing the GFP transgene under each combination of conditions. All cell cultures were examined at 20 hours after transfection, and at 48 hours if no evidence of transgene expression was visible at 20 hours.

4.3. Results

4.3.1. Transfection efficacy and efficiency achieved by PEI transfection of rat primary hepatocytes

As shown in table 4.3, transfection complexes formed using 0.1-4.0 µg DNA and an N:P ratio of 4:1 failed to produce any detectable expression of GFP at either 20 or 48 hours. Using the same amounts of DNA and an N:P ratio of 10:1, a few cells per well were seen to exhibit GFP fluorescence only where the maximum of 4 µg DNA was used.

Further experiments therefore examined the efficacies achieved with at least 6 µg DNA and N:P ratios of at least 10:1. The results obtained are shown in table 4.4. The level of expression was highly variable, as indicated by the high relative standard deviation (RSD) values, but generally low for all combinations of DNA and N:P ratios. At an N:P ratio of 20:1, sporadic cellular dysmorphology and reduced confluence of the monolayer was observed. On the basis of this apparent toxicity, higher N:P ratios were not examined.

Table 4.3: Results of preliminary experiments to transfect rat primary hepatocytes with PEI:DNA complexes containing the GFP-encoding plasmid gWiz[1]

N:P ratio	DNA (μg)	Hepatocytes expressing GFP		Total cells viewed (10^3)	Mean transfection efficacy (%)
		Cell batch			
		Prelim 1	Prelim 2		
4:1	0.5	0	0	3.60	<0.027
	1.0	0	0	3.53	<0.028
	2.0	0	0	3.84	<0.026
	4.0	0	0	4.01	<0.025
10:1	0.5	0	0	3.31	<0.030
	1.0	0	0	3.60	<0.027
	2.0	0	0	3.96	<0.025
	4.0	3	12	3.89	0.386

[1] PEI = polyethylimine; N:P = nitrogen:phosphate; GFP = green fluorescent protein

Table 4.4: Expression of GFP in rat primary hepatocytes at 20 hours post-transfection using PEI:DNA complexes[1].

N:P ratio	DNA (µg)	Proportion of cells expressing GFP (%) Experimental hepatocyte batch[2]				Mean efficacy (%)	Standard deviation	RSD (%)[1]
		A	B	C	D			
10:1	6	0.64	4.33	1.47	1.04	1.870	1.675	90
	8	0.86	5.35	2.4	0.21	2.205	2.289	104
	10	1.48	6.73	3.64	0.18	3.008	2.863	95
	12	0.52	4.50	4.47	0.90	2.598	2.185	84
12.5:1	6	0.28	NT[3]	3.47	0.04	1.263	1.915	152
	8	1.12	NT[3]	4.58	0.16	1.953	2.325	119
	10	0.76	NT[3]	5.2	1.72	2.560	2.336	91
	12	0.15	NT[3]	1.42	0.24	0.603	0.709	118
15:1	6	0.64	3.99	2.36	1.22	2.053	1.476	72
	8	1.28	5.21	3.07	1.12	2.670	1.910	34
	10	1.96	2.42	3.29	0.39	2.015	1.216	60
	12	NT[4]	1.40	0.98	0.88	1.087	0.276	24
20:1	6	1.24	NT[3]	3.87	1.12	2.077	1.554	75
	8	1.28	NT[3]	0.71	0.64	0.877	0.351	40
	10	0.48	NT[3]	0.18	0.28	0.313	0.153	49
	Batch mean	0.91	4.24	2.74	0.68			
	Batch RSD (%)	56	40	56	75			

[1] GFP = green fluorescent protein; PEI = polyethylimine; N:P = nitrogen:phosphate; RSD = relative standard deviation; NT = not tested.

[2] Each batch contained cells from a single liver isolation procedure.

[3] Testing of batch B was limited by total cell numbers and insufficient cells were available for all the tests

[4] This sample was incorrectly tested and did not yield a valid result.

A graphical comparison of average efficacies obtained from PEI-mediated transfection of rat primary hepatocytes is shown in figure 4.6. Overall, there was no correlation between efficacy and DNA μg (correlation coefficient = -0.02) and only a weak negative correlation between efficacy and N:P ratio (correlation coefficient = -0.23). On average, the highest proportion of rat hepatocytes expressing the GFP transgene (i.e. highest efficacy) was obtained using an N:P ratio of 10:1 and 10 μg gWiz plasmid DNA, although the differences observed were not statistically significant and therefore any apparent trend in figure 4.6 should be interpreted cautiously.

A comparison of average transfection efficiencies obtained under the same range of conditions is shown in figure 4.7. The highest proportion of rat hepatocytes expressing the GFP transgene, when expressed per gram of DNA (i.e. highest efficiency) was found using an N:P ratio of 20:1 and 6 μg gWiz plasmid DNA. Although the differences were not statistically significant, and there appeared to be no correlation when the results from replicates were considered separately (correlation coefficient = -0.04), the mean values for efficiency (as shown in figure 4.7) reveal some negative correlation with the amount of plasmid DNA (correlation coefficient = -0.47). The lack of a clear correlation between efficacy and either DNA amount or N:P ratio is, perhaps, not unexpected, given that there must be a concentration of DNA which effectively saturates the potential for membrane penetration by the PEI-DNA complex, and that there must be an N:P ratio above which additional ("free") PEI either cannot enhance the uptake of PEI-DNA complex or may even interfere with it. A representative image of PEI-transfected rat hepatocytes, using 10 μg plasmid DNA at an N:P ratio of 10:1, is shown in figure 4.8.

The data in table 4.4 indicate that the most important variable affecting transfection efficacy was the batch of cells (A, B, C or D) used in the experiments, which may have contributed considerably to the difficulty in distinguishing between the effects of N:P ratio and DNA quantity. Although batch efficacies were very variable (RSD = 40 to 75%), with the exception of batches A and D, the differences between the batch means are significant ($P \leq 0.02$). Thus some unidentified (and hence unquantified) characteristic of the hepatocyte and/or complex populations had a major influence on transfection efficacy. Given that the rats were of a single laboratory strain, of similar age and same sex, and that every effort was made to achieve uniformity in the isolation of the hepatocytes, it may be that the between-batch differences in efficacy arose from inherent differences in the behaviour of hepatocytes from different individual rats towards PEI-DNA complexes. This difference has not been reported by other workers. An alternative explanation may be that small, unnoticeable differences in the physical preparation of the PEI:DNA complexes (such as the style and efficacy of mixing) resulted in variable populations of complex size and charge distribution, thus altering the entire batch efficacy.

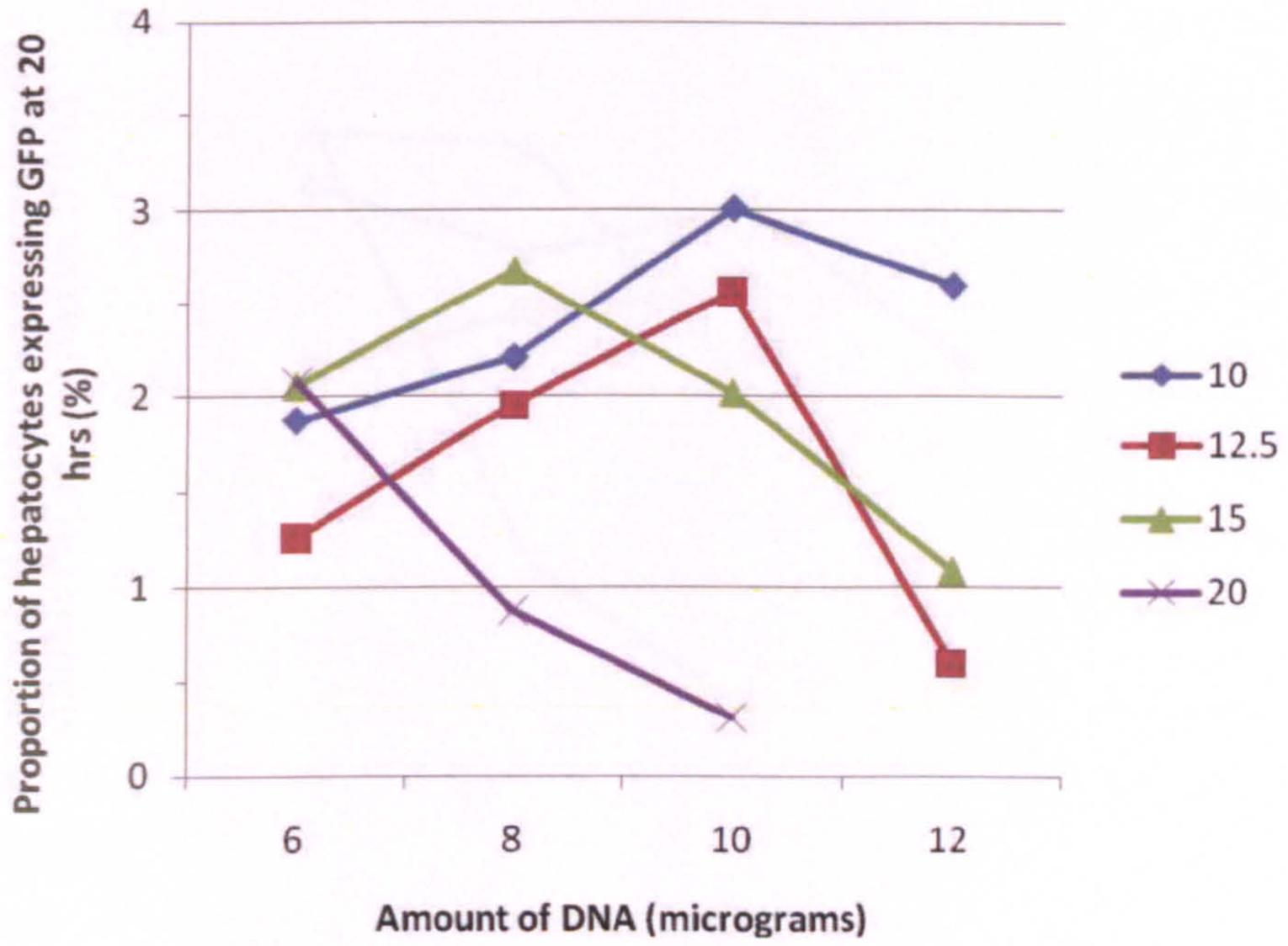


Figure 4.6: Transfection efficacies obtained by PEI transfection of rat primary hepatocyte monocultures. Legend indicates N:P ratios.

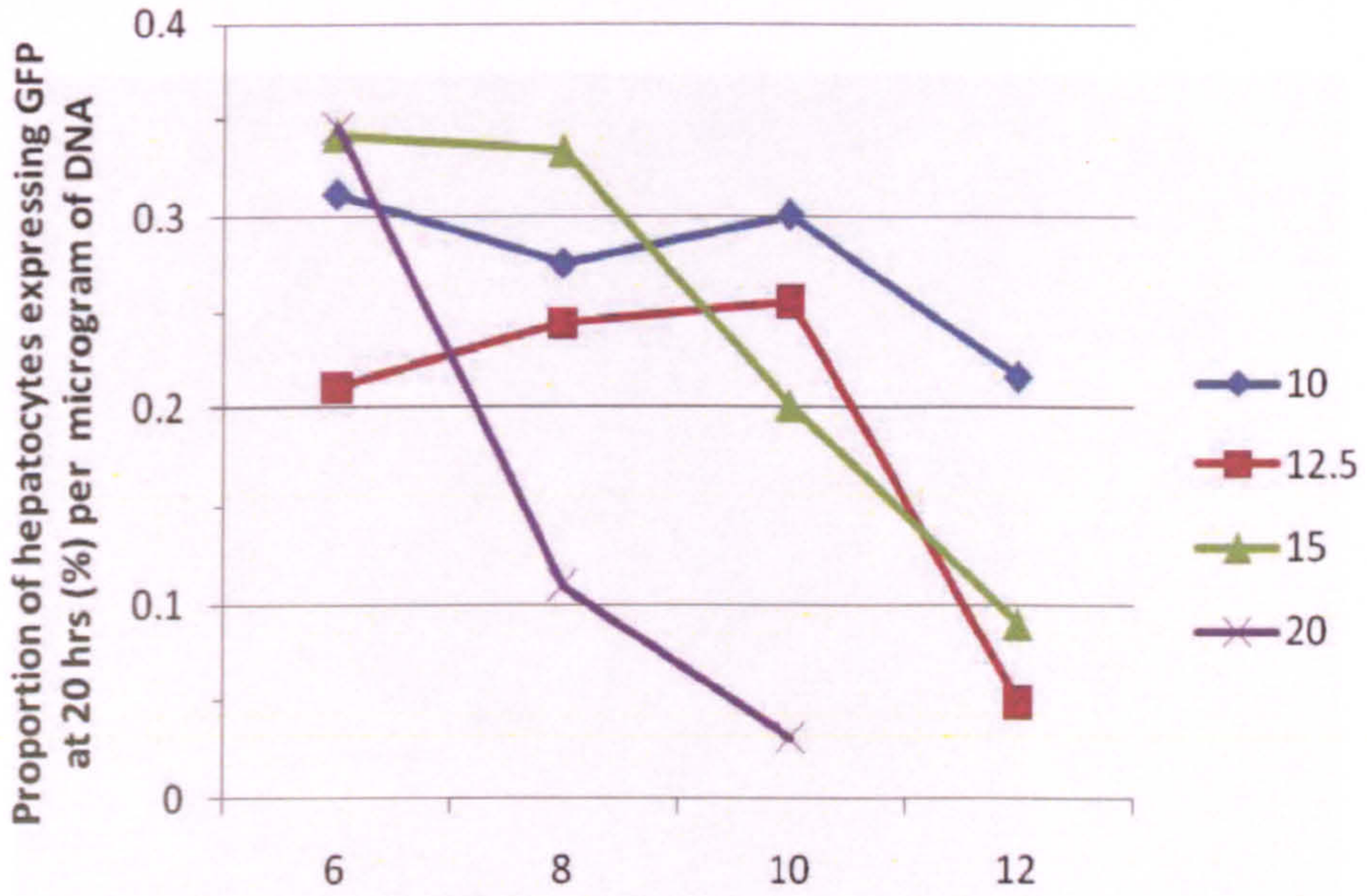


Figure 4.7: Transfection efficiencies obtained by PEI transfection of rat primary hepatocyte monocultures. Legend indicates N:P ratios.

Figure 4.8: Rat primary hepatocytes, visualised using ultra-violet fluorescence microscopy, with those visible having expressed the fluorescent protein GFP, 20 hours after PEI-mediated transfection of the gWiz plasmid.

4.3.2. Transfection efficacy and efficiency achieved by nucleofection of rat primary hepatocytes

The proportions of cells which were found to express the GFP transgene following nucleofection using varying amounts of plasmid DNA are shown in table 4.5.

The viability of control **e** was, on average, 20.9% lower at 15 minutes post-nucleofection, compared to the initial, post-isolation (pre-nucleofection) viability of the hepatocyte batch. Thus roughly 80% of rat hepatocytes survived nucleofection. This effect was evidenced by a visible reduction in the confluence of the hepatocyte monolayer of control d, when examined at 20 hours post-nucleofection, compared to non-nucleofected cells (controls a, b and **c**).

There were no visible signs of cell toxicity due to DNA (control b) or Nucleofector® buffer (control **c**) alone.

No expression of GFP was seen in any of the control wells (**a**-d).

Table 4.5: Expression of GFP in rat primary hepatocytes at 20 hours post-transfection using nucleofection[1].

DNA (µg)	Proportion of cells expressing GFP at 20 hours post transfection (%)				Mean efficacy (%)	Standard deviation
	Experimental hepatocyte batch[1]					
	C	E	F	G		
2	21.81	1.50	10.68	12.88	11.718	5.859
4	NT	9.03	19.89	28.21	19.043	4.761
6	26.42	16.64	21.63	24.09	22.195	3.699
8	NT	20.51	23.32	36.80	26.877	3.360
10	55.96	NT[2]	51.91	54.64	54.170	5.417
Batch mean	34.7	11.9	25.5	31.3		
Batch RSD %	53	71	61	50		
Viability drop %	NT	18.5	23.5	20.7	20.90	2.51

[1] GFP = green fluorescent protein; RSD = relative standard deviation; NT = not tested.

[2] Each batch contained cells from a single liver isolation procedure.

[3] This sample was incorrectly tested and did not yield a valid result.

In contrast with PEI-mediated transfection, there was much less evidence of batch-to-batch variation in efficacy. Batches C, F and G produced very similar levels and ranges of efficacy and, although batch E produced the lowest average efficacy, the differences were only borderline for statistical significance ($p = 0.04$, 0.08 and 0.03, for batches C, F and G, respectively). Most of the hepatocyte batches were different from those used for PEI-mediated transfection and it is possible that the lesser between-batch variation observed with nucleofection arose by chance but it may also be that electroporation, at least in the form of nucleofection, is less prone to variations in efficacy resulting from differences between the livers of individual rats.

Also in contrast with PEI-mediated transfection, nucleofection showed a clear positive correlation between DNA amount and the efficacy achieved in transfection (figure 4.9) (correlation coefficients 0.87 to 0.99 for individual batches). This is logical, given that a higher concentration of plasmids around a membrane pore should increase the numbers entering the cell and, presumably, the nucleus.

Figure 4.10 shows the efficacy of transfection of rat hepatocytes by nucleofection, as compared with the PEI-mediated transfection at N:P ratio of 10:1. The highest proportion of cells expressing the GFP transgene was obtained by nucleofection using 10 μg DNA (54.17%; range 51.91-55.96). The mean difference between the maximum efficacy following nucleofection and the maximum efficacy following PEI-mediated transfection was 51.16%. This difference was highly statistically significant ($p < 0.0001$).

Maximum efficiency of nucleofection was observed using 2 µg DNA, as shown in figure 4.11. However, the difference in efficiency between 2 and 10 µg DNA was not significant ($p = 0.865$). Furthermore, although there was an apparent nadir of efficiency at 6-8 µg DNA, the high variability of batch efficacies obtained using 2 µg DNA mean that the comparatively lower efficiencies at 4, 6 or 8 µg were not statistically significant. However, the increase in efficiency achieved by changing from 6 or 8 µg DNA to 10 µg DNA was significant ($p = 0.033$ and <0.001 respectively). Nucleofection with 10 µg DNA was very significantly more efficient than PEI transfection using the same amount ($p = <0.0001$).

A representative image of nucleofected rat hepatocytes, using 10 µg plasmid DNA, is shown in figure 4.12. The brightness of fluorescence per cell, although variable, was noticeably higher than following PEI-mediated transfection, suggesting the presence of higher levels of the protein within each cell. This may be due to a higher rate of translation (perhaps due to higher copy numbers of the plasmid per cell) or may simply reflect earlier initiation of translation (due to the rapid, and transport-independent, nature of plasmid entry during any form of electroporation) and, consequently, a higher level of GFP at the time of analysis.

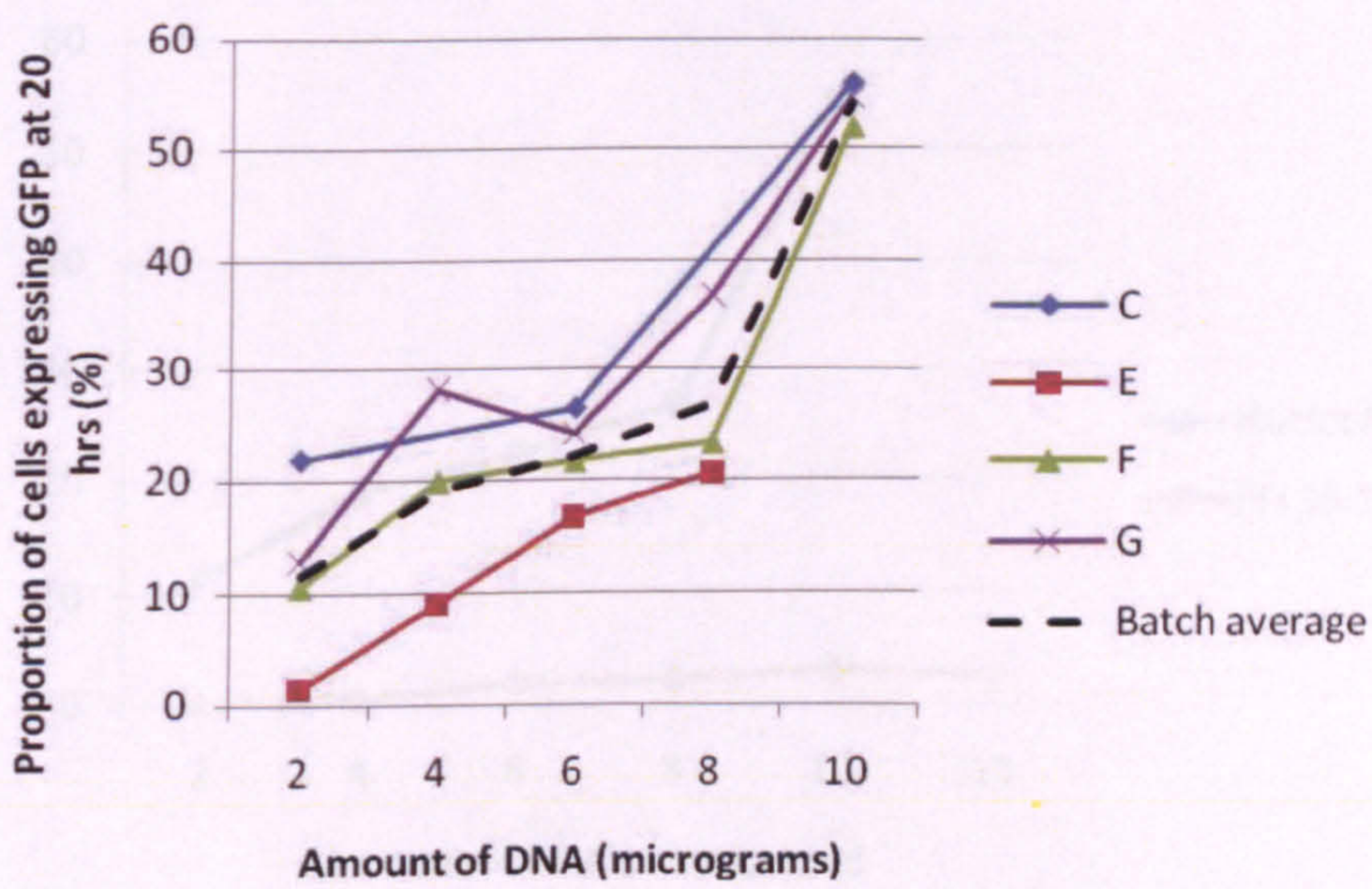


Figure 4.9: Graph showing the influence of plasmid DNA, µg, on the efficacy of transfection by nucleofection. Legend shows batch codes.

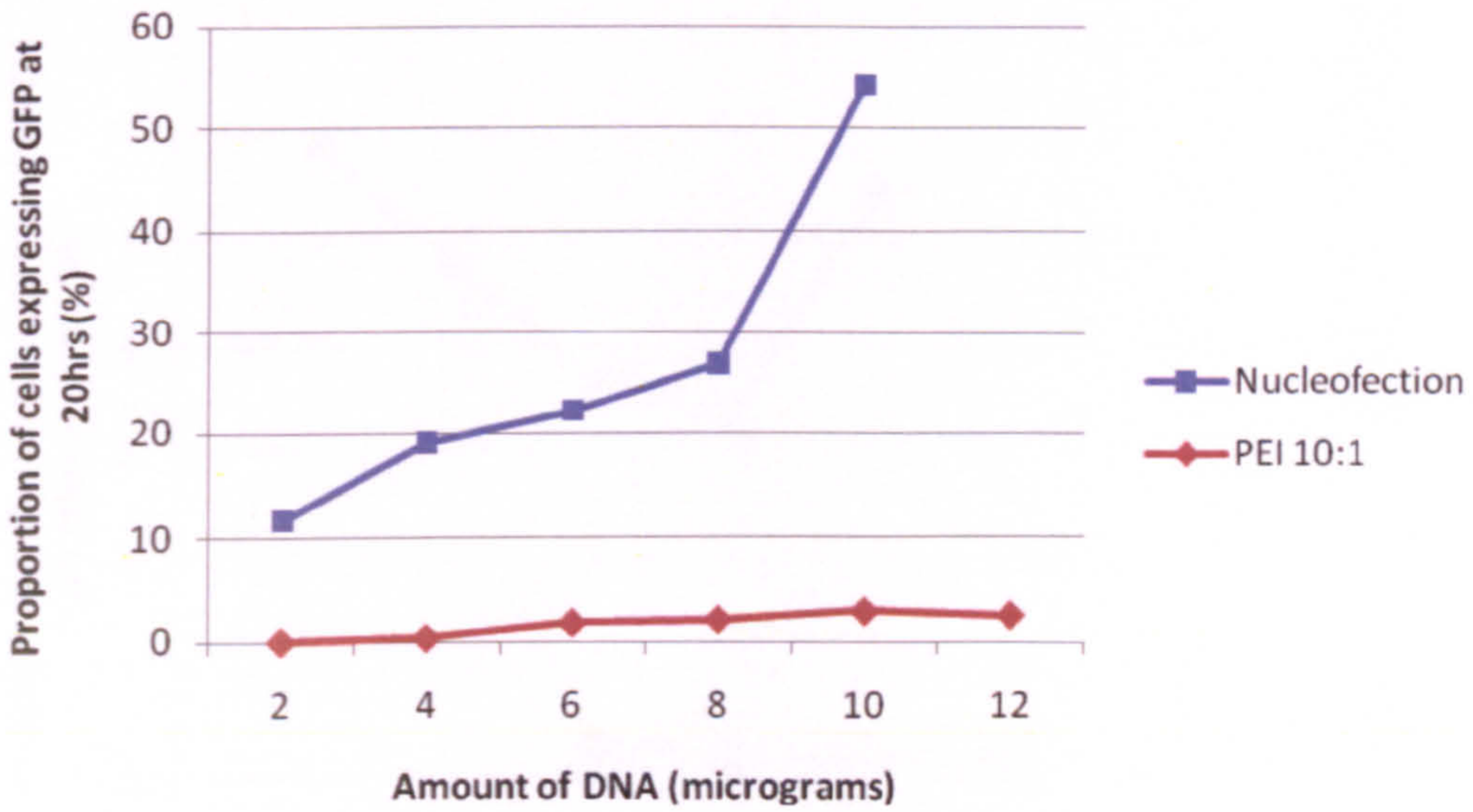


Figure 4.10: The gWiz plasmid is more effectively expressed in rat primary hepatocytes following nucleofection of freshly isolated cells (◆; mean values as shown in table 4.5), in comparison with PEI mediated transfection of 2 hours-old monocultures at an N:P ratio of 10:1 (■; as shown in tables 4.3 and 4.4).

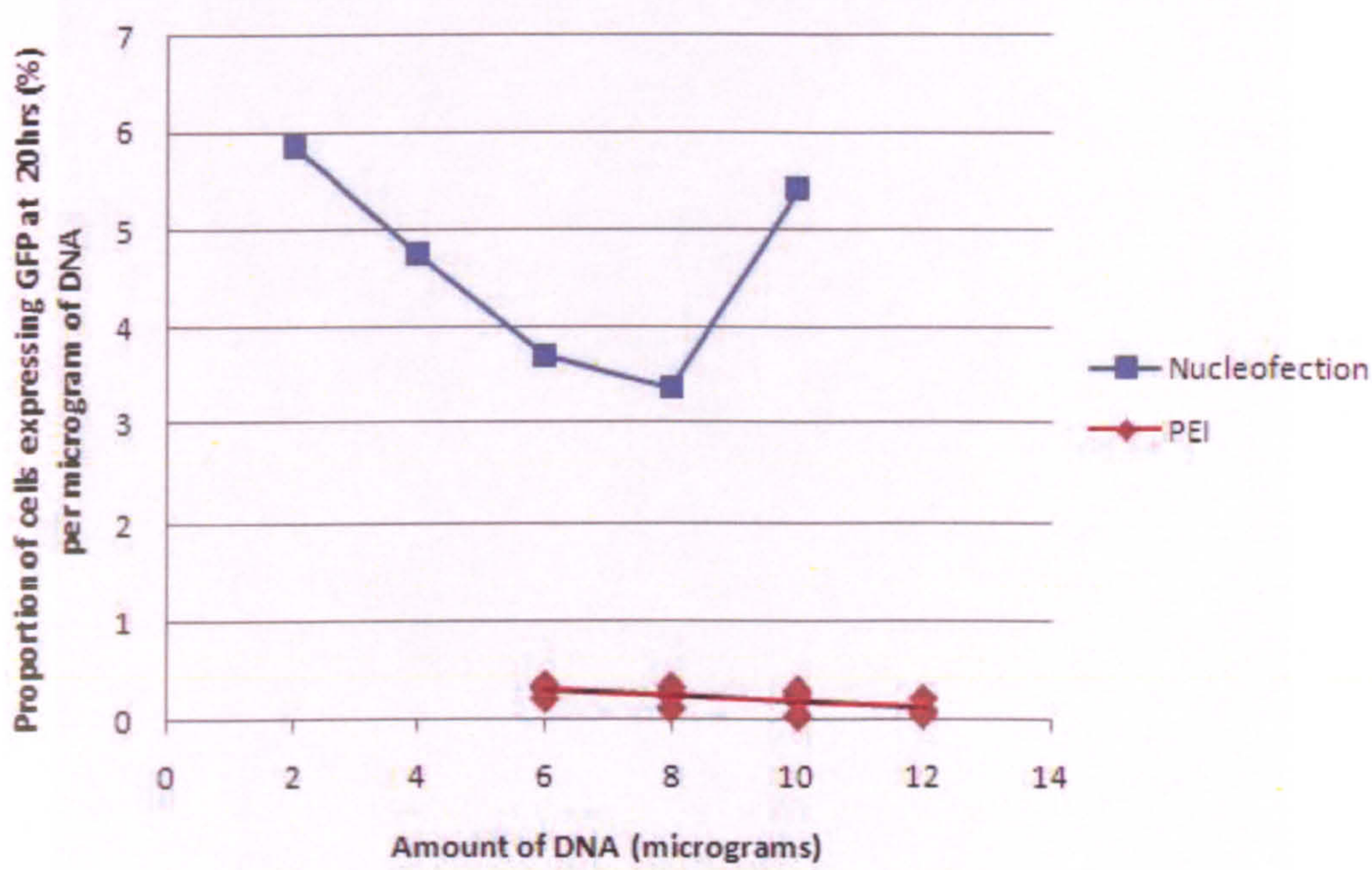


Figure 4.11: The gWiz plasmid is more efficiently expressed in rat primary hepatocytes following nucleofection of freshly isolated cells (◆), in comparison with PEI-mediated transfection of 2-hour-old monocultures at all N:P ratios (■).

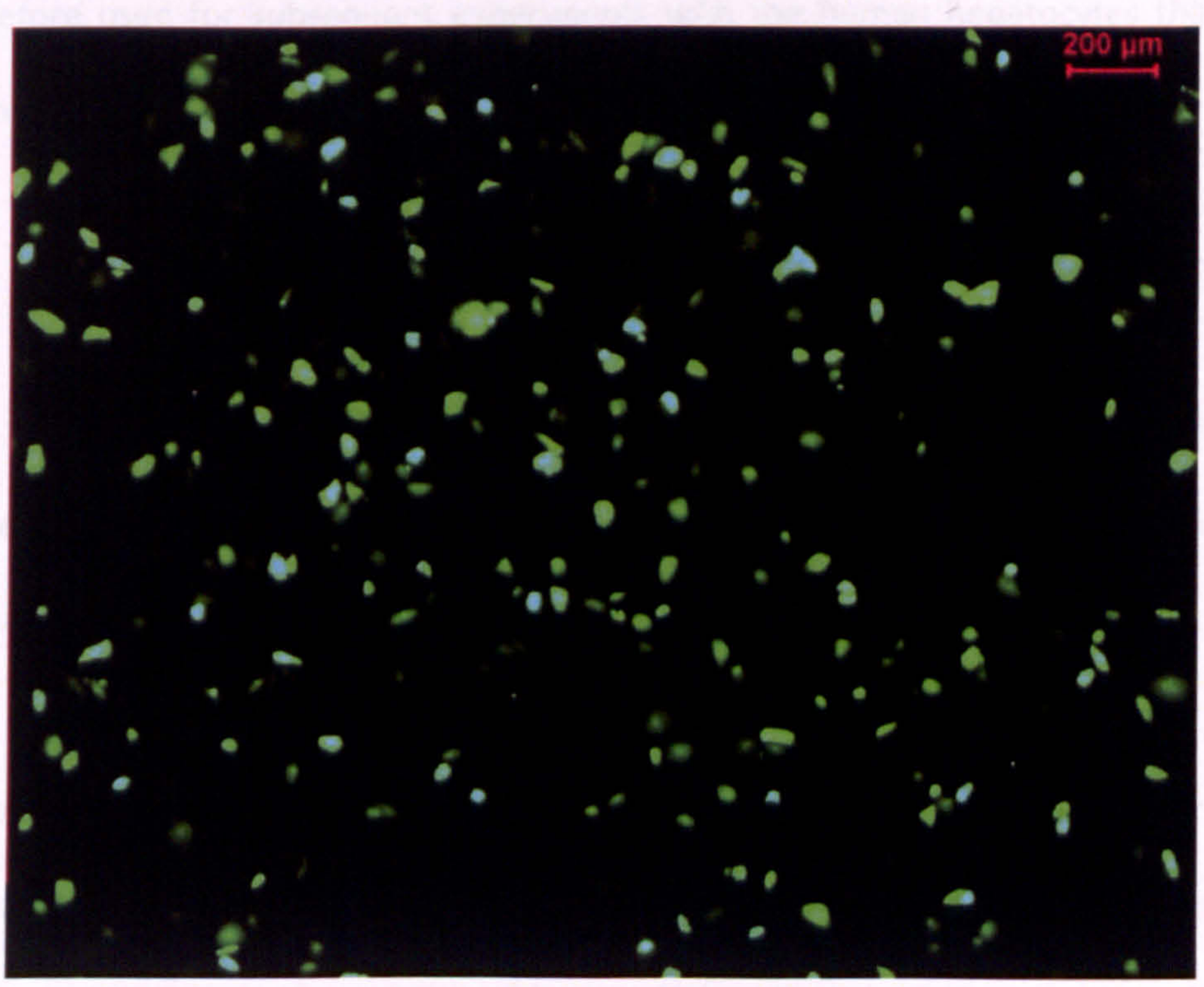


Figure 4.12: Rat primary hepatocytes expressing the fluorescent protein GFP, 20 hours after nucleofection of the gWiz plasmid. The cell monolayer had been washed to ensure that all visible cells were adherent and therefore equidistant from the lens. Note the varied, but generally high, level of fluorescence per cell.

4.3.3. Transfection efficacy and efficiency achieved by nucleofection of human primary hepatocytes

Electroporation, in the form of nucleofection, having proven to give much higher transfection efficacy with rat hepatocytes than PEI-mediated transfection, was therefore used for subsequent experiments with the human hepatocytes that had been isolated.

4.3.3.1. Effect of a post-nucleofection rest period prior to addition of cell culture media

Table 4.6 and figure 4.13 show the results obtained by nucleofection of human primary hepatocytes from a single hepatocyte batch (15), using various amounts of the pMaxGFP™ plasmid, with or without the addition of a 15-minute post-electroporation rest period. Four discharge programmes were used for this assessment, as advised by the kit manufacturer (A24, M23, P20, T28). The addition of the rest period was associated with a significant increase in transfection efficacy ($p < 0.035$ for all DNA values). Efficacy was strongly correlated with DNA amount, without or with the rest period (correlation coefficient 0.98 and 0.91, respectively).

Further experiments, to determine the optimum Nucleofector® programme and DNA quantity for expression of the transgene, were all performed with the addition of a 15-minute post-nucleofection rest period, as indicated by the above results.

Table 4.6: Expression of GFP in human primary hepatocytes at 20 hours post-transfection using nucleofection, either with or without a 15-minute rest period post-electroporation[1].

DNA (µg)	Nucleofection Programme	Proportion of cells expressing GFP at 20 hours post-transfection (%)					
		Plated immediately			Plated after 15-minute rest		
		Results	Mean	SD	Results	Mean	SD
2	A24	0.66	0.55	0.35	1.11	2.47	1.25
	M23	0.63			3.29		
	P20	0.85			3.75		
	T28	0.05			1.74		
4	A24	1.65	1.81	1.13	3.25	6.34	3.12
	M23	3.44			10.68		
	P20	1.00			5.51		
	T28	1.13			5.92		
6	A24	5.00	3.90	1.04	23.04	19.18	5.53
	M23	4.18			24.80		
	P20	3.92			15.06		
	T28	2.50			13.80		

[1] GFP = green fluorescent protein; SD = standard deviation

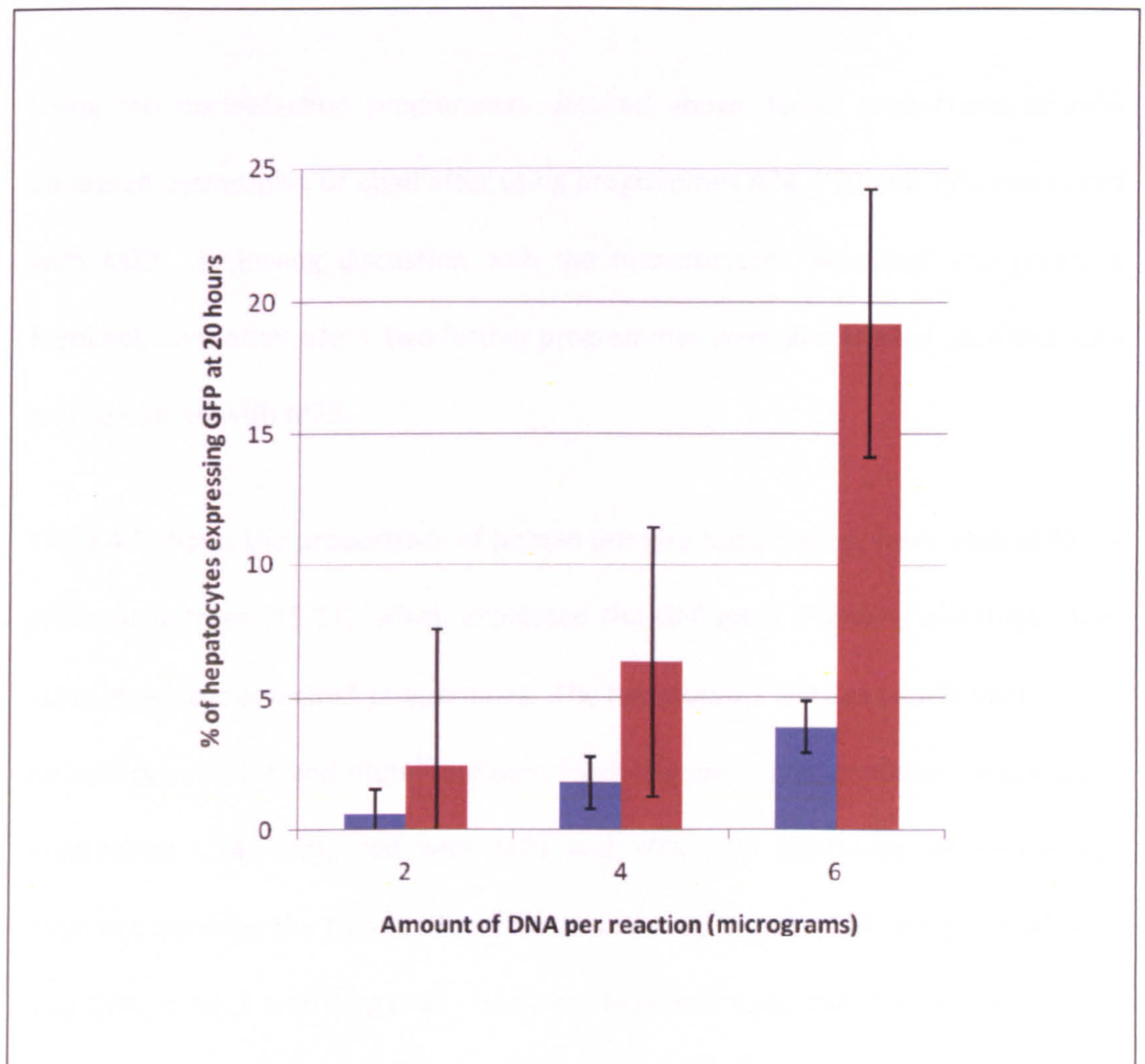


Figure 4.13: *Transfection efficacy of nucleofection of freshly isolated human hepatocytes is significantly increased by allowing a 15-minute incubation period at room temperature after nucleofection and prior to plating (showing standard error bars).* ■ *cells plated immediately following nucleofection;* ■ *cells plated following a 15-minute undisturbed rest period after nucleofection [n = 4].*

4.3.3.2. Use of various, manufacturer-recommended nucleofection programmes for transfecting human primary hepatocytes

Using the nucleofection programmes detailed above, larger proportions of cells appeared dysmorphic or dead after using programmes A24, P20 and T28, compared with M23. Following discussion with the manufacturer, who had also received feedback from other users, two further programmes were also trialled (U14 and X09) and compared with M23.

Table 4.7 shows the proportions of human primary hepatocytes, from each of three different batches (15-17), which expressed the GFP gene following electroporation using three Nucleofector® programmes. The hepatocytes were generally observed to be less dysmorphic and more confluent following overnight incubation when using programme U14, compared with M23 and X09. No significant difference was observed between the transfection efficacies of programmes U14 and X09, with 4 or 6 µg DNA, or M23 with 6 µg DNA. However, M23 with 4 µg DNA showed significantly lower efficacy ($p = 0.001$) than the other combinations, confirming (with different batches of hepatocytes) the observation in table 4.6 that programme M23 showed a correlation between efficacy and DNA amount, over this narrow range. The absence of a similar correlation when using programmes U14 and X09 might have indicated either that transfection efficacy was already maximal using 4 µg DNA in combination with U14 and X09 or that the apparent differences and similarities between all three programmes were actually due to the different hepatocyte batches used for each programme. Nonetheless, on the basis that programme U14 produced the lowest proportion of dead/dysmorphic cells, it was selected for use in the final experiments to optimise the quantity of DNA required.

Table 4.7: Expression of GFP in human primary hepatocytes at 20 hours post-transfection and with a 15-minute rest period post-transfection, using three different nucleofection programmes[1].

DNA (µg)	Proportion of cells expressing GFP at 20 hours post-transfection (%)								
	Programme M23			Programme U14			Programme X09		
	Results	Mean	SD	Results	Mean	SD	Results	Mean	SD
4	10.68	10.74	0.80	16.72	21.90	7.07	10.64	21.10	9.76
	11.56			29.96			29.97		
	9.97			19.03			22.70		
6	24.80	25.00	2.85	19.51	22.33	2.67	13.03	20.41	7.50
	27.94			24.82			28.02		
	22.26			22.68			20.18		

[1] GFP = green fluorescent protein; SD = standard deviation.

4.3.3.3. Transfection efficacy and efficiency achieved by nucleofection of human primary hepatocytes using a range of DNA quantities

The proportions of cells from each of 3 further batches of hepatocytes (23-25), which were found to express the GFP transgene following nucleofection using programme U14 and varying amounts of pMaxGFP™ plasmid DNA, are shown in table 4.8.

These three batches of hepatocytes showed clear correlations between transfection efficacy and DNA amount (correlation coefficients = 0.90 to 0.97), using programme U14. Taking into account the results presented in tables 4.6 and 4.7, this indicates that the presence or absence of such a correlation is at least partly dependent upon the particular batch of hepatocytes.

Maximum efficacy was obtained using 8 µg DNA (the range of DNA values was not extended, in order to conserve reagents) with hepatocyte batches 23, 24 and 25. Using 2 µg DNA was associated with significantly lower efficacy than when using 4, 6 or 8 µg DNA ($p < 0.02$). Maximum efficiency of transfection was obtained using 4 µg DNA. Using 2 µg DNA was associated with significantly lower efficiency than when using 4, 6 or 8 µg DNA ($p < 0.04$). As shown in figure 4.14, the efficacy of the batches was consistently in the order 24>23>25, underlining the fact that efficacy was also batch-dependent, in addition to being dependent on the amount of plasmid DNA used in these three batches. Transfection efficiency showed a similar pattern of batch-dependency. Figures 4.15, and 4.16, show the average efficacy and efficiency, respectively, of nucleofection in batches 23, 24 and 25 of human primary hepatocytes. Figure 4.17 shows human primary hepatocytes that expressed GFP following nucleofection with 8 µg pMaxGFP™ DNA using programme U14.

Table 4.8: Expression of GFP in human primary hepatocytes at 20 hours post-transfection using nucleofection[1].

DNA (g)	Proportion of cells expressing GFP at 20 hours post transfection (%)			Mean efficacy (%)	Standard deviation
	Experimental hepatocyte batch				
	23	24	25		
2	3.29	3.75	1.43	2.823	1.228
4	17.30	22.45	10.68	16.78	5.896
6	19.95	25.13	18.77	21.28	3.383
8	22.14	29.10	20.82	24.02	4.449

[1] GFP = green fluorescent protein

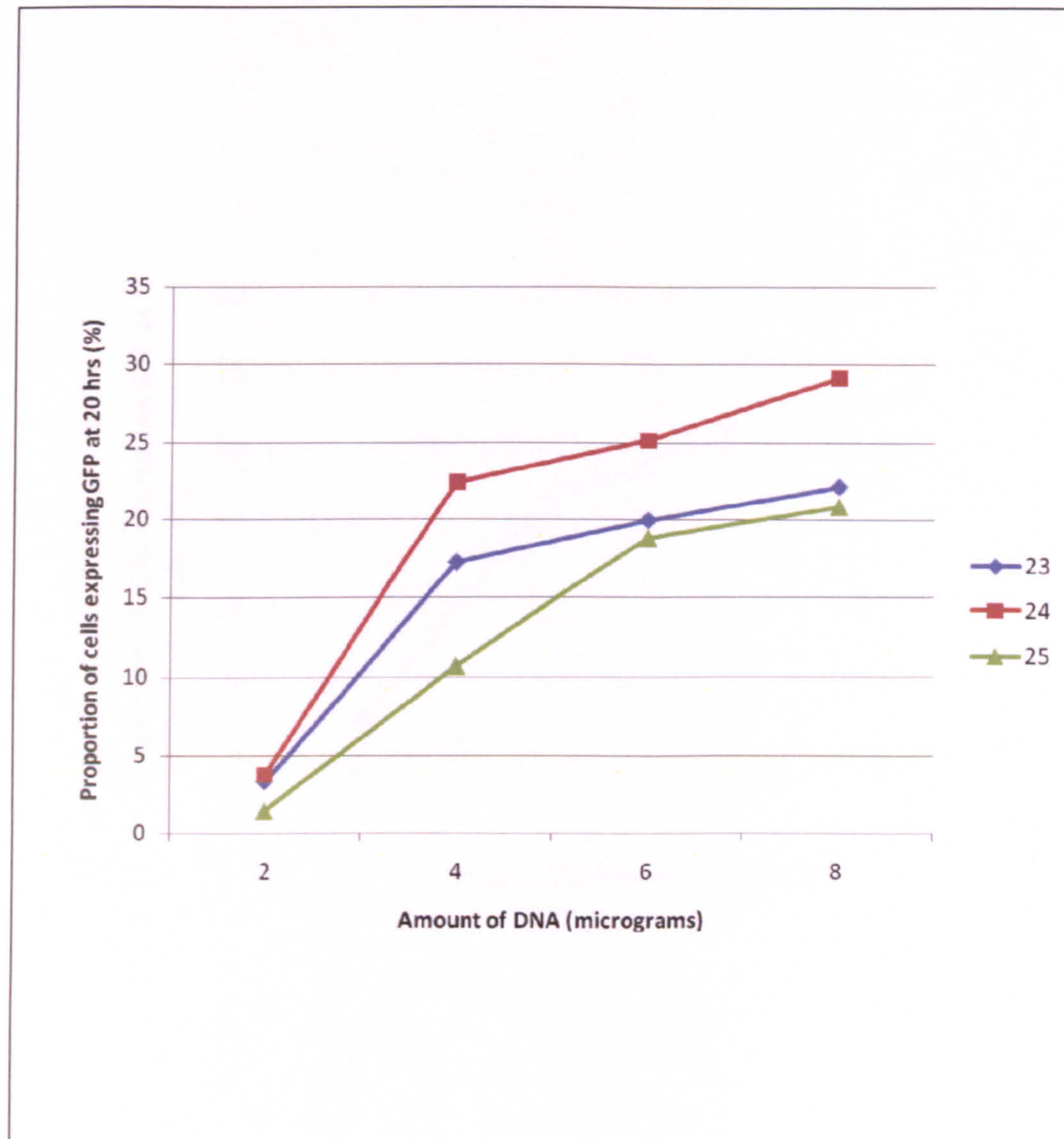


Figure 4.14: Influence of human hepatocyte batch on the efficacy achieved using nucleofection and varying amounts of the pMaxGFP™ plasmid DNA. Legend shows human hepatocyte batch number.

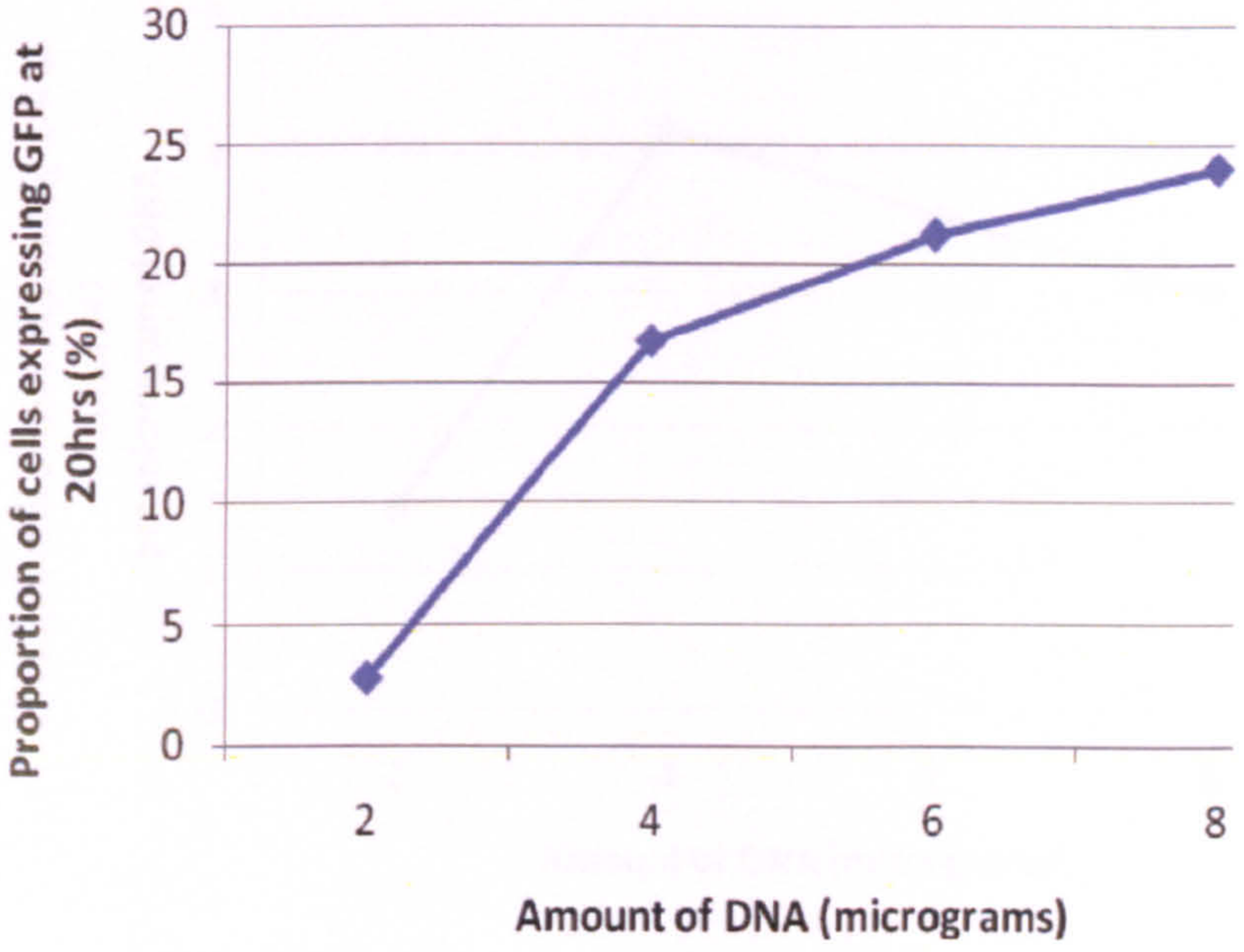


Figure 4.15: Transfection efficacies (average of batches 23, 24 and 25) obtained using nucleofection of the pMaxGFP™ plasmid in freshly isolated human primary hepatocytes.

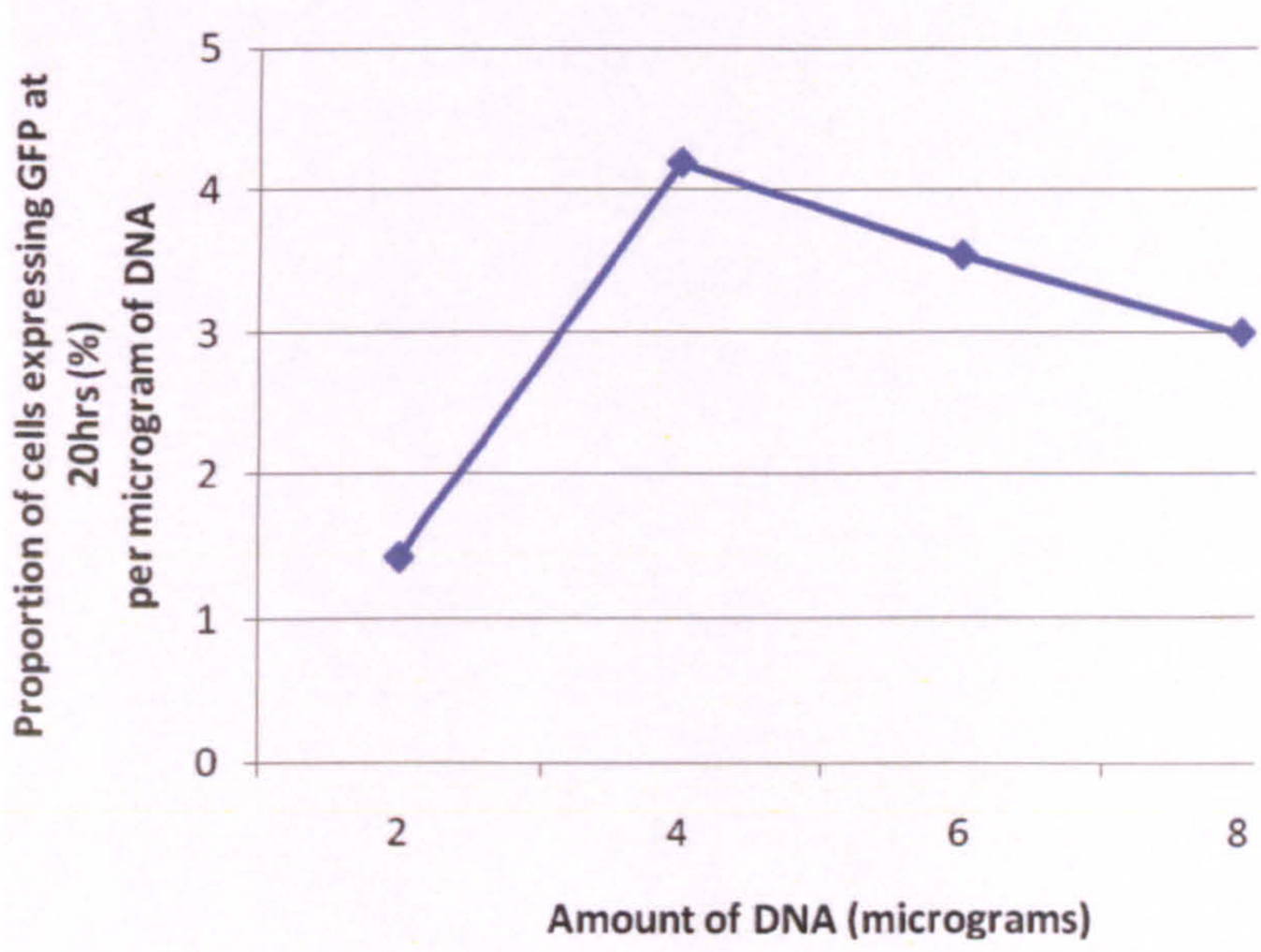


Figure 4.16: Transfection efficiencies (average of batches 23, 24 and 25) obtained using nucleofection of the pMaxGFP™ plasmid in freshly isolated human primary hepatocytes.

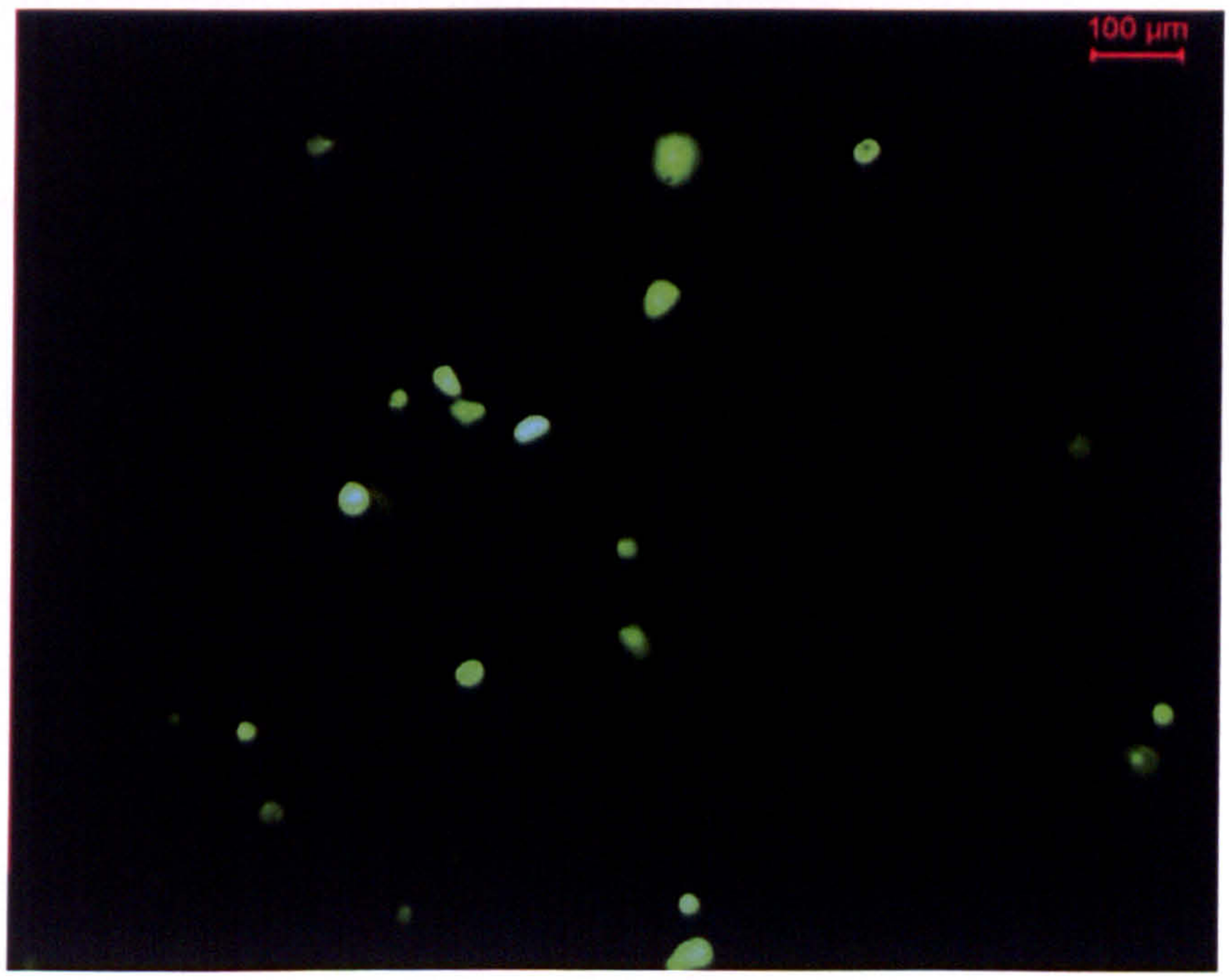


Figure 4.17: Human primary hepatocytes containing the fluorescent protein GFP, 20 hours after nucleofection of the pMaxGFP™ plasmid. The cell monolayer had been washed to ensure that all visible cells were adherent and therefore equidistant from the lens. A variable, but generally high, level of fluorescence is seen per cell.

4.4. Discussion

The selection and optimisation of a method to transfect heterogeneous populations of fragile, non-proliferating cells (isolated from a single piece of liver tissue) requires solutions for various potential problems, the number of which is increased if there is a need, or preference, for more than just a low level of transgene expression. During the experiments described above, two non-viral transfection methods, based on very different principles, were optimised for rat and human primary hepatocytes. Each method had advantages and disadvantages which impacted upon its overall utility and which are discussed further below.

4.4.1. PEI-mediated transfection of rat primary hepatocytes

As expected, PEI-mediated transfection of rat primary hepatocytes was found to be affected by numerous factors; including the N:P charge ratio, amount of DNA applied, composition of the PEI:DNA diluents and composition of the cell culture media. Despite controlled changes in these variables, only low proportions of cells could be induced to express the transgene. Furthermore, most of the transfected cells were only weakly fluorescent under ultra-violet light, suggesting either that few copies of the transgene entered each cell, or that the rate of translation was low (perhaps hampered by any continuing association with PEI).

In the above experiments on rat hepatocytes, maximum efficacy was observed when using an N:P ratio of 10:1, although this conclusion was not statistically proven. It has been shown that polyplexes formed at N:P ratios of >4 generally contain fully condensed DNA and tend towards smaller size as the N:P ratio increases[120]. In these experiments, no expression was seen with N:P ratios of <4, suggesting that

condensation (and perhaps smaller size) is important for successful transfection. Smaller polyplex size has, with some cell and polyplex types, been associated with higher rates of plasmid transfer to the nucleus, and thus transgene expression[120].

No demonstrable changes in the confluence of the hepatocyte monolayers were observed following exposure to the PEI polyplex solution, other than at the highest N:P or DNA values (section 4.3.1), thus toxicity during transfection was not considered to have influenced the results at lower levels. Further experiments, to assess the viability or hepatocyte-specific function of hepatocytes following PEI transfection at a range of N:P ratios and DNA amounts, were not performed to confirm this observation. This would have been an obvious subject for experimental trial, had an acceptable level of efficacy been achieved with PEI-mediated transfection.

The efficacy of PEI-mediated transfection appears to be highly variable depending on hepatocyte source species but, in primary hepatocytes derived from tree shrews and ducks, it has been found to induce expression of a transgene in 10 and 50%, respectively, of observed cells[136]. These levels are higher than the maximum average (3%) found in these experiments on rat primary hepatocytes. There was also a marked variation in the range of efficacies found, under different conditions, using different batches of rat hepatocytes. Each batch was prepared from a different rat, but the animals were of identical strains, kept under identical conditions of husbandry, and the hepatocyte preparations were all of very similar, high viability. The reasons for the high level of between-batch variation in PEI-mediated transfection efficacy are uncertain. Some variation between the cell populations was inevitable, both as a result of differences in the *in vivo* physiology of the hepatocytes

(perhaps due to slight variations in animal age) and due to anatomical variation affecting the liver perfusion procedure. Both of these unavoidable factors would have had some impact on the relative physiological similarity of the resultant hepatocytes *in vitro*. Small, unrecorded variations in the hepatocyte isolation procedure may have caused some level of non-fatal cell damage, thus affecting the subsequent response of the cells to the transfection procedure. Despite very careful preparation, the size, charge distribution and numbers of PEI:DNA complexes applied to the cells presumably also varied, both within and between batches (although these parameters were not quantified during the above experiments). For example, manual mixing would probably produce heterogeneous populations of complexes, and short, unavoidable delays in the application of complexes are thought to result in increased complex size[120], although the exact impact of these variables remains unclear and unpredictable. Nonetheless, as these parameters are thought to be important in optimising the efficacy of PEI-mediated transfection, they could have led to the variations in results seen in the above experiments. Whatever the cause of between-batch variations in PEI transfections, its occurrence is undesirable and creates problems in the interpretation of data from subsequent experiments.

The low efficacy and apparent low level of cellular transgene expression in rat primary hepatocytes resulting from PEI transfection was disappointing and would have created problems in the conduct of later experiments to investigate the replication of JFH1, if the electroporation technique had not been available as an alternative.

4.4.2. Issues affecting the comparison of PEI-mediated transfection and nucleofection

Nucleofection, which has been reported to produce high transfection efficacy and lower levels of cell damage than are commonly associated with electroporation, seemed to offer a possible alternative to PEI-mediated transfection. The relative efficacy and efficiency of PEI and nucleofection in the transfection of rat primary hepatocytes, was therefore studied. However, there are important differences between PEI-mediated and electroporation-mediated (nucleofection) transfection which make it difficult to compare their efficacies directly. Perhaps the most obvious of these stem from the differing physical requirements of each procedure.

The nucleofection method, in common with most platforms for electroporating mammalian cells, requires the target cells to be suspended in a defined solution of electrolytes and enclosed within a cuvette containing metal contacts for conductance of an electrical pulse or pulses. This requirement for use of a cell suspension essentially restricts the use of electroporation to freshly-isolated cells (unless specialised equipment is available), due to the physical intolerance of primary hepatocytes to disturbance, or passage, once established in adherent culture. In contrast, the long duration needed for polyplex contact, together with the numerous wash procedures involved in PEI-mediated transfection, require adherent culture of hepatocytes to have been established before using this procedure. This unavoidable difference in the cells used for transfection gives rise to a number of confounding factors when attempting to compare the outcomes of these methods.

Firstly, differences in the length of time between hepatocyte isolation and transfection can be expected to result in the transgene being transported, transcribed and translated in cells with very different levels of environmental (particularly cell-to-cell) stimuli. Hence, such cells are likely to experience both qualitative and quantitative differences in intra- and inter-cellular signalling, which will subsequently affect many aspects of differentiated cell functions, gene activation and cell cycle control.

Secondly, a relatively high level of cell attrition occurred following nucleofection. The viability of rat hepatocytes was, on average, 20.9% lower at 15 minutes post-nucleofection than before nucleofection. However, due to the nature of electroporation, these measurements are unlikely to afford an accurate representation of cell viability, because the pores created by electroporation would be expected to resolve in many cells once placed into supportive culture conditions. Thus cell viability may have been under-estimated due to penetration of the dye through the induced pores in cells which could have recovered. Furthermore, dead, non-adherent cells were removed by gentle flushing of media over the cell monolayer, following overnight incubation after transfection. The efficacy, as measured by expression of the transgene after overnight incubation, is expressed as a proportion of the remaining, viable cell population; the proportions recorded are thus both statistically, and practically, enriched. PEI-mediated transfection was not observed to produce any excess cell death.

Further variables, which affect any comparative assessment of primary cells, are those which stem from the fact that each batch is derived from a different patient or animal, and may therefore possess different (and unpredictable) levels of nutrition,

function and robustness. Any pre-existing differences will be further complicated by cellular variation arising from the hepatocyte isolation procedure (as presented and discussed in Chapter 3). Therefore, not only is there heterogeneity within hepatocyte populations but also between them, with between-population variability being unavoidable even if the two populations are derived from the same animal (or patient). In any case, due to the limited availability of cells and (in the case of nucleofection) reagents at any one time, it was not possible to perform synchronous comparative experiments on all occasions. This inevitably leads to some uncertainty when drawing conclusions, which could only ever be partially mitigated by increasing the experimental numbers.

There are advantages arising from a transfection protocol which can be applied immediately after isolation of a hepatocyte suspension from the liver tissue. Principal among these is the ability to place the transfected cell population into any culture conditions (and with any other cell types) required for subsequent experiments, whether that might be sandwich mono- or co-culture, free-floating spheroid co-culture, or bio-reactive scaffold culture. Furthermore, it is advantageous to be able to introduce the cells into these systems as soon as possible following isolation, in order to allow maximal retention of hepatocyte-specific cell functions.

4.4.3. Nucleofection of rat primary hepatocytes

Rat hepatocytes displayed visibly higher amounts of fluorescence after nucleofection than after PEI-mediated transfection, suggesting that higher copy numbers of the transgene had been transferred into the nuclei and/or higher rates of transcription and translation had occurred due to the differing ages of the cells (as discussed

above). However, the freshly isolated cells used for nucleofection were unlikely to have been as functional or responsive as the newly-cultured cells used in PEI-mediated transfection, due to the recent mechanical and physiological trauma they had received, and therefore the observed higher levels of GFP protein in nucleofection could have stemmed from its higher efficacy of transfection. However, it must be borne in mind that expression of the GFP gene in both of the plasmid constructs used is driven by the cytomegalovirus intermediate-early (CMV-IE) promoter sequence, which is a common, and highly effective promoter sequence used in many experimental plasmids. Derived from a highly infectious virus, the sequence might enable preferential translation of its associated genes without requiring the host cell to be fully functionally intact and differentiated. Changes in the intensity of fluorescence, following either PEI- or Nucleofector®-mediated transfection, did not occur for up to 3 days post-transfection, which implies that the level of GFP expression in individual cells was influenced, in this case, more by the number of gene copies which reached the nucleus, than by differences in the age and/or differentiation of the host cells (i.e. than the rates of expression). It is unknown whether GFP synthesis ceased, due perhaps to loss of the transfected material, or simply reached a balanced level between synthesis and degradation, and therefore it is not possible to deduce whether there were any differences in these mechanisms between PEI-transfected or nucleofected cells.

In the experiments described in this chapter, nucleofection was found to provide superior efficacy and efficiency of expression of the GFP transgene in rat primary hepatocytes, in comparison with PEI-mediated transfection. Nucleofection produced a maximum efficiency at 10 µg DNA, which was approximately 14-fold higher than that obtained using PEI. The highest nucleofection efficiency required only about

2.3×10^6 plasmids per hepatocyte to effect the transfection of approximately 50% of cells. Thus, nucleofection resulted in the GFP gene being expressed in approximately 40,000 hepatocytes per µg of DNA. Despite the attrition rate of around 21%, this was therefore a very effective means of transfection. Killed cells were largely removed by washing the monolayer established after overnight incubation following nucleofection. However, if transfected cells were to be used in any form of suspension or aggregate culture following transfection, it would probably be necessary to insert a sedimentation step (with or without the addition of a density gradient), in order to remove dead cells before proceeding.

4.4.4. Nucleofection of human primary hepatocytes

The good results obtained by nucleofection of rat hepatocytes in these experiments, and favourable reports of the technique in the literature, supported the trial of nucleofection for the transfection of human primary hepatocytes. At the time these experiments were performed, the manufacturer (Amaxa®), did not offer a commercial protocol for transfection of human primary hepatocytes, but was able to provide some buffer solutions (presumably similar to those already marketed for rat and mouse hepatocytes) for experimental testing. Limited availability of these solutions, and of human hepatocytes (for the reasons detailed in the chapter on hepatocyte isolation), unfortunately restricted the scope of experiments performed. For these reasons, experimental numbers were sub-optimal, and the effects of nucleofection on human hepatocyte cell numbers and viability could not be fully assessed, in contrast to the measurements performed during the rat hepatocyte nucleofection experiments. This was partly due to the restricted amount of cuvettes and buffer available (allowance could not be made for the additional controls

required), but also partly because it was not possible for one person to perform all of the procedures and tests required without compromising the overall speed of the experiment (and therefore adversely affecting hepatocyte viability).

As with rat hepatocytes, the nucleofection procedure was found to be rapid and relatively simple for use with human hepatocytes. The ability to include fetal calf serum in the cell culture medium, without affecting the success of the transfection procedure, was particularly useful for human hepatocytes, as the isolation procedure is invariably longer and thus physiologically more traumatic to the cells than for rat hepatocytes, and serum seems to be important in cell recovery, post-isolation. Using a proprietary technology is very restrictive in terms of the extent to which variables (notably the code-named electroporation programmes and cell suspension buffer) can be tested, and their effects understood, during the optimisation of a method. However, the efficacy and reliability of the method compensated, in part, for this disadvantage.

Nucleofection of human primary hepatocytes resulted in relatively high proportions of cells expressing the GFP transgene. In the same manner as with rat primary hepatocytes, nucleofection appeared to cause the death of a substantial proportion of cells which were, however, easily removed by gentle flushing of media over the cell monolayer, following overnight incubation after transfection. Both the measured efficacy and the measured efficiency were proportionately increased by this removal of dead cells (and therefore reducing the magnitude of the denominator). The creation of a high proportion of transfected cells was a key requirement for the subsequent experiments and, together with the high hepatocyte cell numbers

obtained by optimising the liver perfusion protocol, this enrichment of the transfected cell fraction was extremely beneficial.

In summary, two protocols for the non-viral transfection of primary hepatocytes were optimised. PEI-mediated transfection resulted in low efficacy and efficiency of transgene expression in rat primary hepatocytes and required undesirable modifications to be made to the culture protocol. Furthermore, this method was only applicable to adherent cell layers and therefore imposed limitations on the ongoing usage of the transfected cells. In contrast, nucleofection proved to be a simple and rapid method of transfection which yielded much higher levels of efficacy and efficiency. The ability to apply this method to a suspension of hepatocytes facilitates a wider range of down-stream experimental formats and also permits the removal of damaged cells by a number of methods. Nucleofection was therefore considered to be most suitable method for the transfection of human primary hepatocytes in the experiments presented in Chapter 5.

5. INVESTIGATION OF THE REPLICATIVE CAPACITY OF THE JFH1 CLONE OF THE HEPATITIS C VIRUS WITHIN HUMAN PRIMARY HEPATOCYTES

5.1. Introduction

The *in vitro* expression, replication and propagation of viral pathogens offers the opportunity for researchers to understand the ways in which they infect, and affect, the host cell(s) and, thence, to conceive and develop targeted drug treatments.

Although some conclusions can be drawn simply by comparing a viral genome to those of its phylogenetically-related family members, this information tends to be of more limited scope and value than that derived from specific investigation of the virus in question. The genomic analysis of hepatitis C virus (HCV), which proved so uniquely valuable in its discovery (as described in chapter 1), revealed a relatively simple 9,600 nucleotide sequence of RNA. Patterns of sequence motifs in the HCV genome were found to be similar to those of the Flaviviridae and, as a consequence of these genomic similarities, HCV was assigned to this family. Through comparison to other Flaviviridae, the genome map and the approximate function of many of the HCV genes was deduced. However, HCV is the only known virus with human infectivity within a separate genus of the Flaviviridae family (the other member of the genus being the marmoset virus GB virus B) . Thus, there are no closer relatives from which more precise aspects of its pathogenesis could be deduced. For further insights, and thus progress towards targeted therapeutics, specific investigation of HCV itself was required.

Other Flaviviridae are able to infect many different types of human (and animal) cells, so therefore the first question to be answered was: which cells are naturally infected

by HCV? *In vivo* studies of cells and tissue from infected patients demonstrated that HCV proteins and/or whole virus are associated with hepatocytes and peripheral blood mononuclear cells (PBMCs). Subsequent *in vitro* studies found that some T- and B- cell lines could indeed be infected by wild type HCV, but the infection was usually transient and only occurred at a low level[140]. Similar results were found in studies of human biliary epithelial cells[141]. Most studies of the pathogenesis of HCV performed to date have therefore investigated the effects of expression of its gene(s) in hepatocytes of some form, as outlined in figure 5.1.

Both *in vivo* animal and *in vitro* cell culture models can be useful for furthering the understanding of viral pathogenesis. The former can provide an excellent means for studying host-pathogen interactions over a longer time period and across a range of physiological systems, rather than being restricted to the target organ (i.e. the liver). Animal hosts also provide an intermediate model, between molecular and/or cellular systems and humans, for the trial of therapeutic agents. However, unless the animal model is particularly easy (and cheap) to obtain and maintain, detailed study at the cellular level may be costly and impractical. Furthermore, animal models are usually subject to some uncertainty surrounding their physiological similarity (and, by deduction, relevance) to a human host. There may also be ethical constraints on this type of research. In contrast, *in vitro* cell culture models are an obvious choice where detailed cellular analysis is required, often being both cheap and readily available. However, the complex cell-cell interactions of the *in vivo* environment are usually lost and, even if the cultured cells are human, the cell types used may function very differently to human primary cells *in vivo*. Both animal and cell culture models may therefore show less relevance to the *in vivo*, clinical situation than is ideal.

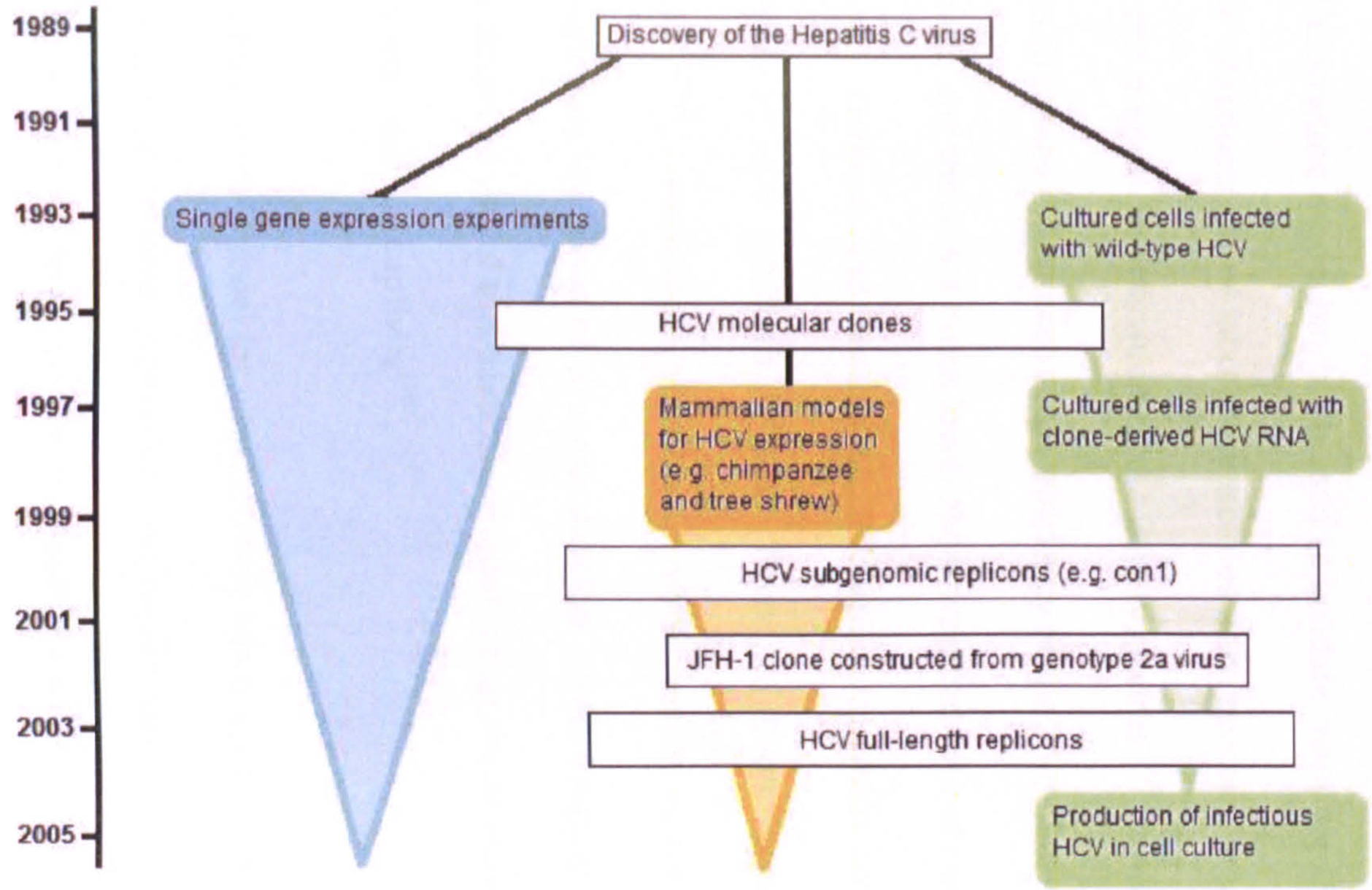


Figure 5.1: Schematic diagram of the main approaches, and some key discoveries, in the historical investigation of HCV in hepatocyte or liver models, with particular reference to the JFH1 clone.

The only known natural reservoir for HCV is man. Two other species have been found to be permissive for infection under laboratory conditions: the chimpanzee (*Pan troglodytes*) and a subspecies of tree shrew (*Tupaia belangeri chinensis*). Neither animal exhibits a typically anthropoid clinical course following infection[142,143], but both have still contributed, enormously, to laboratory studies of HCV.

The chimpanzee model has been used to validate many significant discoveries, such as the infectiousness of laboratory isolates and replicons, treatment strategies, and even the original identification of the wild-type virus[142]. However, there have been shown to be key differences in the efficiency of the chimpanzee immune response to infection, and there is a much lower-than-expected rate of chronic infection in these animals. Cirrhosis and hepatocellular carcinoma, which are significant long-term sequelae of HCV infection in man, have not been found to occur in either animal[142,143]. Persistent infection is more readily established in tree shrews, although only low levels of viraemia develop. Unfortunately, the main limitation to the widespread use of this particular model is that the animals are difficult to breed, and to work with, in captivity[142].

An artificial animal model for viral infection has been also been used to examine the effects of HCV genome expression and replication. Chimeric mouse models are essentially designed to support human cells (hepatocytes, for example) within an *in vivo* (murine) environment. Mice with severe combined immunodeficiency (SCID) are bred with transgenic mice which carry the urokinase plasminogen activator gene with an albumin promoter (*alb-uPa*). Expression of the *uPa* gene, which is targeted towards hepatocytes due to its albumin promoter, causes over-expression of

urokinase, hypofibrinogenaemia and accelerated cell death. Human hepatocytes, harvested from an uninfected adult donor, are implanted intra-hepatically and experience a survival advantage as the pre-existing murine hepatocytes are depleted by their expression of the *uPa* gene[144]. The engrafted human hepatocytes can then support *in vivo* infection with HCV.

Early work in this field demonstrated the ability of the chimeric mouse model to support, and to some extent sustain, productive infection of wild-type HCV following injection of serum from HCV-infected human patients[144]. Later studies have demonstrated infection of a similar model with infectious HCV clones[145].

Naturally, the immune system of the animals must be severely deficient, in order to allow persistence of the foreign cells[144], and therefore those aspects of pathogenesis which derive from the host's immune response are unlikely to be replicated. At least 21 human-specific proteins are produced by this model, but unusual patterns of glycogen storage are seen in the hepatocytes[146]. It is possible that normal regulatory cell-cell signalling is inhibited by the species differences and, if so, this has further implications for the study of disease pathogenesis. Furthermore, the mechanism of chimera creation means that significant numbers of hepatic progenitor cells are present in the liver and many cells are actively proliferating[146]. This undoubtedly results in a liver phenotype which is very different from the usual *in vivo* situation.

An alternative previous approach has involved taking adult, primary hepatocytes from human patients already naturally infected with HCV, to allow them to be cultured and characterised *in vitro*[147]. Further studies have examined the novel

infection, with wild-type HCV from patient sera, of human primary hepatocytes from uninfected donors[74,80]. Such studies seem to offer valuable similarities for comparison with natural, *in vivo*, HCV infection. Unfortunately, the level of replication of serum-derived HCV in primary hepatocytes has been found to be highly variable and generally very low[148]. Furthermore, there are at least three potential drawbacks to this approach. Firstly, the presence of HCV quasi-species within infected patients means that both the viruses and the infected cells will be heterogeneous and difficult or impossible to characterise or reproduce. Secondly, the distribution and amount of viral genetic material will be unpredictable and difficult to identify accurately. Thirdly, when using naturally-infected hepatocytes, the low numbers of cells (either derived from biopsy specimens or from end-stage cirrhotic livers removed for transplantation) and their short durability of differentiated function in culture tend to limit the scope of investigations.

Adult human hepatocytes can be immortalised, *in vitro*, by transformation (whether spontaneous or due to the transfection of a gene such as SV-40 T-ag). Immortalisation is usually accompanied by manifestation of a proliferative (and even neoplastic) phenotype which prolongs the available experimental period. Unfortunately, this loss of hepatocyte senescence is also associated with loss of differentiated function during prolonged culture. Furthermore, the level of permissivity for wild-type HCV replication in immortalised human hepatocytes appears to be just as low as that of primary cells[148].

Studies of the mechanisms of binding, and entry into host cells, of HCV have been greatly facilitated by the use of two successful model systems: HCV pseudoparticles (HCVpp; formed by expressing the HCV envelope glycoproteins that are normally

present on the surface of HIV virions[149]) and virus-like particles (HCV-LP; formed by recombination of baculoviruses to express the envelope and core protein of HCV[79]). Both types of construct have been used, in hepatic and non-hepatic cells *in vitro*, to explore the mechanisms of attachment and cell entry of HCV. However, these systems are of little utility for studying the intracellular pathogenesis of the virus and will not be discussed further in this review.

Until recently, the majority of studies of HCV pathogenesis have been performed using permanent cell culture lines *in vitro* in the study of single genes or viral replicons (either subgenomic or full-length), to examine particular aspects of the viral life cycle. By definition, such studies have made use of relatively homogeneous laboratory clones of the virus, rather than wild-type heterogeneous virus populations, in order to improve the specificity and reproducibility of results by working with a defined viral sequence. Inevitably, this reduces somewhat the physiological relevance of these experiments. In fact, it is the heterogeneity of HCV, derived from the mutability of its RNA genome during replication, which has created most problems for HCV researchers.

On the other hand, just occasionally, the heterogeneity of HCV has also helped towards achieving certain solutions. The highly important JFH1 genome was characterised from the dominant strain of an HCV population found in just one infected patient in Japan[150]. Its serendipitous isolation, and the subsequent discovery that it is able, uniquely, to infect common *in vitro* cell lines, is an example of just such a solution.

5.1.1. Viral requirements for HCV expression and replication *in vitro*

Consensus sequences, and thence clones, of numerous different viral strains have now been derived from all of the major genotypes of HCV. HCV replicons, created from these consensus sequence clones of wild-type viruses, have been constructed using the non-structural genes of genotypes 1a (e.g. H77), 1b (e.g. con1) and 2a (e.g. JFH1). Some schematic examples of HCV replicons are shown in figure 5.2. These constructs, which are described as either sub-genomic or full length, have recently been widely used with *in vitro* cell cultures to study the viral and cellular requirements for HCV replication.

Studies, using these replicons to investigate the pathogenesis of HCV, began with a focus on the non-structural proteins, which effect viral processing and packaging inside the host cell. Subgenomic replicons generally comprise the NS3 – NS5B genes of the HCV genome in question, flanked by its 5' and 3' non-translated regions (NTRs). The NS2 sequence has been found not to be required for *in vitro* replication and its *in vivo* function remains unclear[151].

Originally, all such replicons were dicistronic: containing the HCV 5' internal ribosome entry site (IRES); an inserted gene followed by a second IRES; and either the non-structural or both structural and non-structural genes from the HCV consensus sequence, together with its 3' repeat region. The 5' NTR contains the HCV IRES, and the 3' NTR is thought to conserve and protect the RNA from intra-cellular degradation. It is therefore desirable to retain both NTRs in any derived construct.

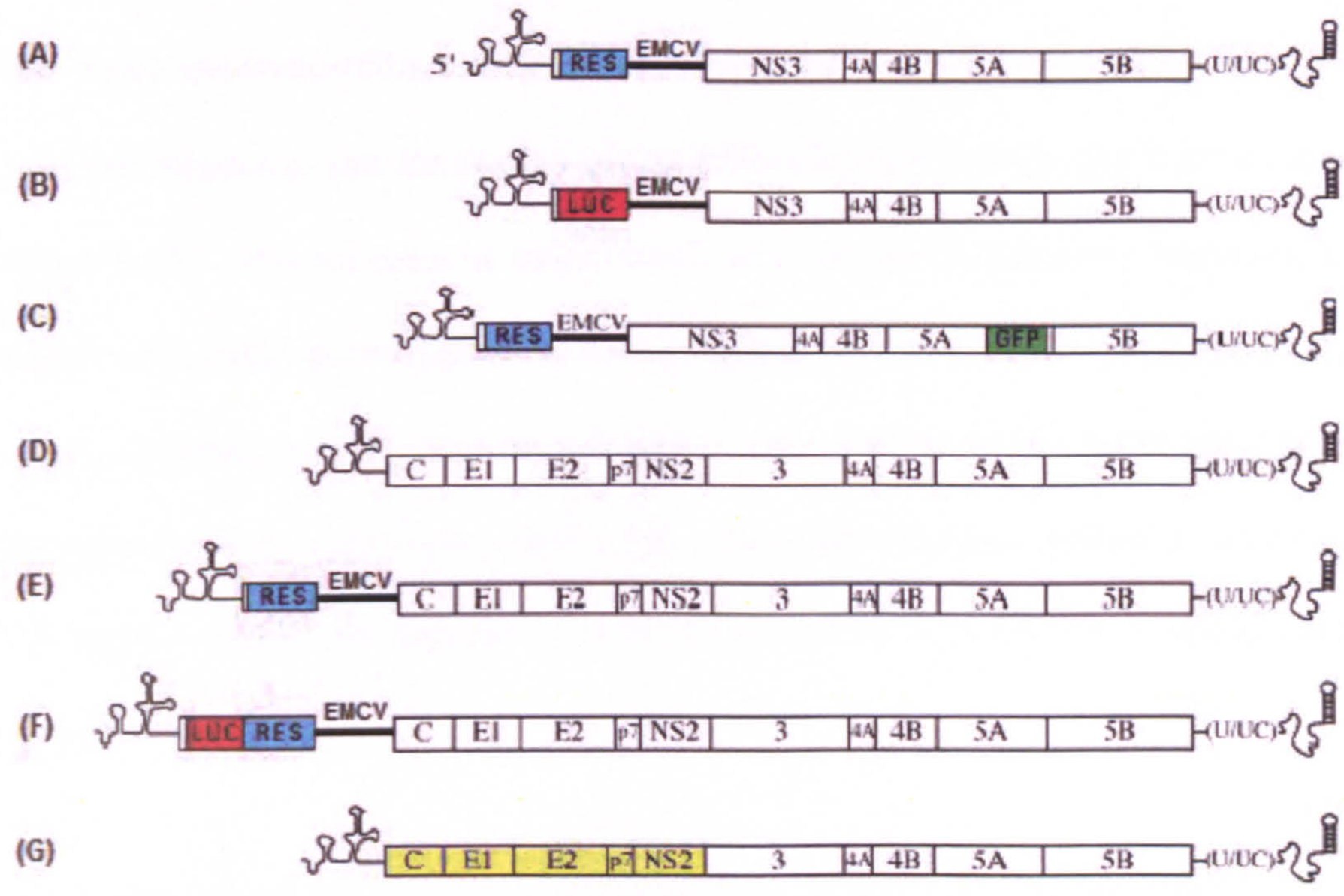


Figure 5.2: Some examples of HCV replicons: (A) subgenomic dicistronic replicon carrying a resistance gene (e.g. neo) for selection and the EMCV IRES; (B) subgenomic dicistronic replicon carrying the firefly luciferase gene for quantification of replicon translation; (C) selectable subgenomic dicistronic replicon carrying the green fluorescent protein (GFP) gene for detection of translation in individual cells; (D) monocistronic full length infectious replicon/wild type virus structure; (E) dicistronic full length infectious replicon with selectable resistance gene; (F) dicistronic full-length infectious replicon with selectable resistance and luciferase marker of translation; (G) monocistronic full-length infectious replicon chimera, with structural proteins of one clone (shown in yellow) and non-structural, replication efficient, genes of a second clone.

In order to select for cells harbouring a replicating sequence, a second cistron is also created by the insertion of a selectable resistance gene (such as the neomycin phosphotransferase gene) following the HCV 5' NTR, plus an external IRES (such as the encephalomyocarditis (EMCV) IRES), to ensure continued association with the host cell ribosome and translation of the following HCV non-structural genes (see figure 5.2A). The subsequent modification of successful subgenomic replicons, to express a reporter gene (such as luciferase) instead of the resistance gene, allows the relative effects of mutations to be assessed as a function of the replicative capacity of the replicon in question (see figure 5.2B). Naturally infectious replicons, which do not require cell-culture adaptation, may be monocistronic in the same way as wild-type HCV.

In the absence of an ideal, *in vivo*-like cell line or other cell culture model, these constructs were transfected into Huh-7 cell clones for examination of their replicative ability[49,140,152]. Most produced no, or very little, evidence of expression or replication. However, those replicons which did replicate successfully were frequently found to have developed cell-culture adaptive mutations, as described below. The sites, and extent of conservation, of these mutations provide useful insights into the function and importance of the peptides where they occur. Interestingly, however, there is some evidence that a high proportion of proteins and protein complexes derived from non-structural genes in infected cells do not contribute to the replication of HCV and the function of these "excess proteins" is not yet known[44].

Cell-culture adaptive mutations have been found to occur in all non-structural genes, whereas the NTRs remain highly conserved[49]. Some mutations increase replicative

capacity only when occurring in synergistic clusters; others are independently functional. An early report from Blight *et al.* described clusters of mutations, in the NS5A gene, which mapped to a region associated with *in vivo* sensitivity to interferon treatment in wild-type viruses[51]. Many further mutations, associated with increased levels of genome replication, have been found to reduce the number of potential phosphorylation sites [151-153]. It has since been found that a hyperphosphorylated form of the NS5A protein reduces replication of the genome and that, if the Huh7 cells are treated with an inhibitor of cellular kinases to block this hyperphosphorylation, these adaptive mutations do not occur[154].

The protein derived from the NS4B gene has been shown to associate with intracellular membranes, inducing a so-called "membranous web", and its gene is another site where adaptive mutations occur during *in vitro* cell culture. NS4B protein is thought to anchor the rest of the viral replication complex to cellular membranes and selected mutations are presumed to increase the affinity of this association in a non-natural host cell environment[44].

Mutations, throughout the region of non-structural genes, tend to map to areas unrelated to the active sites of the resultant enzymes, thus the proteins' functions are commonly unaffected[36]. Furthermore, most viral mutations arising through cell culture are not found in wild-type HCV viruses and many are common to different genotypes. These cell-culture-adaptive mutations are therefore likely to act as specific adaptations to the Huh7, or other, cell line and to affect the interactions between cellular and viral proteins. Importantly, most highly adapted replicons which replicate successfully in cell cultures fail to infect, or to replicate efficiently, in

in vivo chimpanzee models[50] and therefore the findings obtained using these constructs must be interpreted with caution.

Even the highly replication-competent JFH1 clone has been shown to develop cell-culture adaptive mutations under certain culture conditions. These particular mutations are associated with increased production of virions in cell culture, which retain infectivity for the uPa-SCID chimeric mouse model. However, it was found that these mutations are quickly lost in the new host and there is evidence that such mutations confer a reduction in *in vivo* fitness of HCV[153]. It has been suggested that adaptive mutations shift viral function towards continuing replication and translation of viral RNA, and away from RNA release in mature viral particles but, again, the reasons for this remain unclear[140].

5.1.2. Host cell requirements for the expression and replication of HCV RNA

The near-exclusive specificity of HCV for human cells has been a dominant factor in the development of cell culture models, as already mentioned. Although there is evidence for the infection of a number of non-hepatic cells *in vivo* (peripheral blood mononucleocytes, lymphocytes and dendritic cells), *in vitro* research has focused on the apparent main site of infection, and its consequences: i.e. the liver. The most permissive cell line identified is the Huh7 cell line, including several adapted sub-lines (such as Huh7.5 and Huh7 Lunet cells).

Permissivity for HCV infection of hepatocytes has been shown to be associated with a number of cell surface membrane proteins, namely CD81, SR-B1, claudin-1 and LDL-R[40,77,155,156]. These proteins have been found to associate with the envelope glycoproteins of HCV prior to cell entry, using the HCVpp and HCV-LP models as

described in section 5.1. Other cell-surface molecules, notably the mannose-binding C-lectins liver/lymph node-specific Intercellular adhesion molecule-3-grabbing nonintegrin (L-SIGN) and dendritic cell-specific Intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), have also been demonstrated to bind the HCV envelope glycoproteins, but they are not present on hepatocytes and thus not directly involved in their infection[157].

If RNA (either genomic or sub-genomic) is inserted into the target cells by transfection, a number of further cellular determinants affect the ability of the genome to be expressed and/or replicated. Lipid droplet formation and transport within the host cell appears to be key to the transport and interactions of viral proteins[35], as outlined in 1.1.2, and viral replication is inhibited by a reduction in the level of saturated or mono-unsaturated fatty acid synthesis[44]. Thus it may be hypothesized that intra-cellular synthesis, transport and storage of lipids and lipoproteins is likely to be a key requirement of a highly successful *in vitro* cell culture model. Other work, using primary hepatocytes, has shown that creation of a (cytokine-induced) proliferative environment increases the rates of serum-derived HCV virus infection and replication[81].

It has been shown that replicative capacity for HCV RNA varies between different stocks of Huh7 cells in different laboratories and also between batches of different passage number in the same laboratory[49,151]. Furthermore, these studies have also shown that in a normal, unselected population of Huh7 cells, only a small proportion will show permissivity for HCV replication, suggesting that these cells are heterogeneous with regard to the (largely undetermined) properties which support HCV infection. Production of infectious HCV particles also decreases as cells are

serially passaged. The reasons for this gradual loss of permissivity are as yet unknown.

In the case of a selected sub-clone of the Huh7 hepatocyte cell line known as Huh7.5, increased permissivity for HCV replication has been shown to be related to the loss of a cytoplasmic protein produced by retinoid-inducible gene 1 (RIG1). The RIG1 protein functions by recognising structured RNA within the cell's cytoplasm and triggers the production of type 1 interferon via interferon regulatory factor 3 (IRF-3) and NF-κB, thereby suppressing infection. Huh 7.5 cells are derived from a clone of Huh-7 cells which possesses a mutated and inefficiently expressed RIG1 gene[158]. This clone was selected after being identified as harbouring HCV subgenomic replicons, and was subsequently "cured" by interferon, thereby offering the prospect of a known permissive environment for full-length HCV infection.

Interestingly, it has also been shown that methamphetamine down-regulates IRF-5 (and thus the innate interferon response), which results in increased replication of HCV in Huh7.5 cells[159]. This finding not only underlines the importance of the innate immune response during HCV infection, but also correlates with the clinical observation that recreational abuse of methamphetamine has been found to be associated with chronic HCV infection[159]. A different study, of Huh7 cells expressing a genotype 1b replicon, showed that exposure to ethanol increased replication and translation of HCV RNA and that this was associated with an oxidative stress pathway response, via the up-regulated expression of cyclo-oxygenase 2 (COX-2)[160].

All of the above evidence therefore suggests that the level of *in vitro* HCV replication results from complex interplay between individual cells and specific replicon

sequences. This interaction may offer advantages and disadvantages to the researcher: on the one hand, it adds yet another source of variation and may render comparative interpretation of the results somewhat unreliable; on the other, if homogeneous clones of HCV can be selected, this variation may be explored to determine the exact requirements of infection.

There is some evidence that HCV infectivity and replication is enhanced when the host cells exist in an organised, three-dimensional (3D) culture, rather than a monolayer. A study performed using immortalised hepatocytes showed some evidence that cells which maintained differentiated function and polarised cuboidal morphology were more susceptible to serum-derived HCV infection[161]. In the same experimental model, down-regulation of the interferon response pathway increased the infectivity of HCV; underlining the importance of the innate immune response as a key factor in hepatocyte susceptibility to HCV infection.

Interestingly, research using 3D cultures of an Huh7 sub-clone infected with a genotype 1b infectious replicon showed that, although cell growth (and viral protein expression) occurred at a lower level than in 2D monolayer cultures, release of infectious particles was enhanced[162]. A further, short-term study also showed that, after 48 hours, expression and replication of a subgenomic replicon in Huh7 cells was significantly lower in 3D culture in comparison to 2D monolayers[163]. Unfortunately, longer-term studies have not been carried out to explore further the temporal profile of expression in 3D culture in Huh7s; most probably because these cells are a rapidly proliferating line which quickly require repeated passage in culture (which would disrupt or destroy any three-dimensional aggregates). However, the apparently altered profile of HCV expression in 3D cell cultures may begin to offer some

explanations for the general discrepancy between the susceptibility to infection found *in vivo* versus *in vitro* cells.

Production of the JFH1 replicon from a genotype 2a virus proved to be a breakthrough against the previous background of replicon research. Uniquely, JFH1 replicons are replicated efficiently in Huh7 cells without cell-culture adaptive mutations. Full length JFH1 replicons also produce virus which is infectious, both to cell cultures and to the chimpanzee model. Although the clinical presentation (of acute, fulminant hepatitis followed by clearance of infection) of the patient from whom JFH1 was derived does suggest that this is a somewhat unusual strain of HCV[57], it is unclear why this particular clone is capable of *in vitro* replication without mutations[164].

5.1.3. Expression systems permissive for JFH1 to date

The JFH1 sub-genomic replicon has been investigated mainly in the Huh7 cell line or sub-clones, as discussed further in section 5.1.3.1. This has enabled findings to be compared and/or correlated with previous findings in these cell types using single gene transfection or other, cell-culture adapted, replicons. However, a number of additional human cell types have been shown to be permissive to replication, albeit at a lower level. HepG2 cells, which are a liver cancer cell line, and IMY-N9 cells, which are a cell line produced by fusion of primary hepatocytes with HepG2 cells, were both shown to be permissive for subgenomic replication of JFH1[150]. HeLa and 293 cells, which originate from human cervical cancer and embryonic kidney tissue, respectively, are non-hepatocyte cell lines which also, nonetheless, have been shown to permit replication of JFH1 without requiring adaptive mutations[165]. In some ways

this raises questions as to the true equivalence of the JFH1 clone to "wild-type" viruses because, as far as is known, this behaviour is not representative of the normal, *in vivo*, infectivity of HCV. On the other hand, these results only reinforce evidence of the clone's high level of infectivity and replicative capacity, which is usually so difficult to reproduce by *in vitro* culture of the virus.

5.1.3.1. Dynamics of JFH1 expression and replication in Huh7s

As previously noted, the JFH1 replicon has been investigated, primarily, in the Huh7 cell line. Elegant work, using a bi-cistronic subgenomic replicon containing the firefly luciferase gene *Luc* (see figure 5.2), has enabled relative quantification of the levels of the replicon's expression over time, following transfection of Huh7 cells with *in vitro* transcribed mRNA[166] (see figure 5.3). By comparison with the levels of expression produced by a mutated, replication-deficient version of the replicon, used as a control, increases in luciferase activity were shown to result from replication of the replicon RNA, thus providing higher levels of the mRNA template. Luciferase expression was found to rise between 4 and 48 hours post-transfection and then plateau until 72 hours (further measurements were not reported); whereas replication-deficient mutants displayed levels which declined from 4 hours onwards. The rise in luciferase expression was correlated with measured increases in both replicon RNA and NS5a protein in transfected cells; whereas this protein became undetectable after 24 hours in cells which had been transfected with the replication-deficient mutant. Translation of both competent and mutated replicons could be inhibited by treating the cells with interferon-α[166].

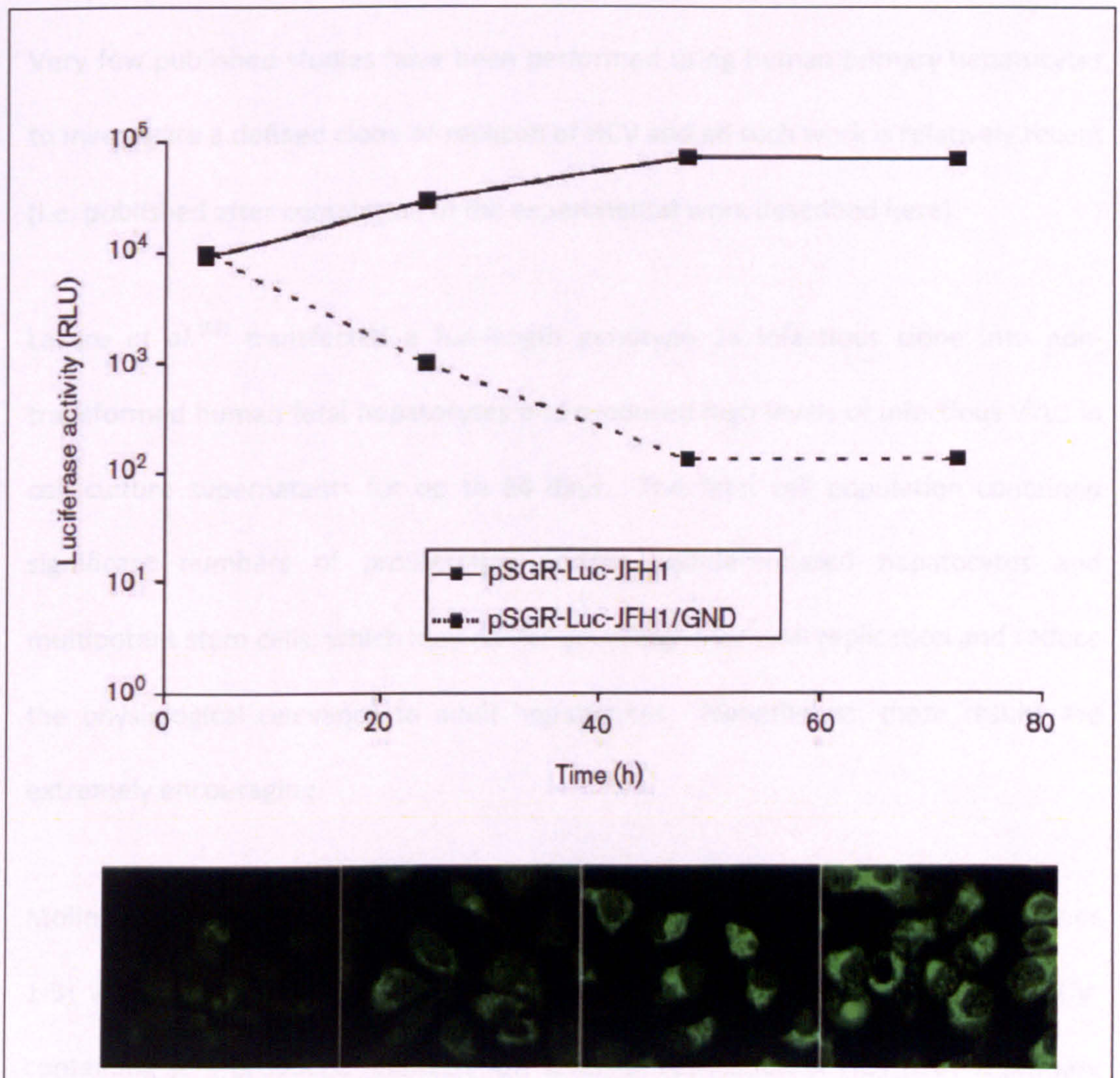


Figure 5.3: ***Top:*** *Replication of JFH1 , in Huh7 cells; as indicated by production of the luciferase marker enzyme at 4, 24, 48 and 72 hours following transfection with in vitro transcribed RNA of the subgenomic JFH1 replicon (solid line), as compared with a relication-deficient replicon containing a GND mutation in the NS5B gene (dashed line). RLU = relative light units.* ***Bottom:*** *Huh7 cells stained with an anti-NS5A antibody at time points corresponding to those in the graph above (adapted from Targett-Adams et al.[166]).*

5.1.3.2. JFH1 and other replicons in primary hepatocytes

Very few published studies have been performed using human primary hepatocytes to investigate a defined clone or replicon of HCV and all such work is relatively recent (i.e. published after completion of the experimental work described here).

Lazaro *et al.*[82] transfected a full-length genotype 1a infectious clone into non-transformed human fetal hepatocytes and produced high levels of infectious virus in cell culture supernatants for up to 64 days. The fetal cell population contained significant numbers of proliferating and/or undifferentiated hepatocytes and multipotent stem cells, which may confer advantages for viral replication and reduce the physiological relevance to adult hepatocytes. Nonetheless, these results are extremely encouraging.

Molina *et al.*[75] compared the infectivity of serum-derived wild-type virus (genotypes 1-3) with that of JFH1 virus in adult human hepatocytes. They found that HCV-containing sera produced relatively low levels of replication of HCV RNA in primary hepatocytes (0.18 genome equivalents (Geq) per cell), compared with JFH1 virus (3.3– 9.7 Geq/cell). However, the latter values remained an order of magnitude lower than those generated by JFH1 infection of Huh7.5 cells. The same study produced data showing that entry of both JFH1 and serum-derived viruses into primary hepatocytes was associated with expression of the CD81 receptor but the authors noted that, although the use of anti-CD-81 monoclonal antibodies inhibited serum-derived HCV infectivity by 90%, JFH1 infection of primary hepatocytes was reduced by only 60%. These observations further suggest that the efficacy of HCV

infection is dependent on heterologous combinations of both viral and host cell factors.

Another study, by Lan *et al.*[167], investigated infectious JFH1-derived virus in both primary hepatocytes and Huh7.5 cells, to examine its effects on apoptosis. They showed that apoptosis was increased by expression of the non-structural viral genes and that this increase was mediated by sensitization of the cells to the TNF-related apoptosis-inducing ligand (TRAIL)[167]. Data on the dynamics, or relative efficacies, of infection were not presented.

No published studies of the translational and/or replicative dynamics of HCV in primary human hepatocytes are available as of this date. Certainly, the results of such studies would be of the utmost interest, both for the novel data and insights they would provide and for the ability for comparisons to be made with the enormous amount of work already performed in Huh7 cell lines. There are undoubtedly many factors to be optimised in the individual laboratory before experimental studies using primary human hepatocytes can be performed satisfactorily and with confidence and these have been addressed (to some extent) in the preceding chapters of this thesis. The remainder of this chapter presents an outline of work performed to build on the established protocols for isolating and transfecting human primary hepatocytes by using the methods to investigate the replicative capacity, and dynamics, of the JFH1 clone in primary hepatocytes.

5.2. Materials and methods

5.2.1. Materials

Nile Red stain was obtained from Sigma and an aqueous stock solution was prepared at 0.5 mg/ml.

Plasmids containing HCV replicons (SGR-Luc-JFH1 and SGR-Luc-GND-JFH1) were kindly provided by Dr Paul Targett-Adams (MRC Virology Unit, Glasgow) with the kind permission of Dr Takaji Wakita (National Institute of Infectious Diseases, Tokyo). The pMaxGFP plasmid DNA was obtained from Amaxa® GmbH, Germany. Midiprep kits were obtained from Qiagen©.

Nucleofection® kits were obtained from Amaxa®, as described in chapter 4. The XBaI restriction enzyme kit (containing Buffer H and XBaI enzyme) and mung bean nuclease enzyme kit were obtained from New England Biolabs©. T7 Megascript kits and RNaseZap® were obtained from Ambion©. The luciferase assay kit (E1500) was obtained from Promega©. Water, pipette tips and Eppendorf tubes, used at all stages, were certified, and maintained, sterile and free of DNAse and RNAse.

For gel electrophoresis, ethidium bromide solution (10 mg/ml), agarose powder and 10x tris-acetate-EDTA buffer were obtained from Sigma. Working-strength buffer (1x tris-acetate-EDTA) was obtained by dilution of the 10x stock solution with sterile, de-ionised and nuclease-free water. DNA and RNA concentrations were determined using a NanoDrop© ND-1000 UV-Vis spectrophotometer.

5.2.2. Demonstrating the presence of lipid in three-dimensional co-culture spheroids of human primary hepatocytes and rat hepatic stellate cells

The production, transport and storage of lipid has been shown to be extremely important for the intracellular processing and replication of HCV, as described earlier and in 1.1.2, therefore it was desirable to ascertain whether or not lipid droplets were present in the hepatocytes of the chimeric human/rat spheroids prepared. As lipid is lost during alcohol- or aldehyde-based methods of fixation, the staining and microscopy for lipid droplet was performed on live, unfixed cells. A disadvantage of this is that cross-sections of the cell aggregates could not be made and only cells at the surface of the spheroids could be observed.

Nile Red dye (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) is strongly fluorescent in a hydrophobic environment (such as intracellular lipid droplets) but is also soluble in aqueous solution and thus is suitable for use on live cells[168].

Intracellular lipid was stained using Nile Red as follows. Culture medium was aspirated from the cell culture well using a sterile 5 ml syringe fitted with a blunt-ended needle. PBS (2 ml), pre-warmed to 37°C and containing Nile Red stock solution at a dilution of 1:5000 v/v, was added to the well and the mixture incubated for 10 minutes. The culture wells were then examined and the spheroids photographed using a Leica TCS confocal microscope (excitation 485 nm; emission 525 nm).

5.2.3. Methods for expression of the subgenomic JFH1 replicon in Huh7 cells and human primary hepatocytes

5.2.3.1. Preparation and *in vitro* transcription of pSGR-Luc-JFH1 and pSGR-Luc-GND-JFH1

Stocks of SGR-Luc-JFH1 and SGR-Luc-GND-JFH1 plasmid DNA were obtained by lysis of transformed, competent E. coli and purified using the Midiprep kit. The DNA was re-dissolved in water, to a measured concentration of 1 µg/µl, and stored at -20°C until required.

In order to prepare mRNA for transfection, the plasmids were linearised using the restriction enzyme XBaI kit, so that an *in vitro* transcription reaction could be performed. Twenty microlitres of 10x Buffer H, 10 µl XBaI enzyme and 70 µl water were added to 100 µl of plasmid DNA solution, to make a total volume of 200 µl, which was vortex mixed and then incubated for 4 hours at 37°C.

The linearised DNA was then precipitated by the addition of approximately 1 ml 100% ethanol, the mixture was centrifuged, the supernatant discarded and the pellet was re-suspended in 85 µl water. Mung bean nuclease was used to remove any single-stranded overhangs (so-called "sticky ends", which can reduce the efficacy of transcription). The DNA suspension was mixed with 10 µl mung bean nuclease buffer and 5µl mung bean nuclease and incubated at 30°C for 30 minutes.

The linearised DNA was then further purified by precipitation, using the phenol-chloroform method, and stored at -20°C until further use. The integrity of the linearised DNA and completeness of linearisation were ascertained by ethidium

bromide gel electrophoresis, as described in 5.2.3.2. Only DNA which showed as a single, clear band and running slower than the original circular plasmid was used as a template for production of mRNA.

RNaseZap® was wiped over working surfaces and equipment to minimise RNase contamination in the working environment. The T7 Megascript® kit was used to prepare an RNA transcription mixture according to the manufacturer's recommendations, which was added to the DNA templates as recommended. The transcription reactions were allowed to proceed for 2 hours at 37°C.

Following the DNase step to remove the DNA template, the resultant RNA was examined for integrity using RNase-free ethidium bromide gel electrophoresis (as below) and quantified by Nanodrop©, as before. Only RNA which showed as a single, clear and un-smeared band was used for cell transfections. RNA was then stored at -80°C until use.

5.2.3.2. Visualising DNA or RNA by ethidium bromide gel electrophoresis

To assess the quality of DNA and/or RNA, following plasmid or RNA preparation, these nucleic acid products were subjected to gel electrophoresis. All plastic-ware was cleaned with RNaseZap prior to use.

A 1% agarose gel was made as follows. Briefly, 1 g agarose powder was suspended in 100 ml 1x tris-acetate-EDTA buffer and heated in a microwave oven until liquified. This solution was allowed to cool to approximately 40°C, ethidium bromide solution was added (final concentration 0.5 µg/ml) and the solution was mixed thoroughly by manual swirling motion. While still molten, the agarose-ethidium bromide gel was

poured into an 8 cm x 10 cm gel casting tray with gel comb and allowed to cool until set.

Once set, the comb was removed and the gel placed into an electrophoresis tank containing further 1x tris-acetate-EDTA buffer and ethidium bromide at 0.5 µg/ml. The nucleic acid solution of interest (2 µl) was mixed with 10 µl loading dye and pipetted into a well in the gel, alongside 5 µl of a molecular weight ladder (0.5-5 kb). The gel was then exposed to current at 90 V for 30 minutes, or 100 V for 45 minutes, for the separation of DNA or RNA respectively, following which it was examined under ultraviolet light for evidence of distinct band(s) of DNA or RNA. See figure 5.4 for examples.

5.2.3.3. Transfection of Huh7 cells with subgenomic JFH1 RNA

Each prepared batch of SGR-Luc-JFH1 RNA and SGR-Luc-GND-JFH1 RNA was tested to confirm intact translational and/or replicative efficacy by electroporation into Huh7 cells and assay of luciferase activity at 4, 24 and 48 hours (as described below). After preparing and counting a single-cell suspension of Huh7 cells in PBS (as described in Chapter 2), the required number of cells was pelleted by centrifugation and resuspended at 1 million/100 µl in PBS containing 5% sucrose. SGR-Luc-JFH1 RNA and SGR-Luc-GND-JFH1 RNA samples were allowed to defrost before use but maintained on melting ice.

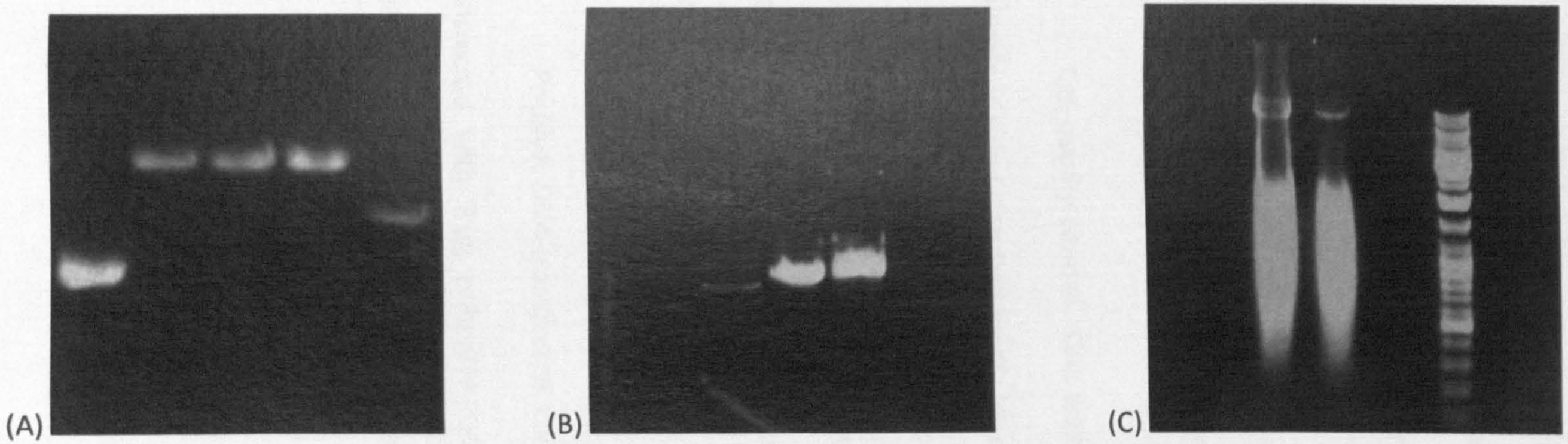


Figure 5.4: *Example images of pSGR-Luc-JFH1 DNA and RNA as viewed under ultraviolet light, following ethidium bromide gel electrophoresis (origin at the top in each case). (A) Left-hand lane showing undigested circular plasmid; middle three lanes showing linearised plasmid; right-hand lane showing DNA ladder. Note that although the plasmid products are of the same molecular weight, the original circular DNA has a close, coiled structure and therefore migrates through the gel more quickly than its linearised DNA counterpart. (B) Left-hand lane showing ladder; middle lane showing SGR-Luc-JFH1 RNA; right-hand lane showing SGR-Luc-GND-JFH1 RNA. (C) Example showing two lanes of mainly degraded RNA, illustrating how the small, randomly-sized fragments of RNA produced by RNase activity disperse ahead of the remaining bands of intact RNA, which are seen nearer the top of the picture; molecular weight ladder is shown on the right.*

Working quickly, 5 µg SGR-Luc-JFH1 RNA or SGR-Luc-GND-JFH1 RNA and 100 µl cell suspension was transferred to each Nucleofector® cuvette. Each cuvette was capped and the mixture was nucleofected immediately, using programme T14. The cell suspension was then immediately but gently added to pre-warmed 6-well cell culture plates containing 2 ml medium 4, using a fine-tipped pipette. Plates were gently agitated to distribute the cells evenly and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Control cell cultures were also prepared for each time point, as follows.

a. *Cell quality control.* One well of non-nucleofected Huh7 cells, prepared and maintained using standard media (as described in Chapter 2).

b. *Negative control.* One well of nucleofected Huh7 cells, prepared as above but with no RNA added to the cuvette; nucleofected and maintained as described for the test cultures.

c. *Positive DNA-transfected control.* One well of nucleofected Huh7 cells, transfected with 2 µg pMaxGFP DNA (as suggested by the manufacturer) and maintained as described for the test cultures.

5.2.3.4. Transfection of human primary hepatocytes with subgenomic JFH1 RNA

Human primary hepatocytes were prepared in suspension as described in Chapter 3 and sedimented by centrifugation (5 minutes; 50 g; 4°C). Working quickly, the supernatant was discarded and the hepatocyte pellet was resuspended in Nucleofector® working buffer solution, to produce a suspension with a measured concentration of 10^6 cells per 100 µl.

For each reaction, 5 µg SGR-Luc-JFH1 RNA or SGR-Luc-GND-JFH1 RNA and 100 µl cell suspension were transferred to each Nucleofector® cuvette. The cuvette was immediately capped and placed into the machine for nucleofection, in order to avoid sedimentation and unnecessary warm ischaemia of the hepatocytes. Following nucleofection, using program U14 as described in Chapter 4, the cuvette was removed and incubated at room temperature for 15 minutes before proceeding.

5.2.3.5. Post-nucleofection procedures for transfected hepatocytes in monoculture

Pre-warmed Medium 5 (500 µl) was then added to the Nucleofector® cuvette and, working quickly, the entire cell suspension was then gently aspirated by pipette and transferred to a collagen-coated cell culture well containing 1.5 ml Medium 5, which had been pre-warmed to 37°C. The culture plate was gently agitated by planar reciprocating motion, to distribute the hepatocytes evenly, and incubated at 37°C in a humidified 5% CO_2 atmosphere.

Control cell cultures were also prepared for each time point, as follows.

a. *Cell quality control.* One well of non-nucleofected hepatocytes, prepared and maintained using standard media (as described in Chapter 2).

b. *Negative control.* One well of nucleofected hepatocytes, prepared as for the test nucleofected cells but with no RNA or DNA added to the cuvette; nucleofected and maintained as described for the test cultures.

c. *Positive DNA-transfected control.* One well of nucleofected hepatocytes, transfected with 4 μg pMaxGFP DNA and maintained as described for the test cultures.

Separate culture wells of were assayed for production of luciferase enzyme at 4, 24 and 48 hours after transfection. If measurement was to occur at or after 24 hours of incubation, non-adherent hepatocytes were removed from the monolayer culture following overnight incubation, by aspiration of the culture medium and washing of the layer with PBS (pre-warmed to 37°C), after which the cultures were incubated with medium 6 (prepared as described in chapter 2) until examined as described below.

5.2.3.6. Measurement of SGR-Luc-JFH1 translation and replication in monocultured cells

The luciferase assay kit was prepared according to the manufacturer's instructions. Cell culture medium was aspirated from the culture plate wells and the adherent cell layer was washed with PBS to remove non-adherent cells and cell debris. Then

100 µl of 1x luciferase assay lysis buffer was added and spread over the cell layer before pipetting the lysed cell mixture into a Microfuge® tube. Lysates were centrifuged at 13000 g for 1 minute and the relative luciferase activity of 40 µl supernatant was determined by mixing with 100 µl luciferase reagent in a scintillation vial (Hughes and Hughes®; 1116) and assaying immediately in a luminometer (Turner® TD-20e; delay 5 s, integration 10 s) to determine the amount of light produced.

A vial containing a mixture of 100 µl luciferase reagent and 40 µl luciferase assay lysis buffer was used to calibrate zero response on the machine reading. The amount of light produced from, and recorded for, the test samples was assumed to be proportional to the amount of luciferase enzyme present in the cells which had been lysed. The results are presented in section 5.3.

5.2.3.7. Post-nucleofection procedures for transfected hepatocytes in three-dimensional co-culture

In order to examine the potential effects of cuboidal morphology and interaction with hepatic stellate cells on the expression and replication of the JFH1 HCV replicon, three-dimensional spheroid co-cultures were formed using freshly transfected human primary hepatocytes. These spheroids were formed as described by Thomas *et al.*[71], but with human, rather than rat, hepatocytes.

Briefly, 10^6 freshly isolated human hepatocytes which had been electroporated in the presence of either SGR-Luc-JFH1 RNA or SGR-Luc-GND-JFH1 RNA, as described in 5.2.3.3, were placed in a single low-adhesion coated culture well (prepared as described in chapter 2), containing 1.2 ml pre-warmed medium 6. A single-cell

suspension of 2-week old rat hepatic stellate cells (obtained and cultured as described in section 2.2.1) was prepared in medium 6 and adjusted to 1 million cells per ml. A volume of 0.3 ml of this suspension, containing 300,000 HSCs, was added to the hepatocyte-containing well. The cell solutions were gently mixed, with each other and the medium, by planar reciprocating motion and incubated at 37°C in a humidified 5% CO_2 atmosphere. The cell culture medium was not exchanged during the subsequent experimental period, as spheroids have been shown to require undisturbed physical conditions during the first 48 hours of culture[71].

5.2.3.8. Measurement of SGR-Luc-JFH1 translation and replication in three-dimensional co-culture spheroids of human primary hepatocytes and rat hepatic stellate cells

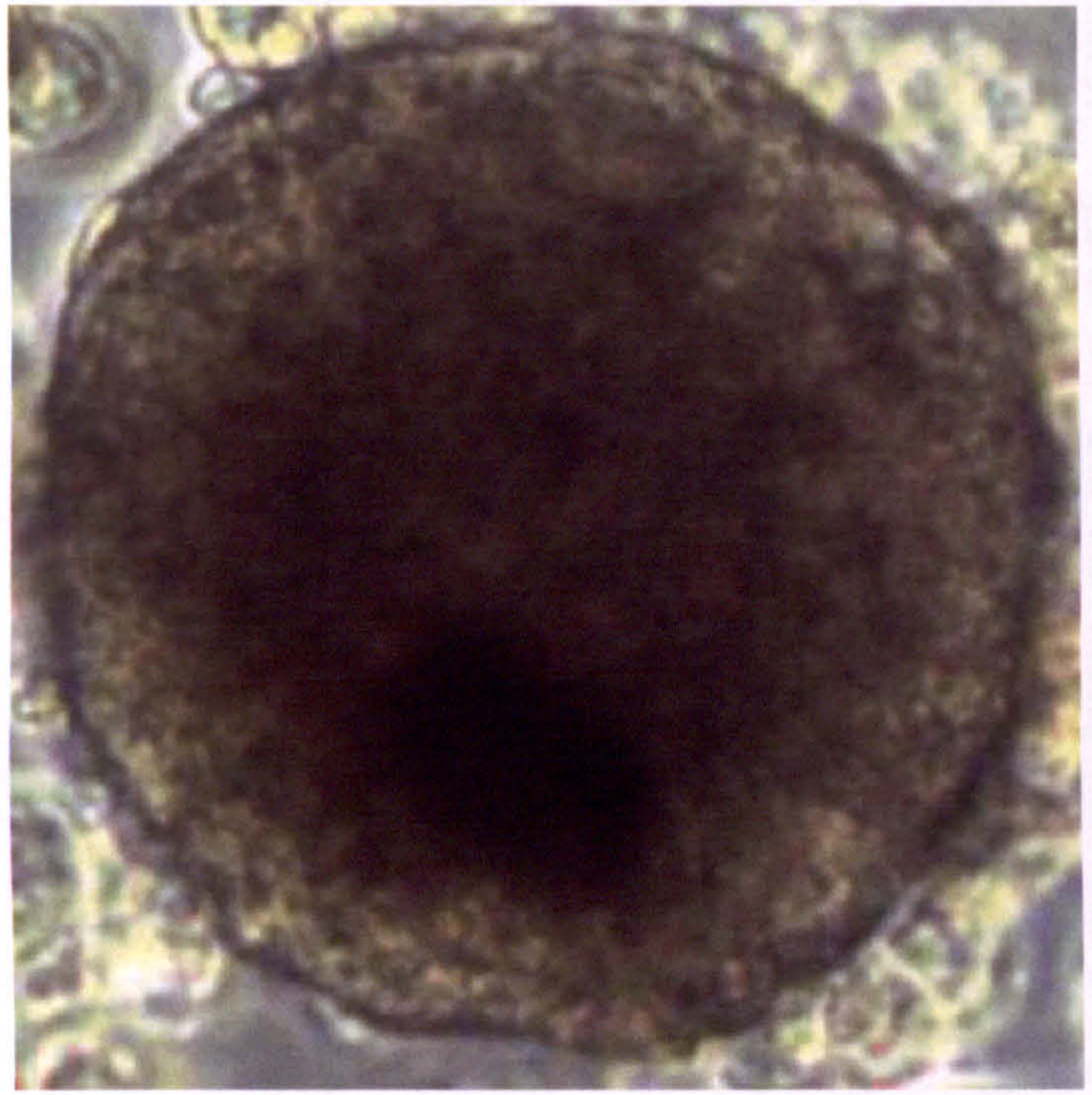
To measure expression of the transfected replicon, the cell culture medium (containing spheroids and non-adherent cells) was aspirated from the culture plate wells and pelleted by centrifugation for 5 minutes at 50*g*. The supernatant was discarded and 100 µl of 1x luciferase assay lysis buffer was added and mixed by pipetting to lyse the cells and cell aggregates. This lysate was then also spread over any remaining adherent cells in the culture well before pipetting the lysed cell mixture into a Microfuge® tube. Luciferase activity was then quantified as described in 5.2.3.6. The results are presented in section 5.3.

5.3. Results

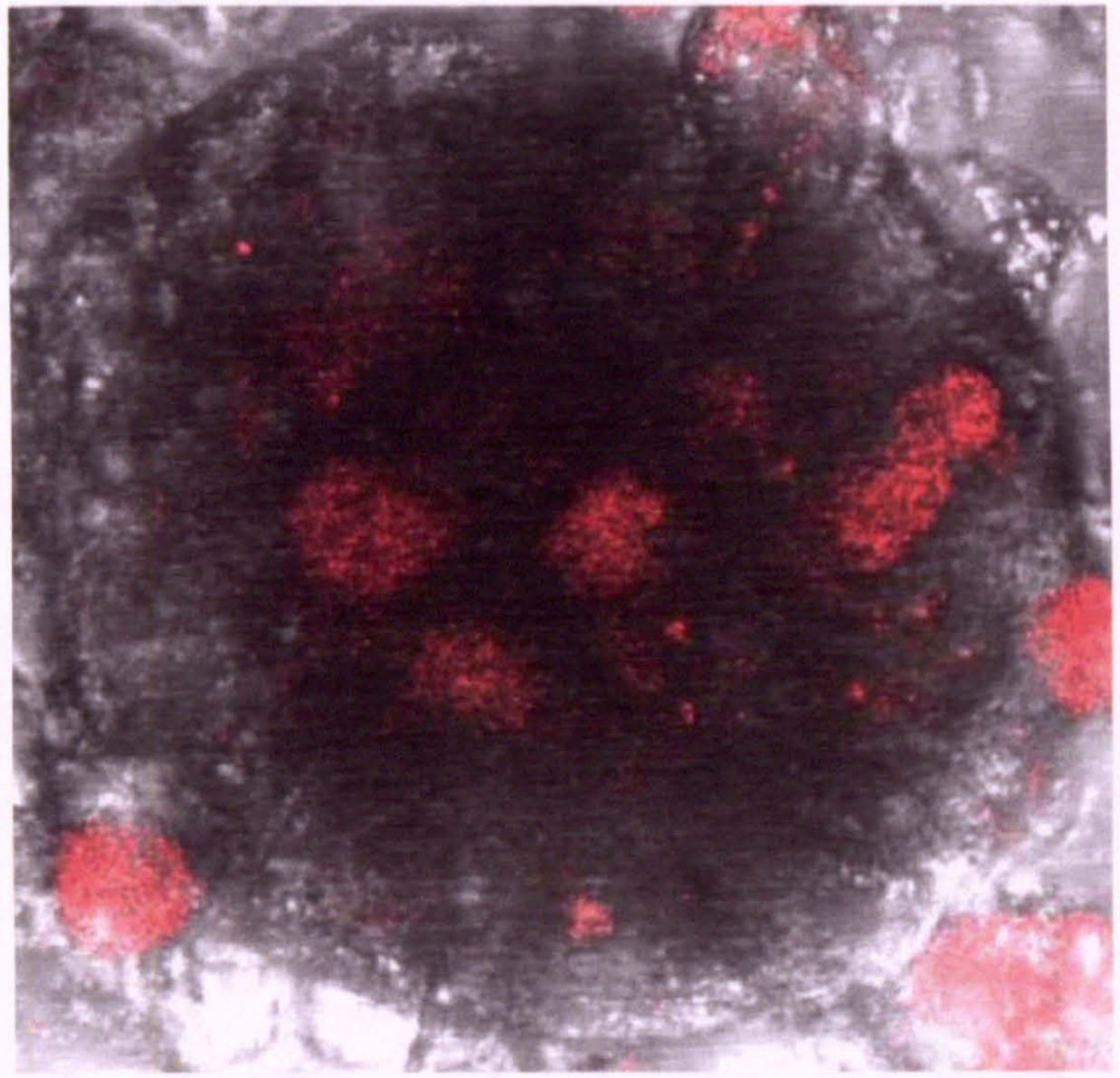
5.3.1. Visualisation of lipid in three-dimensional co-culture spheroids of human primary hepatocytes and rat hepatic stellate cells

A representative image of a spheroid (not transfected with replicon) stained with Nile Red is shown in figure 5.5. Large numbers of red-stained lipid inclusions can be seen in a number of cells on the surface of, and around the edge of, the spheroid (note that Nile Red only fluoresces in a hydrophobic environment[168] and thus it is not excited in either the aqueous cytoplasm or the serum-free, extracellular culture medium). Previous work at the University of Nottingham has shown that HSCs maintain an extremely slender morphology and activated cytochemical profile during spheroid co-culture[71], thus it seems clear that these rounded cells containing the lipid inclusions are indeed hepatocytes.

This provides encouraging evidence that human primary hepatocytes in spheroid co-culture with hepatic stellate cells contain intracellular lipid droplets, thus apparently fulfilling one of the requirements for HCV infection as described in 1.1.2. and 5.1.2.



(A)



(B)

Figure 5.5: *Images of spheroid co-cultures of human primary hepatocytes with rat hepatic stellate cells. (A) light microscopy image of single, organised spheroid after 48 hours of co-culture. (B) confocal laser microscopy image focused at the surface of same spheroid, showing uptake of Nile Red stain into lipid droplets of hepatocytes, both within and adjacent to the spheroid. Measured diameter of spheroid = 270 µm.*

5.3.2 Transfection of Huh7 cells with subgenomic JFH1 RNA

For each experiment, the untransfected control cultures showed no visible signs of toxicity or contamination, by light microscopy. GFP-protein was observed by UV-microscopy in all DNA-transfected controls after 20-24 hours, as described in chapter 4. No luciferase activity was detectable in the mock-transfected controls (**b**), thus there was no evidence of unintended contamination of cultures with RNA and spectrophotometric light emission was specific to luciferase-transfected test cultures.

Table 5.1 and figure 5.6 show the results of experiments to transfect Huh7 cells with subgenomic JFH1 replicon. These findings essentially confirm the published results of other groups (see figure 5.3[166]) and demonstrate an increase in replicon translation for at least 48 hours following transfection with replication-competent mRNA, whereas translation of the replication-deficient mutant declined progressively from 4 hours post-transfection onwards. Three batches of each RNA replicon were produced. Figures 5.7 and 5.8 shows that the change in average luciferase production, from the 3 batches of the two different types of RNA used, was more or less exponential over the 4 to 48 hour period (data for each separate batch showed similar curves with similar R^2 values to those of the averages). Coincidentally, perhaps, the doubling- (t_2) or halving-time ($t_{0.5}$) for luciferase activity in JFH1- or GND mutant-transfected cells, respectively, occurred over a period of about 12 hours.

The pattern of luciferase decrease observed in the GND mutant-transfected cells was broadly similar to that shown in figure 5.3[166] (note: y-axis in figure 5.3 is exponential) but $t_{0.5}$ was only about 4 h in that particular case. The decrease in luciferase activity in GND mutant-transfected cells was presumably due to enzyme

and/or RNA template loss by degradation during Huh7 cell proliferation. The longer $t_{0.5}$ seen in the present study may reflect slower rates of cell proliferation and/or luciferase degradation, in the particular Huh7 cell batches used.

The pattern of luciferase increase observed with the JFH1-transfected cells in the present study was also broadly similar to that shown in figure 5.3[166] (note: y-axis in figure 5.3 is exponential), in which $t_2 \approx 14$ h during the first 24 h, although in that earlier study a plateau in luciferase production was clearly reached at about 48 h (the cut-off time in the present study), possibly due to exhaustion of the nutrient supply. The net increase in luciferase (over losses incurred by enzyme and/or RNA template degradation) observed in the JFH1-transfected cells presumably reflected replication of the RNA template.

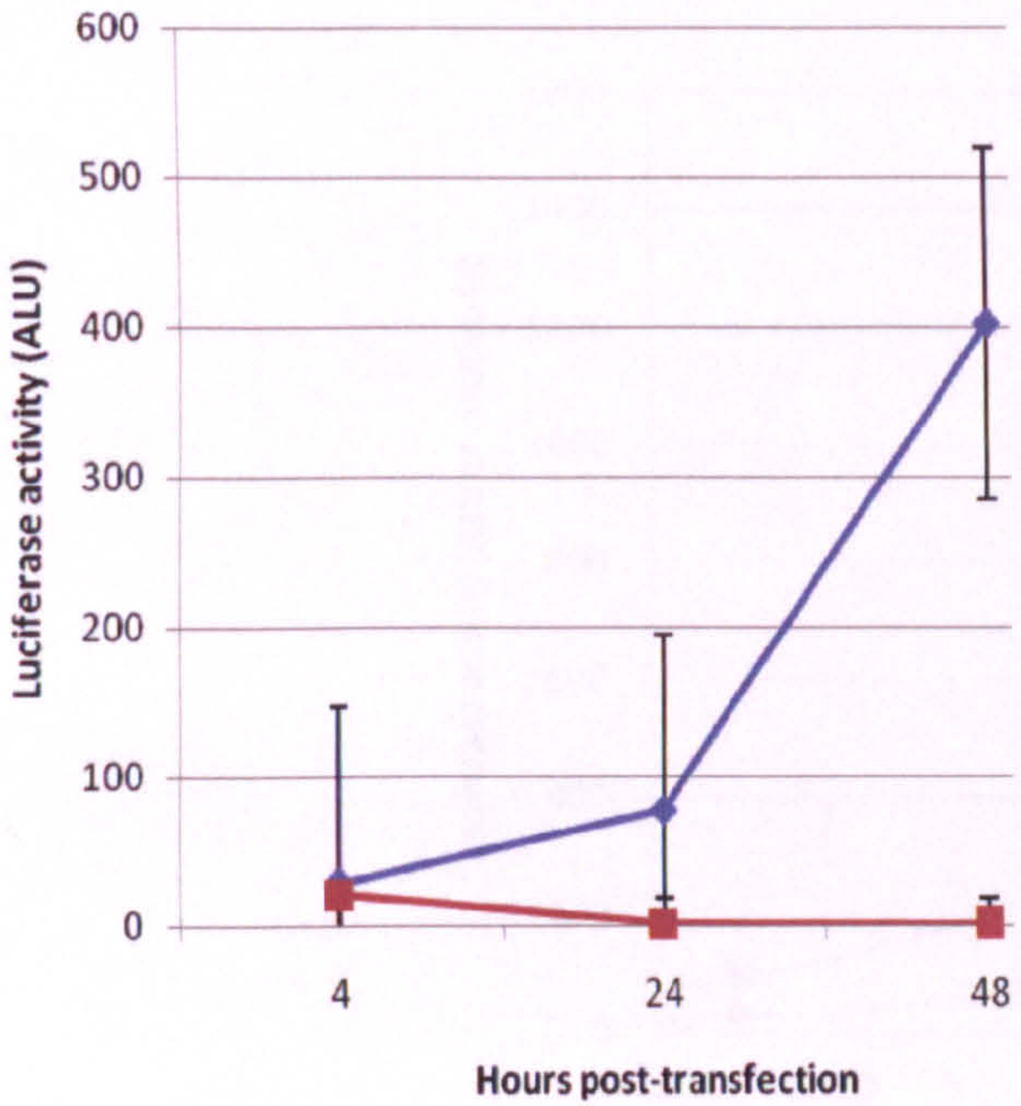
Irrespective of the slight differences between the results of the two studies, the results of the present study indicated clearly that the RNA used in subsequent experiments was intact, functional and capable of replication as expected and that the methodology was working reliably.

Table 5.1: Expression of the subgenomic JFH1 replicon, or a replication-deficient version, in monocultures of Huh7 cells.

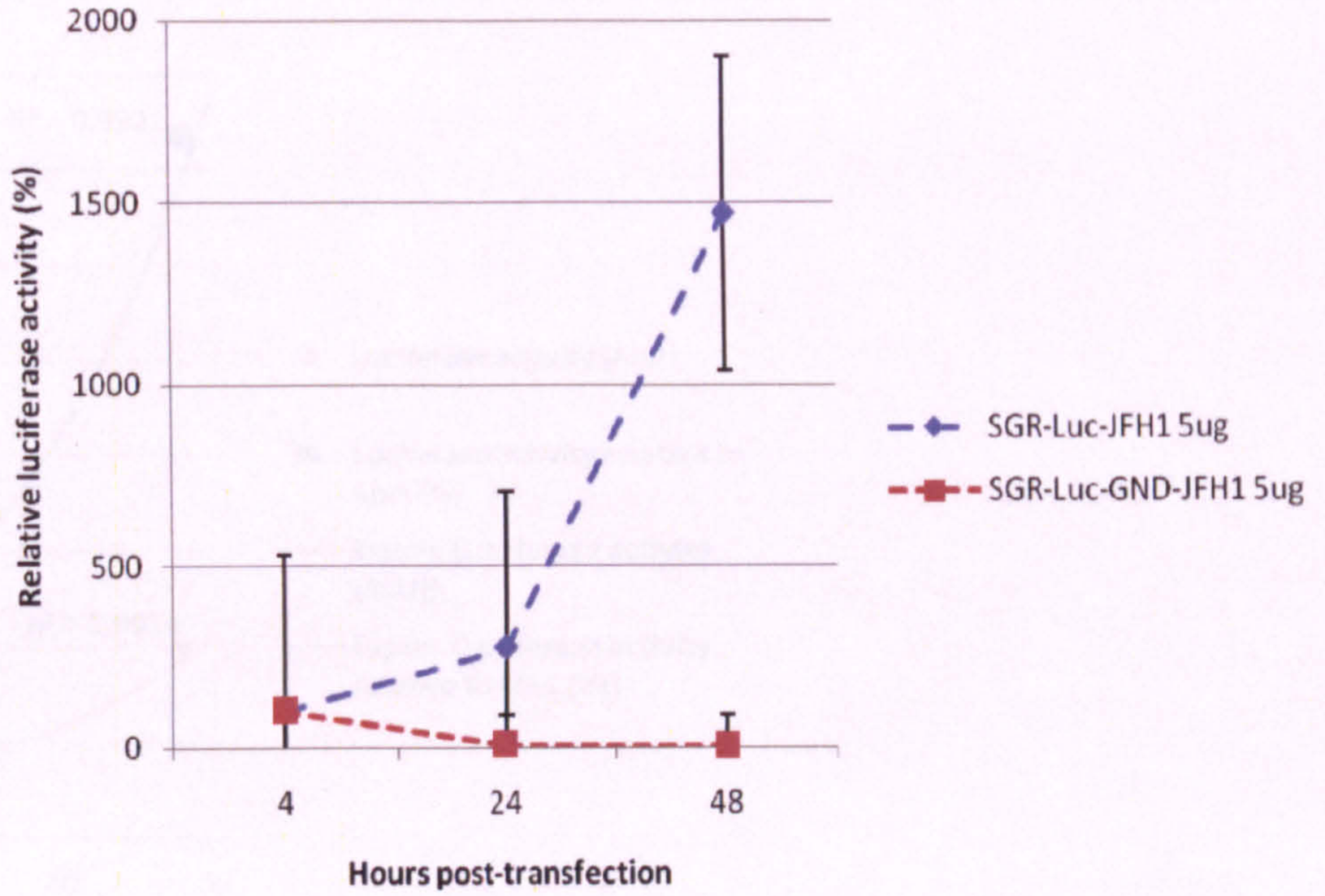
Replicon	Amount (µg)	Hours post-transfection	Luciferase activity (ALU)[1] / relative to 4 hour activity (%)						Mean luciferase activity (ALU)[1]	RSD of mean luciferase activity (%)[2]	Mean relative luciferase activity (%)	RSD of relative luciferase activity (%)[2]
			RNA batch 1		RNA batch 2		RNA batch 3					
SGR-Luc-JFH1	5	4	39.920	100.00	25.470	100.00	21.170	100.00	28.85	34.0	100.00	0.0
		24	93.150	233.34	76.260	299.41	61.920	292.49	77.11	20.3	275.08	13.2
		48	444.180	1112.58	377.800	1483.31	384.100	1814.36	402.03	9.1	1470.12	23.9
SGR-Luc-GND-JFH1	5	4	29.090	100.00	25.650	100.00	7.704	100.00	20.82	55.2	100.00	0.0
		24	2.170	7.46	0.748	2.92	0.651	8.45	1.19	71.5	6.28	47.0
		48	0.603	2.07	0.426	1.66	0.243	3.15	0.42	42.5	2.30	33.6

[1] ALU = arbitrary light units

[2] RSD = relative standard deviation



(A)



(B)

Figure 5.6: *Expression of the luciferase marker gene in Huh7 cells transfected with the subgenomic JFH1 replicon. (A) Showing the absolute luciferase activity of the SGR-Luc-JFH1 replicon and its replication-incompetent GND mutant, measured in arbitrary light units (ALU). (B) Showing the luciferase activity of both replicons relative to the ALU baseline level (100%) at 4 hours post-transfection (%). Standard error bars are shown.*

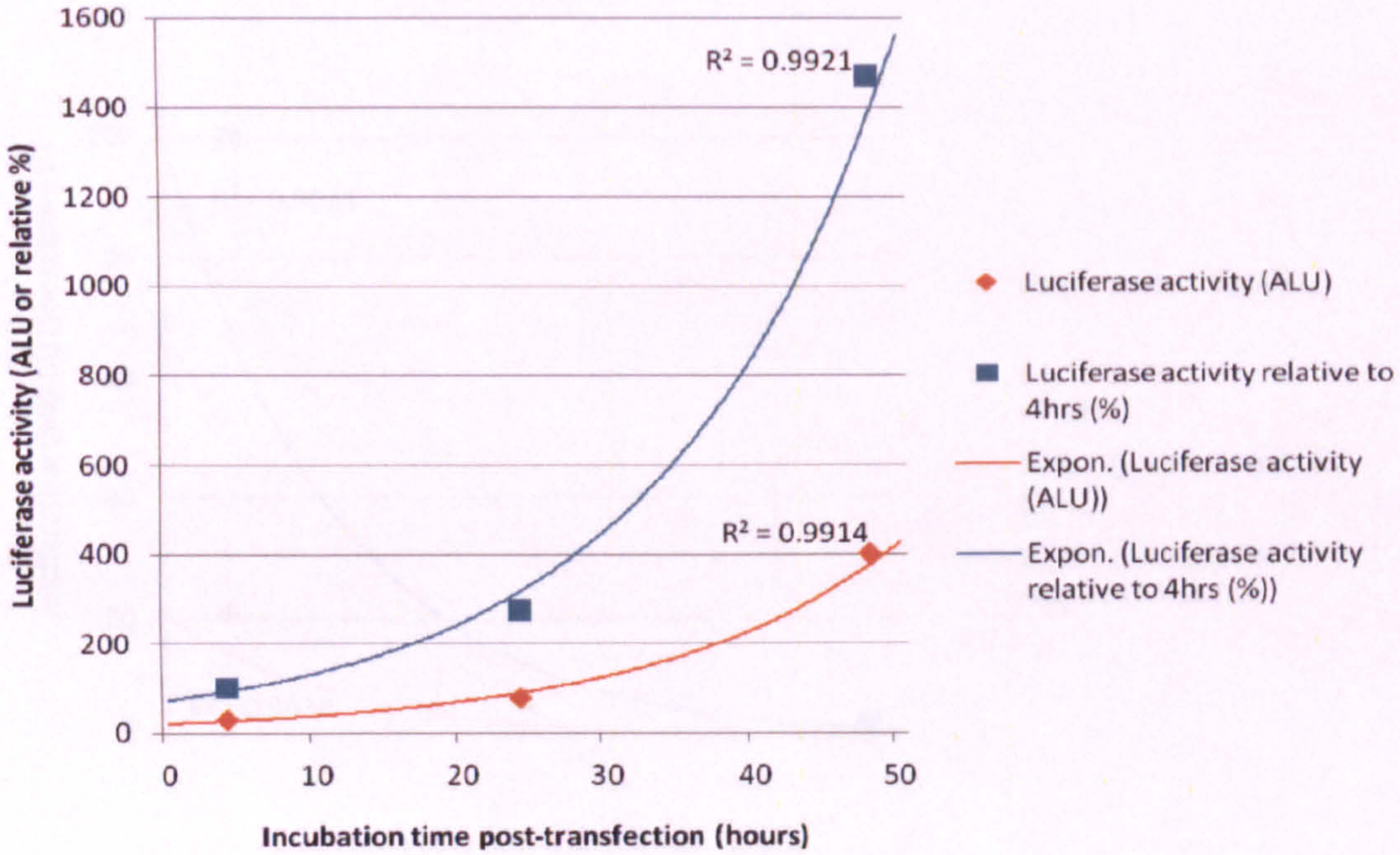


Figure 5.7: *Average luciferase activity (ALU and relative %) observed in Huh7 cells transfected with 3 batches of SGR-Luc-JFH1 RNA.*

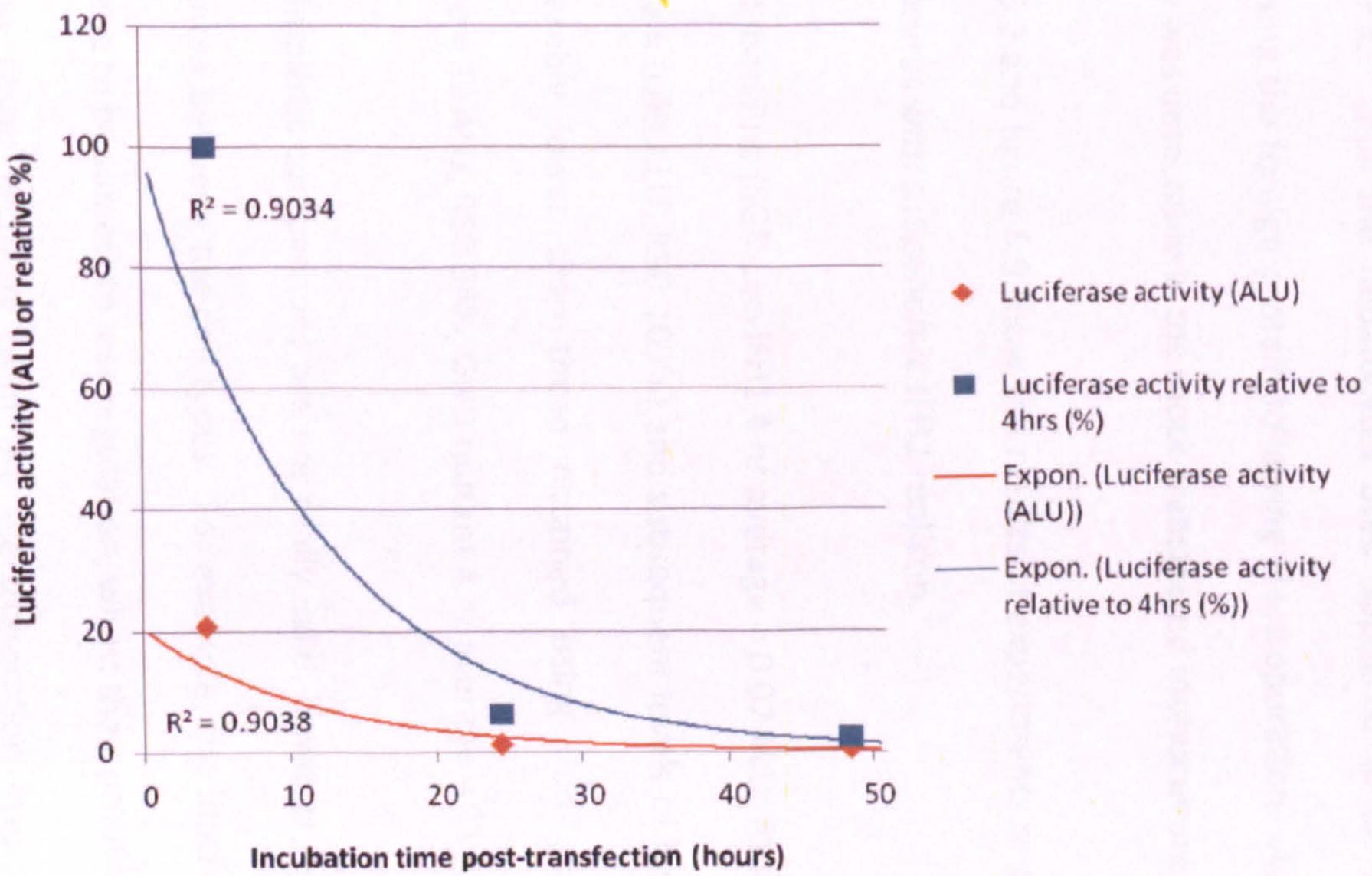


Figure 5.8: Average luciferase activity (ALU and relative %) observed in Huh7 cells transfected with 3 batches of the SGR-Luc-GND-JFH1 RNA.

5.3.3. Translation and replication of the subgenomic JFH1 replicon in human primary hepatocytes

For each experiment, the untransfected control cultures showed no visible signs of toxicity or contamination, by light microscopy. GFP-protein was observed by UV-microscopy in all DNA-transfected controls after 20-24 hours, as described in chapter 4. Thus the hepatocytes used appeared to be healthy and capable of expressing the foreign protein following electroporation. As expected, no luciferase activity was detectable in the mock-transfected monoculture controls (**b**).

Table 5.2 and figure 5.9 show the results of experiments to transfect human primary hepatocytes with subgenomic JFH1 replicon.

Overall, baseline (SGR-Luc-JFH1 4 hr average = 0.02 ALU, RSD 143%; GND mutant 4 hr average = 0.08 ALU, RSD 109%) and subsequent levels of luciferase expression were considerably lower than those obtained using Huh7 cells (SGR-Luc-JFH1 4 hr average = 29 ALU, RSD 34%; GND mutant 4 hr average = 21 ALU, RSD 55%).

Such simplistic comparisons are not really valid, however, because of the numerous differences between the cell types. For example, the intra-cellular processes of cell lines tend to be subject to less regulation, whilst their proliferative phenotype is likely to allow faster recovery from the electroporation insult as well as subsequent increase in cell numbers, compared to primary cells. Due to the differences in functional phenotype, there is no defined mechanism by which the recovery of function of the Huh7 cells (which existed in optimum culture conditions up until 5-10 minutes before electroporation) can be compared with recovery of function of primary hepatocytes (which may have been disrupted from optimum conditions *in*

vivo some 3-6 hours prior to electroporation). Measurement of DNA content, in order to quantify cell numbers and thereby determine the luciferase activity per cell, would have been problematic due to the multinucleated nature of some hepatocytes and was not performed due to the very limited availability of materials and experimental time.

In the absence of these data, it is not clear how much of the difference in luciferase expression between the Huh7 cells and human primary hepatocytes was due to differing cell numbers (due to electroporation-associated cell death and/or subsequent proliferation of the Huh7 cells) and how much was due to a real difference in translational efficacy or permissivity between the two cell types.

As shown in figure 5.9, there appeared to be a tendency for a higher level of baseline (4 h) translation of the GND mutant as compared to the replication-competent JFH1 RNA. However (and unsurprisingly, given the high RSD values), this difference was not found to be statistically significant, even when baseline luciferase activity was expressed as a function of the amount of RNA added prior to electroporation (average for GND mutant = 0.080 ALU/µg; average for JFH1 = 0.028 ALU/µg; $p = 0.15$ by unpaired two-tailed t-test). There is thus no evidence that the low level of translation by primary hepatocytes, compared with Huh7s, was associated with either the presence or absence of replicative capacity or function of the viral RNA, as the level was similarly low for both replication-competent and replication deficient JFH1 RNA, at the 4 h baseline.

The luciferase expression data might be taken to show that SGR-Luc-JFH1 expression in monocultures of human primary hepatocytes does not, on average, follow the same pattern as that found in Huh7 cells, especially as, after 48 hours, luciferase

activity was almost entirely lost. When the results from all batches were averaged, and compared as ratios of baseline levels of translation at 4 hours post-transfection, there was an apparent rise in the level of translation of SGR-Luc-JFH1 after 24 hours (both with 5 and 10 μg), compared with the replication incompetent mutant.

However, comparison of the means in this way may be misleading, as some batches of primary hepatocytes gave a transient increase in expression whilst others did not. In any case, due perhaps to this variability, the average differences were not statistically significant ($p = 0.227$ and 0.308, respectively, by unpaired t-test) and therefore, if the means are examined, these results provide no evidence that replication of the JFH1 replicon occurred in monocultures of human primary hepatocytes, between 4 and 48 hours post-transfection.

As already noted above, the results from individual batches of hepatocytes showed a wide variation in the pattern of luciferase expression. Three batches showed a decline in luciferase activity at 24 hrs, whereas 2 showed evidence of a transient increase at the same time point. This suggests that the presence or absence of a transient increase in luciferase expression (and perhaps, therefore, RNA template replication), may be highly batch-dependent. Furthermore, it seems that some batches of hepatocytes may indeed be permissive for transient replication of the SGR-Luc-JFH1 replicon within the first 48 hours post-transfection. Unfortunately, due to the small numbers of "permissive batches", meaningful statistical analysis to prove this hypothesis is not possible.

In the case of the replication-incompetent GND mutant, the general pattern of decline in luciferase expression in the human primary hepatocytes was very similar to that seen in the Huh7 cells. Given that there would have been very little, if any, loss

of template RNA due to proliferation in primary hepatocytes, this suggests that these cells may be better at either eliminating the viral RNA or degrading the luciferase than are Huh7 cells (as may be expected in healthy, normal cells with intact innate immune responses). Unexpectedly, 3 of the 5 batches of human primary hepatocytes, transfected with the replication-competent subgenomic JFH1 replicon and maintained in monoculture, also showed this pattern of declining luciferase expression. This suggests that some donors' hepatocytes may be more resistant to RNA transfection, translation, or replication and/or more able to eliminate the RNA template or its resultant protein(s), than those of others. These possibilities will be addressed further in section 5.4.

Table 5.2: Expression of the subgenomic JFH1 replicon, and a replication-deficient version, in monocultures of human primary hepatocytes

Replicon	Amount (µg)	Hours post-transfection	Luciferase activity (ALU)[1] / relative to 4 hour activity (%)										Mean luciferase activity (ALU)[1]	Mean relative luciferase activity (%)	RSD of mean % change from 4hr baseline[2]
			Batch 18[3]		Batch 19[3]		Batch 20[4]		Batch 21[4]		Batch 26[5]				
SGR-Luc-JFH1	5	4			0.513	100.0	0.015	100.0	0.096	100.0	0.123	100.0	0.187	100.0	0.0
		24			0.173	33.7	0.050	343.5	0.029	30.2	0.413	335.8	0.166	185.8	95.6
		48			0.017	3.3	-	-	<0.001	<1.0	0.021	17.1	0.019	10.2	95.5
	10	4	0.121	100.0	0.037	100.0					0.079	100.0	0.079	100.0	0.0
		24	0.053	43.8	0.027	73.0					0.660	835.4	0.247	317.4	141.4
		48	0.018	14.9	0.009	24.3					0.005	6.3	0.011	15.3	58.8
SGR-Luc-GND-JFH1	5	4			1.224	100.0	0.025	100.0			0.445	100.0	0.565	100.0	0.0
		24			0.267	21.8	0.009	52.2			0.214	48.1	0.163	40.7	40.5
		48			0.157	12.8	-	-			0.001	0.2	0.079	6.5	137.1
	10	2	0.524	100.0	0.138	100.0					0.731	100.0	0.464	100.0	0.0
		24	0.071	13.5	0.016	11.6					0.147	20.1	0.078	15.1	29.7
		48	0.028	5.3	0.034	24.6					<0.001	<0.1	0.021	10.0	128.8

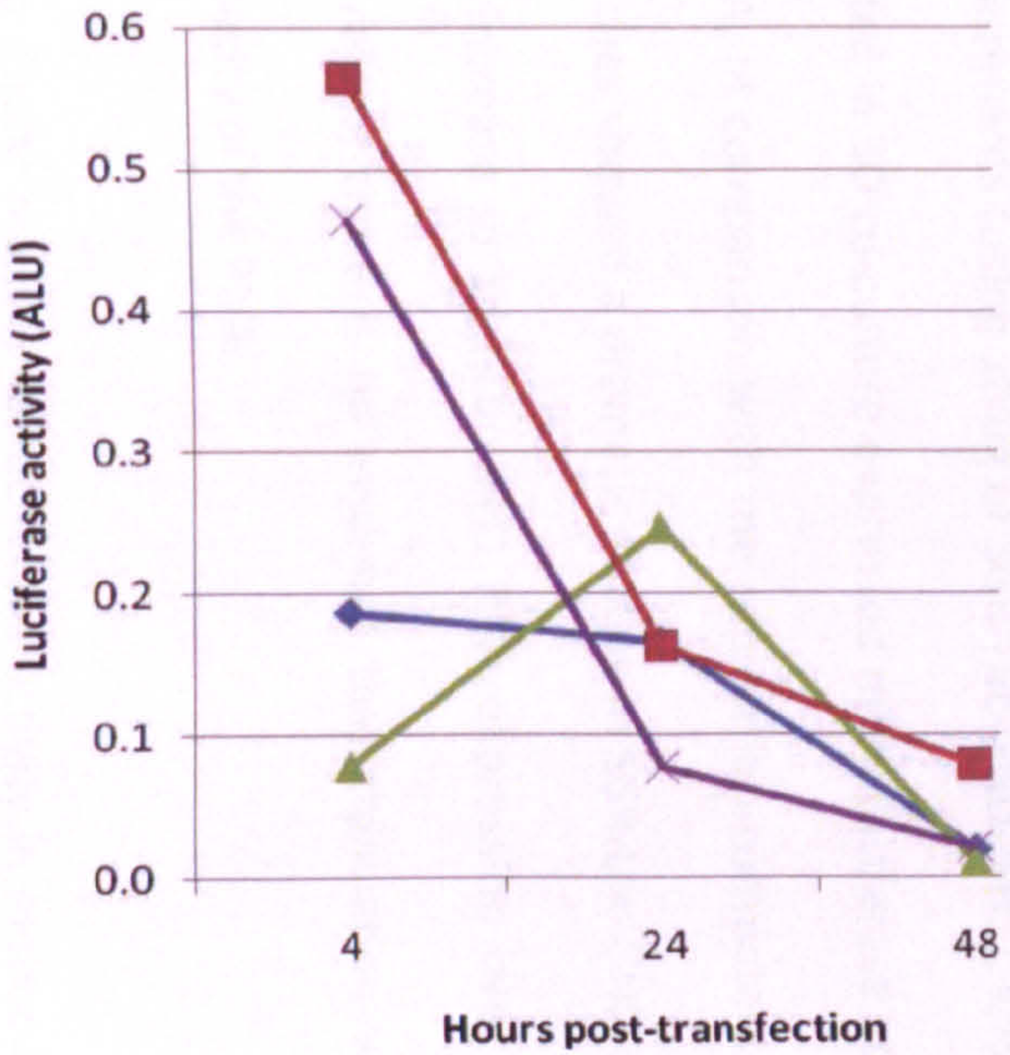
[1] ALU = arbitrary light units

[2] RSD = relative standard deviation

[3] Experiment performed using RNA batch 1

[4] Experiment performed using RNA batch 2

[5] Experiment performed using RNA batch 3



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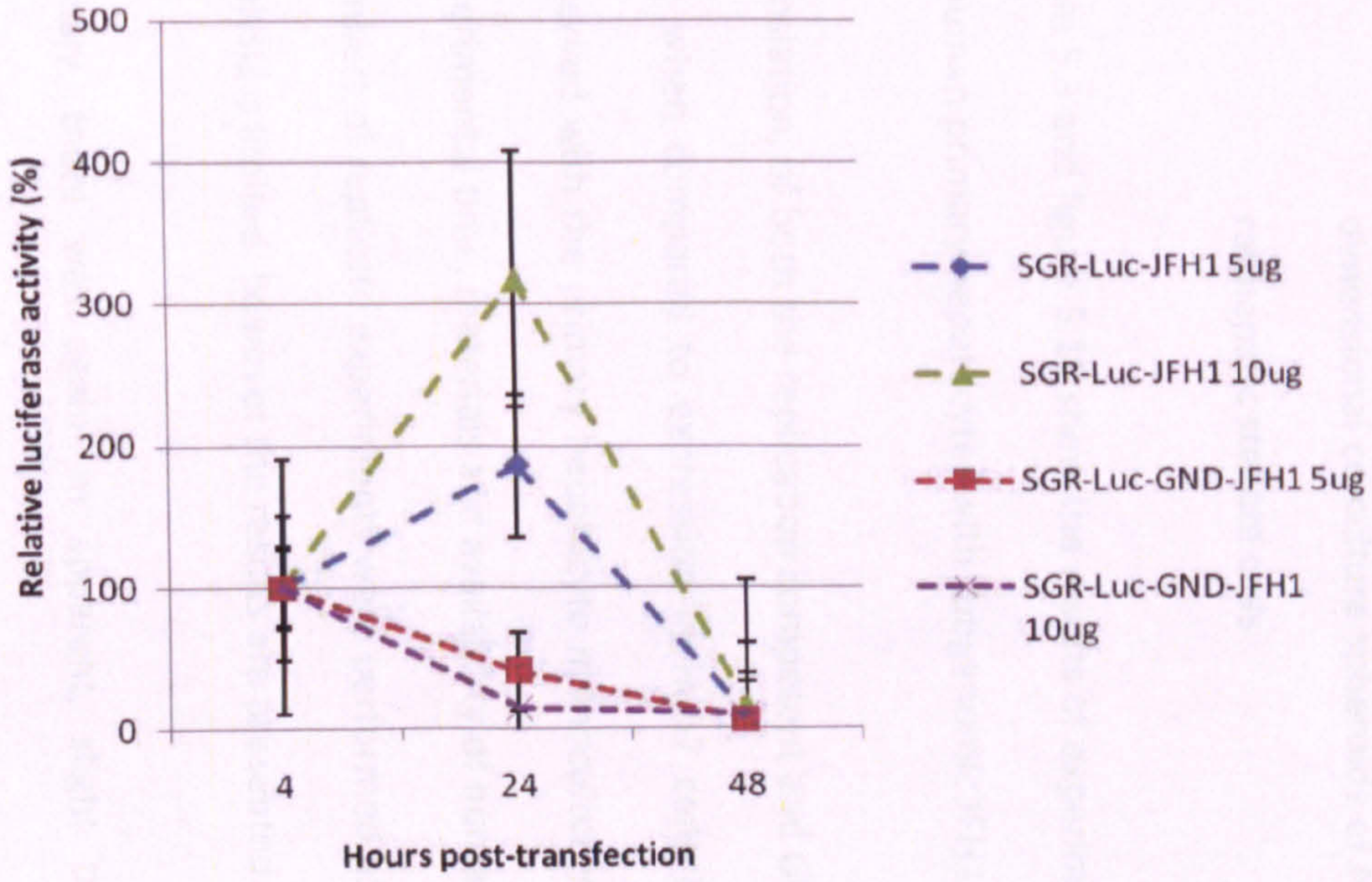


(B)

Figure 5.9: *Expression of the luciferase marker gene in monocultures of human primary hepatocytes transfected with the subgenomic JFH1 replicon. (A) Showing the absolute luciferase activity of the JFH1 replicon and its replication-incompetent GND mutant in arbitrary light units (ALU). (B) Showing the luciferase activity of both replicons relative to the baseline level found at 4 hours post transfection (%). Standard error bars are shown only in B, for clarity.*

5.3.4. Translation and replication of the subgenomic JFH1 replicon in three-dimensional co-culture spheroids of human primary hepatocytes and rat hepatic stellate cells

Table 5.3 and figure 5.10 show the results of experiments to transfect 3D co-cultures of human primary hepatocytes with subgenomic JFH1 replicon.

Translation, of both the replication competent and GND-mutant replicons, was again low when compared to expression in Huh7 cells but was comparable with that observed with the primary hepatocyte monocultures. Unfortunately, constraints of experimental time, materials and availability of human tissue meant that sub-optimal numbers of replicate experiments were performed and therefore potential statistical analysis is limited; however the results are presented for completeness.

Initially, there was again an apparent, slight translational advantage of the replication-deficient mutant over the intact JFH1 replicon, at 4 hours. Although this impression might be biased by a particularly high result from one of the two experiments (using 10 μg of SGR-Luc-GND-JFH1), it is clear that both batches of cells tested in 3D co-culture expressed more luciferase at baseline from the GND mutant RNA, in comparison with the replication-competent JFH1 RNA. Furthermore, both batches showed a dramatic decline in SGR-Luc-GND-JFH1 expression after 24hrs, but an increase in SGR-Luc-JFH1 RNA expression over the same time period. Again therefore, there is no evidence that translation was inhibited by the replicative capacity of the RNA.

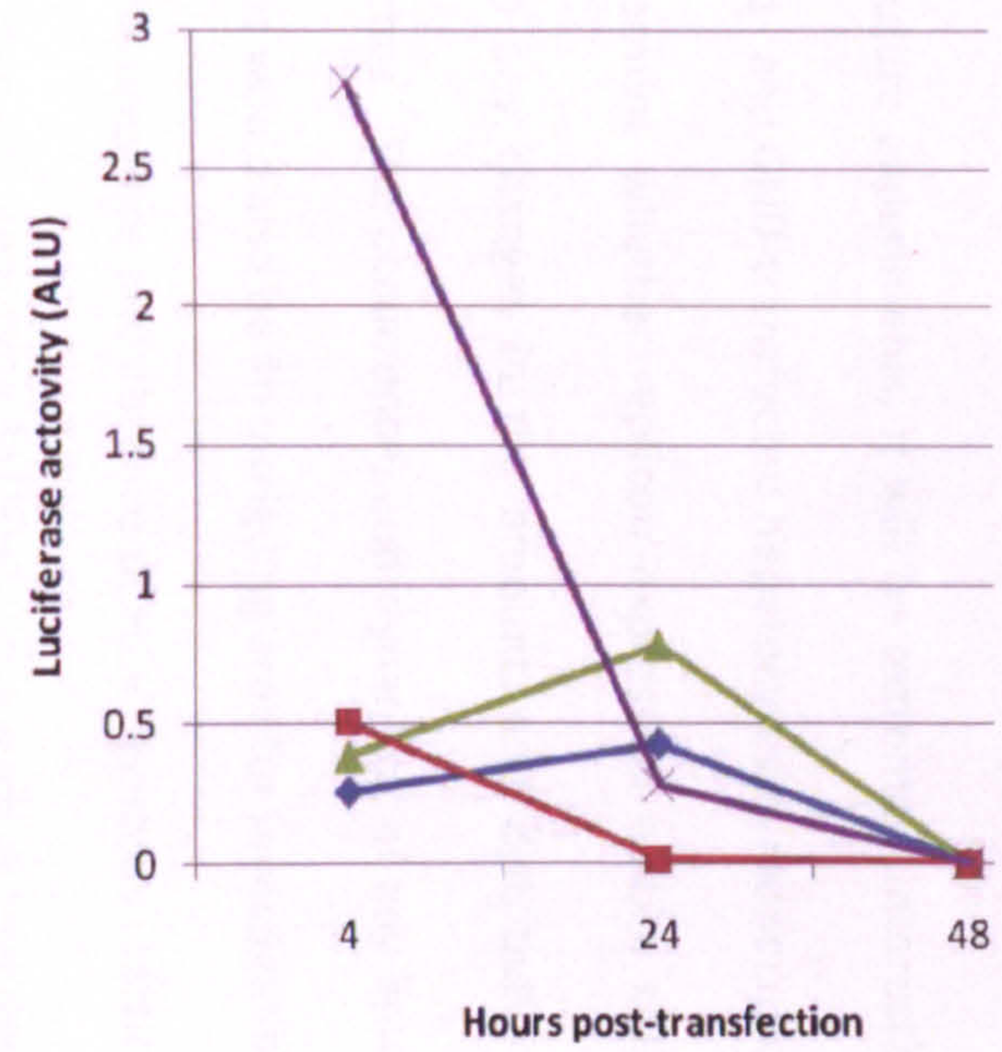
In a similar manner to luciferase activity in monocultures, detectable expression of the transfected RNA was lost by 48 hours post-transfection, for both intact and

replication-deficient RNA. When expressed as a ratio of the baseline levels of translation at 4 hours post-transfection, there was (again) a rise in luciferase expression at 24 hours, in cultures transfected with the intact JFH1 replicon. When using 5 μg SGR-Luc-JFH1 RNA, this increase was highly significant (159% vs. 3%; $p = 0.003$ by unpaired t-test). However, given the low level of replication of these experiments, the results must be interpreted with caution and will be discussed further in section 5.4.

Table 5.3: *Expression of the subgenomic JFH1 replicon, or a replication-deficient version, in 3D co-cultures of human primary hepatocytes with rat hepatic stellate cells*

Replicon	Amount (µg)	Hours post-transfection	Luciferase activity (ALU)[1] / relative to 4 hour activity (%)				Mean luciferase activity (ALU)[1]	Mean relative luciferase activity (%)	RSD of mean % change from 4hr baseline[2]
			Batch 21[3]		Batch 26[4]				
SGR-Luc-JFH1	5	4	0.012	100.00	0.503	100.00	0.258	100.0	0.0
		24	0.018	150.00	0.841	167.20	0.430	158.6	7.7
		48	<0.001	<8.33	<0.001	<0.20	<0.001	<0.4	<1337.2
	10	4			0.388	100.00	0.388	100.0	
		24			0.788	203.09	0.788	203.1	
		48			<0.001	<0.26	<0.001	<0.3	
SGR-Luc-GND-JFH1	5	4	0.031	100.00	0.989	100.00	0.510	100.0	0.0
		24	0.001	3.23	0.031	3.13	0.016	3.2	1.9
		48	<0.001	<3.23	<0.001	<0.10	<0.001	<1.7	<133.1
	10	2			2.811	100.00	2.811	100.0	
		24			0.283	10.07	0.283	10.1	
		48			<0.001	<0.04	<0.001	<0.04	

[1] ALU = arbitrary light units
[2] RSD = relative standard deviation
[3] Experiment performed using RNA batch 2
[4] Experiment performed using RNA batch 3



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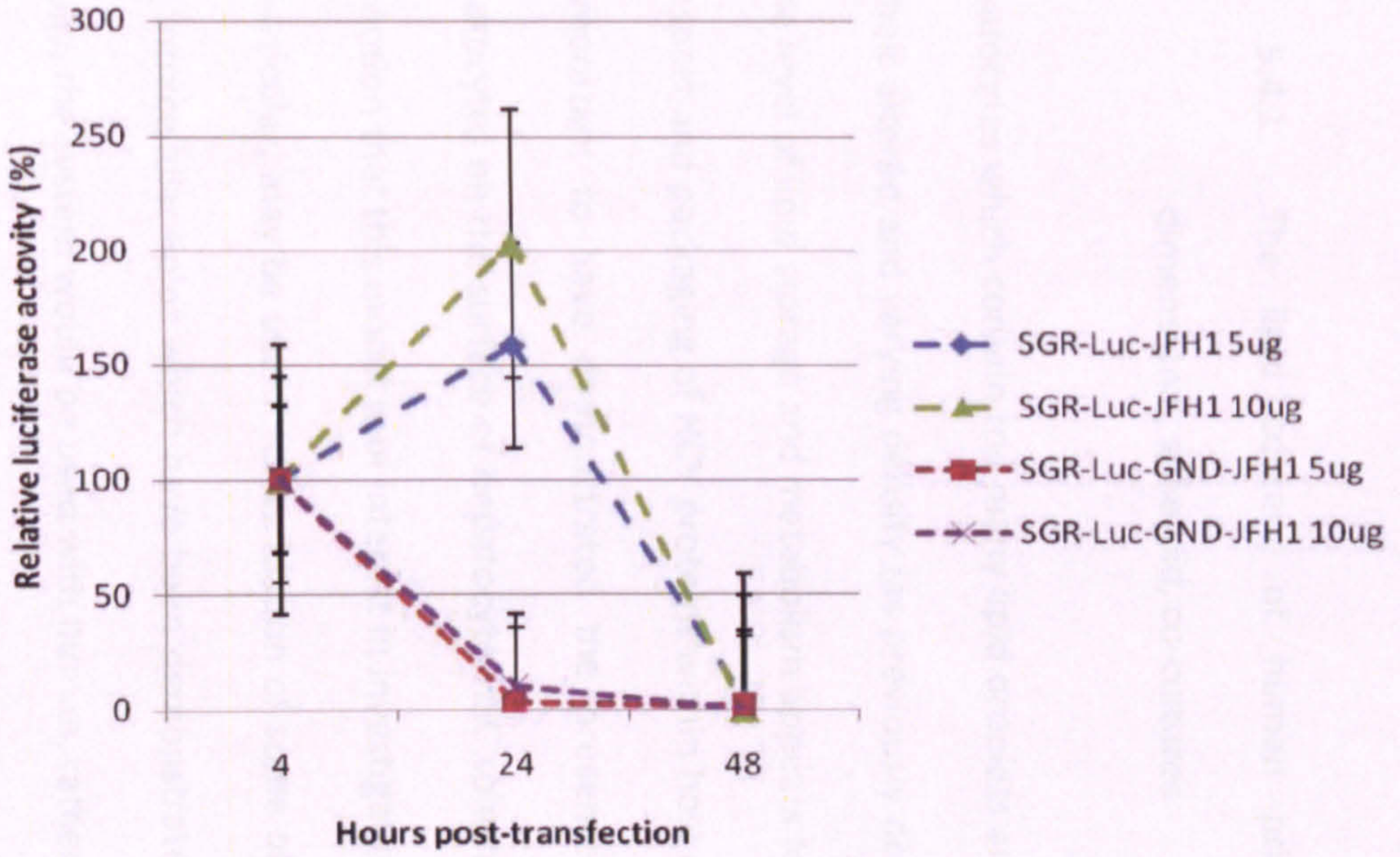


(B)

Figure 5.10: Expression of the luciferase marker gene in spheroid co-cultures of human primary hepatocytes, transfected with the subgenomic JFH1 replicon, and rat stellate cells. (A) Showing the absolute luciferase activity of the JFH1 replicon and its replication-incompetent GND mutant, in arbitrary light units (ALU). (B) Showing the luciferase activity of both replicons relative to the baseline level found at 4 hours post-transfection (%). Standard error bars are shown only in B, for clarity.

5.4. Discussion

5.4.1. The lipid content of human primary hepatocytes in three-dimensional, spheroid, co-cultures

Hepatocytes which contain too many lipid droplets are difficult to isolate *in vitro* due to their altered and varying density (as previously described in chapter 3); however, some level of lipid storage and metabolism appears to be required for the anchoring, transport and packaging of HCV proteins within host cells (1.1.2.1 and 5.1.2). Thus it is important to have demonstrated the presence of lipid droplets within the hepatocytes on the surface of hepatocyte-HSC spheroids. This observation supports the notion that this model is of interest in investigating the pathogenesis of HCV and, in particular, may be useful for validation of some of the associations between HCV and intracellular lipids which have been demonstrated in the Huh7 cell model[35,169]. Ideally, the system would be used with human, rather than rat HSCs. However it was not possible to isolate and durably culture sufficient numbers of human HSCs within the timeframe of this project, though it may be possible in future studies.

In future experiments, it will be extremely interesting to examine lipid droplets in JFH1- and GND-transfected hepatocytes in monocultures and co-cultures, in order to determine whether replicon expression and/or culture conditions, are associated with any changes in the amount, and distribution, of lipid in "permissive" cell batches. The occurrence, and dynamics, of any lipid transfer between the two cell types would also be an intriguing area for investigation in the co-culture system, both in the presence and absence of HCV replicons (particularly, perhaps, in the presence of a chimeric genotype 3a/JFH1 virus, such as that described by Gottwein *et al.*[170]).

5.4.2. Translation and replication of subgenomic JFH1 RNA

Replicon systems, and particularly the JFH1 replicons, have previously proven to be extremely useful tools for investigating the pathogenesis of hepatitis C. The SGR-Luc-JFH1 plasmid that was created using the JFH1 clone offers a simple, yet effective means to monitor the level of HCV expression in a cell culture system[166]. Furthermore, as it derives directly from the RNA which is inserted into target cells, expression of the luciferase marker gene is not affected by the variables of viral attachment, endocytosis or endolysosomal escape.

In the above experiments, the RNA which was derived from the SGR-Luc-JFH1 and GND-JFH1 sequences proved to be stable, and functional, prior to and following transfection, when handled and stored under the conditions described above. Expression in unmodified Huh7 cells resulted in the expected pattern of RNA replication, as shown by the increase in luciferase levels in SGR-Luc-JFH1 transfected cells over 48 hours and a corresponding decrease within cells transfected with a replication-incompetent sequence. These control experiments also showed that nucleofection, and the Nucleofector® kits, were compatible with (and did not inhibit) transfection of subgenomic JFH1 RNA.

However, it is noteworthy that such experiments provide no conclusive evidence that the replicon's RNA was replicated within *undivided* Huh7 cells. The proliferation rates of transfected Huh7s were not measured during the present study. Whilst the data in table 5.1 imply that, given an Huh7 cell doubling time of 35-40 hours[48], relative translation of the replicon increased by around five-fold during this same period, it is possible that such an increase (whether due to replication of the

transcript or higher rates of translation) was facilitated by the physiological changes accompanying mitosis and would not occur in any undivided cells. In other words, it is therefore possible that most, if not all, of the increase in luciferase in the Huh7 cells was due to (or at least facilitated by) cell proliferation. If so, this has important implications for the expected pattern of behaviour of the JFH1 replicons in primary hepatocytes.

5.4.2.1. Translation and replication of JFH1 RNA within monocultures of human primary hepatocytes

SGR-Luc-JFH1 mRNA was transfected into freshly isolated human hepatocytes, which were then placed into two-dimensional monoculture. In comparison with Huh7 cells, the initial average level of luciferase activity at 4 hours post-transfection was approximately 100-fold lower, using the same initial numbers of cells and amounts of RNA.

This lower level is to be expected for a number of reasons. Firstly, healthy primary cells are likely to have much tighter control of transcriptional and translational processes than a tumour cell line which, almost by definition, has lost some of the regulation of such functions. Secondly, electroporation has been shown to cause significant cell damage and may induce apoptosis in some cases[171]; the Huh7 hepatocyte cell line could be expected to recover from, or resist, such effects more robustly than primary cells. Thirdly, HCV replication has been shown to vary in extent, depending upon genotype and quasispecies, but previously has been maximal only at about 3-5 days after inoculation of human primary hepatocytes[74]. In addition, the number of HCV copies per hepatocyte may normally be low under *in*

vitro conditions[75]. Lastly, it has previously been shown that IRES-dependent translation is not subject to the same limitations, during the mitotic phase of the cell cycle, as cap-dependent translation[172]. Other studies have indeed demonstrated that HCV (and its replicons) are translated much more readily in dividing cells than in resting or quiescent cells[173,174].

This evidence clearly indicates that the level of replicon translation should be expected to be much higher in a rapidly proliferating cell line than in largely quiescent primary cells, due both to the subsequent proliferation of the replicon within those cells and to the overall increase in the total number of replicon-bearing cells. The findings of previous studies, to investigate replication of serum-derived HCV in primary hepatocytes, have been consistent with this hypothesis, generally demonstrating very low levels of replication[148]. A lower level of translation and/or replication of JFH1 in primary hepatocytes is therefore to be expected and should not be viewed as an insurmountable flaw because, although the generation of high levels of HCV proteins and/or RNA may be useful for some *in vitro* investigations of viral processes, a primary cell experimental system will offer greater potential insights into the balance of factors affecting host permissivity and overall pathogenesis in a more *in vivo*-like situation.

Untransfected cells produced no measurable luciferase activity, as expected (data not shown). However, interpretation of the low levels of luciferase detected in the transfected primary hepatocytes remains doubtful, because measurement uncertainty was much greater than with the Huh7 cells. Between different hepatocyte batches, the levels of luciferase activity at the 4 h baseline were found to be much more variable than with Huh7 cells, with a further 10- or 50-fold difference

between the highest and lowest levels of baseline luciferase activity, produced by intact or GND-mutant RNAs, respectively.

It would have been desirable, had there been sufficient experimental reagents, time and opportunity, to have repeated the experiments and reported the relative luciferase activity per cell, by determining the DNA content of each cell lysate and using this to calculate the number of cells present for each reaction. Due to the multinucleate nature of hepatocytes, calibrated standards would have had to be created for each individual batch and at each separate time point, using non-transfected and mock-transfected cells. This would then have provided some extremely useful and detailed information on the amount of cell death occurring due to electroporation and the extent to which lower levels of translation in primary hepatocytes reflected a correspondingly higher rate of cell death.

Given that primary hepatocytes are expected to be largely non-proliferating unless subject to specific hormonal stimulation *in vitro*, and that the translational capacity of the viral RNA had been proven in the Huh7, the low level of luciferase activity in primary hepatocytes may have had several further causes, as follows (*i-vii*) and as shown in figure 5.11.

i. Firstly, much of the RNA may have been degraded by some aspect of the experimental process before or during transfection (figure 5.11a). However, given that the reagents and materials were all single-use, certified RNase-free and caused no such problem when used for the transfection of Huh7 cells, this seems an unlikely explanation. The experimental procedure and timeframes (at least from the point of RNA-use onwards) were identical when using either Huh7s or primary hepatocytes.

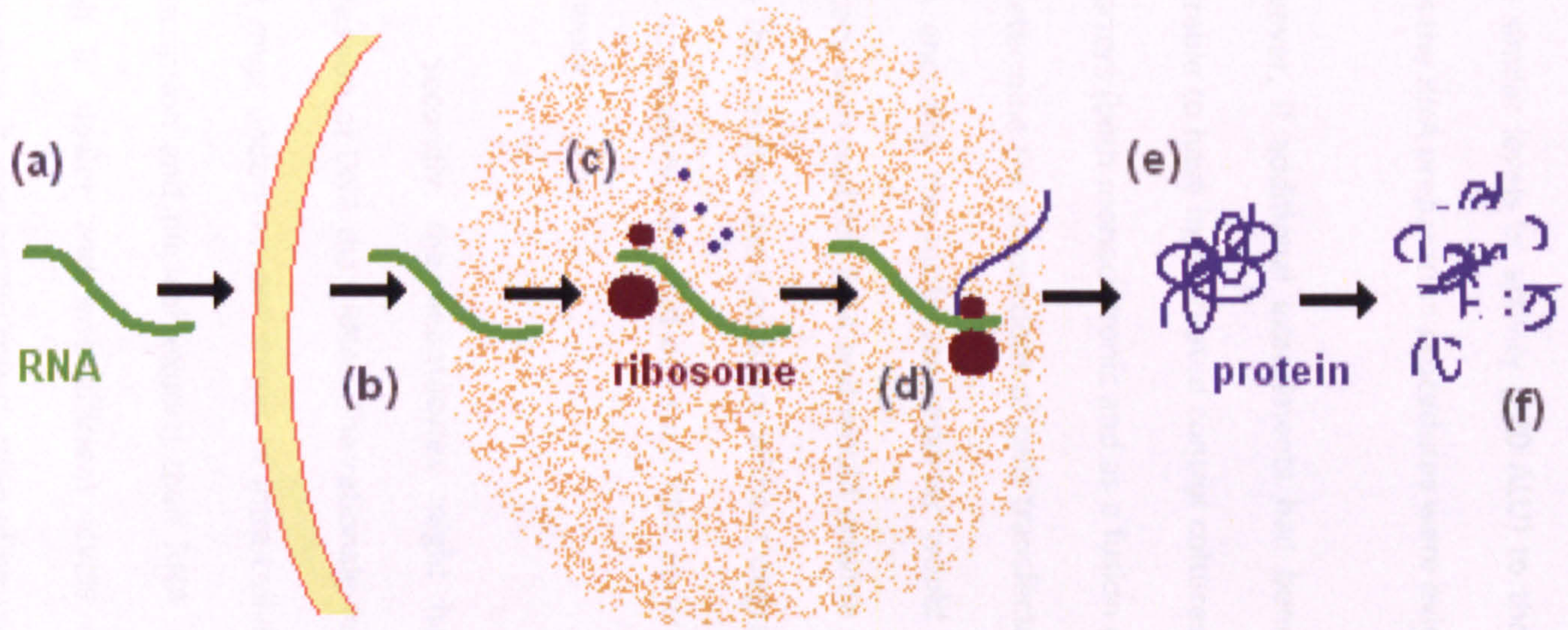


Figure 5.11: *A simplied, schematic diagram, showing the some of the key processes underlying the production of functional protein from transfected RNA. (a) RNA must reach the cell, and pass through its membrane, intact; (b) RNA must travel to an area containing ribosomes (usually the endoplasmic reticulum) without degradation; (c) ribosomes must be recruited for initiation of translation and amino acids must be available; (d) translation must be completed without disruption and the polypeptide released; (e) processing and folding of the polypeptide are usually required to produce the mature, functional protein; (f) the mature protein must perform its function before degradation. Some of the mechanisms which may disrupt these processes are detailed in the main text (section 5.4.1 i – vii).*

Due to the relatively small yields of RNA from each batch of *in vitro* transcription, it was not possible to re-test every batch to check that degradation had not occurred during storage; however batches 2 and 3 were re-tested, by transfection into Huh7 cells and subsequent luciferase assay at 4 hours post-transfection, and were found to give similar levels of activity (>20 ALU) to those obtained when initially screened. Thus the RNA preparation procedures were evidently robust and reliable.

However, if additional experiments had been possible, it would also have been desirable to have incorporated control cultures transfected with RNAs encoding GFP reporters (both monocistronic and as a fusion gene in a JFH1 HCV replicon), in order to determine the proportions of cells transfected. Furthermore, the use of a control RNA encoding the luciferase reporter would be more suitable for comparison of efficacy with replicon RNA and would provide some quantification of the amount of RNA that reaches the cytoplasm without degradation. Unfortunately there was not time to produce, test and use such RNA controls during the experimental period of this work.

ii. Secondly, the hepatocytes might have been more permissive for the expression of DNA than RNA. The rationale for this would be difficult to explain, as DNA must undergo more stages of intra-cellular processing (such as nuclear entry, transcription and nuclear export) than RNA, and therefore might be expected to result in slower and less efficient levels of transgene expression. A direct comparison, based on the transfection of equal copy numbers of a DNA template and its own RNA transcript, would be required to investigate this issue further.

iii. Thirdly, there may have been poor transport of RNA in the cytoplasm of nucleofected hepatocytes. It is conceivable that only endogenous RNA is directed to the endoplasmic reticulum, or recognised by the ribosomes, of primary hepatocytes (figure 5.11b). However, these explanations seem unlikely, given that the successful entry and translation of non-host RNA in primary hepatocytes has been reported in a number of published studies[133,175].

iv. Fourthly, innate cellular responses of primary hepatocytes may identify and remove the transfected RNA before significant translation and/or replication can occur. It is thought that the 5'NTR region of HCV RNA has a protective "cap" function, thus should have some resistance to intra-cellular RNases. However, it is well recognised that the presence of double-stranded RNA usually induces a complex process of antiviral mechanisms within the host cell, involving the activation of, for example, toll-like receptor 3 (TLR-3) and the subsequent up-regulation of the transcription factors nuclear factor κB (NF-κB) and interferon regulatory factor 3 (IRF-3)[176-178]. These mechanisms result in an increase in type-1 interferon production and a TRAIL-induced, pro-apoptotic response[177,178].

Despite this, specific innate anti-viral mechanisms seem unlikely to be activated prior to translation of the transfected RNA and the formation of replication complexes. Studies of a Huh7-derived cell line have demonstrated that, although a replicon is clearly translated at four hours, dsRNA is not detected at this time and its synthesis (indicating the start of RNA replication) begins somewhere between the 4 and 24 hour time points[179]. This observation is consistent with results, obtained using the subgenomic JFH1 replicon in Huh7 cells in the present study, showing similar levels of luciferase expression in both intact JFH1 and the replication-deficient GND mutant;

suggesting that expression at this time is largely due to translation of the original RNA.

However, the possibility remains that, unlike Huh7 cells[180], primary hepatocytes retain expression of TLR-3 and therefore possess more intact and *in vivo*-like responses to dsRNA, through which to repress the overall translational and replicative capacity of JFH1. If this hypothesis is correct, it could also account for the variability of expression dynamics seen between different batches of hepatocytes, as it is highly likely that the efficacy of such responses will vary (as a result of both nature and nurture) from person to person and may also be affected by differences in the time taken to regain specific functionalities and/or total functional capacity post-isolation and *in vitro*. There is also likely to be spatial and temporal variation of these cellular responses within each liver, as a consequence of exposure to nutrients, oxygen and insults (whether toxic, infectious or traumatic), which would further prejudice the ability of the researcher to replicate results, even within the same cell batch. Such variations may seem problematic but, in fact, must be essential to a closer understanding of the dynamics of natural HCV infection.

Interestingly, there is some evidence that the NS3/4 protease complex interferes with some aspects of the innate anti-viral response of the host cell and may entirely abrogate it in chronically infected cells[181,182]. It may be that innate physiological differences, as well as the loss of the RIG1 response, make Huh7 cells more permissive than primary hepatocytes to this interference, at least compared with the early phases of primary hepatocyte infection by HCV.

v. Fifthly, the HCV 5' IRES and/or EMCV IRES may not have high affinity for the ribosomes of primary hepatocytes (figure 5.11c). Again, this seems unlikely, given that HCV infection becomes well established *in vivo* and that the EMCV IRES is commonly used as a particularly highly active ribosome binding sequence (clearly, it functions well in the Huh7 cells). It seems unlikely that ribosomes of primary hepatocytes would have a different structure (and hence affinity) to those of Huh7 cells, but there might be significant differences in transcriptional regulation. Ideally then, further RNA controls, driven by each of these IRESs, would have been transfected into each batch in order to exclude this possibility.

vi. Sixthly, human primary hepatocytes may translate replicon RNA, but the some of the luciferase enzyme may not be active (figure 5.11d and 5.11e). This could occur if the protein was not correctly folded to attain a functional tertiary structure. Such folding is mainly dependent on the amino acid sequence of a protein and there is ample evidence that the polypeptide develops activity in the Huh7 cells. However, the environment in which the protein is formed (and particularly whether it is associated with a mainly membranous or cytoplasmic environment) will also affect its ultimate conformation so it is possible that cellular factors could be responsible for a lack of function.

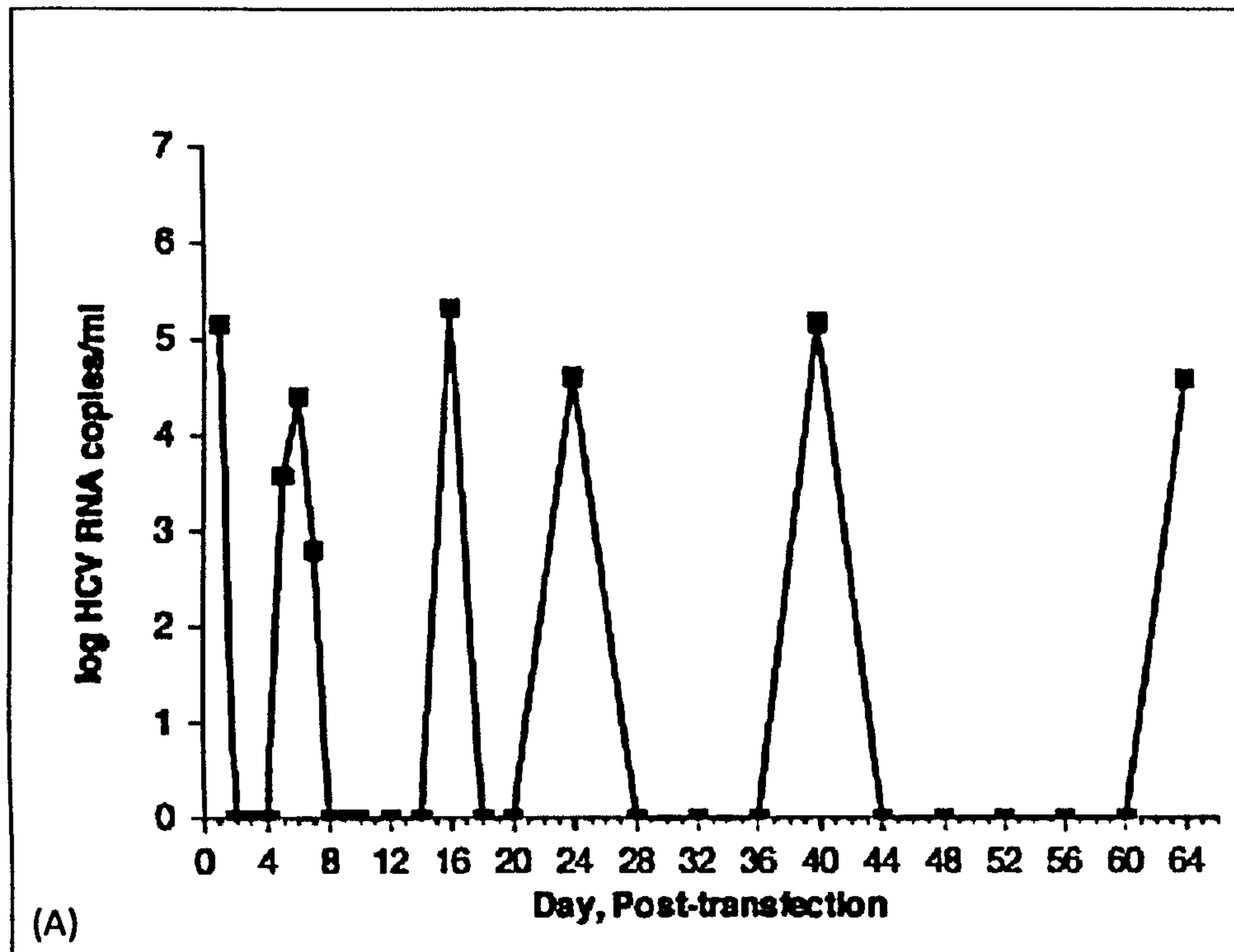
Alternatively, the luciferase enzyme may be functional, but simply rapidly degraded by primary hepatocytes (figure 5.11f). Interestingly, a study by Garmo *et al.* showed that, although luciferase was functionally expressed following cationic lipid-mediated transfection of a non-replicating construct into adherent rat hepatocytes, the levels of activity peaked between 3 and 24 hours and declined after 24-31 hours[130]. Unfortunately, the data set of that study was limited, and also complicated by the

fact that the dynamics of expression varied depending upon the time at which hepatocytes were transfected, post-isolation.

Ideally, in the present study, both hypotheses would have been disproven by additional experiments to transfect monocistronic luciferase-bearing control RNA (perhaps with the CMV-IE promoter, which had been shown to function during DNA transfection) into the primary hepatocytes. The accuracy of measurement of luciferase activity could also be improved by using a more sensitive luminometer.

vii. Lastly, it is possible that the kinetics of HCV replicon expression in primary hepatocytes simply differ from those seen in Huh7 cells. A study by Lazaro *et al.*[82], showed evidence that fetal hepatocytes transfected with RNA derived from an infectious genotype 1a replicon released HCV virions in a cyclical manner post-transfection (see figure 5.12). This variation was independent of the means of cell entry and it is possible that it resulted from fluctuating levels of replication and/or translation.

Notably, HCV virion production was undetectable at 48 hours, having been readily measurable at 24 hours. This is consistent with the results of the present study. Furthermore, this fluctuation was noted to occur with variable magnitude and timing between different experiments and hepatocyte batches and was even absent on some occasions[82]. It therefore seems likely that the host cell physiology strongly influences these fluctuations, although the underlying mechanisms involved and the basis of the fluctuations remain unclear. Thus it seems very likely that certain aspects of primary hepatocyte physiology, and their interactions with viral processes,



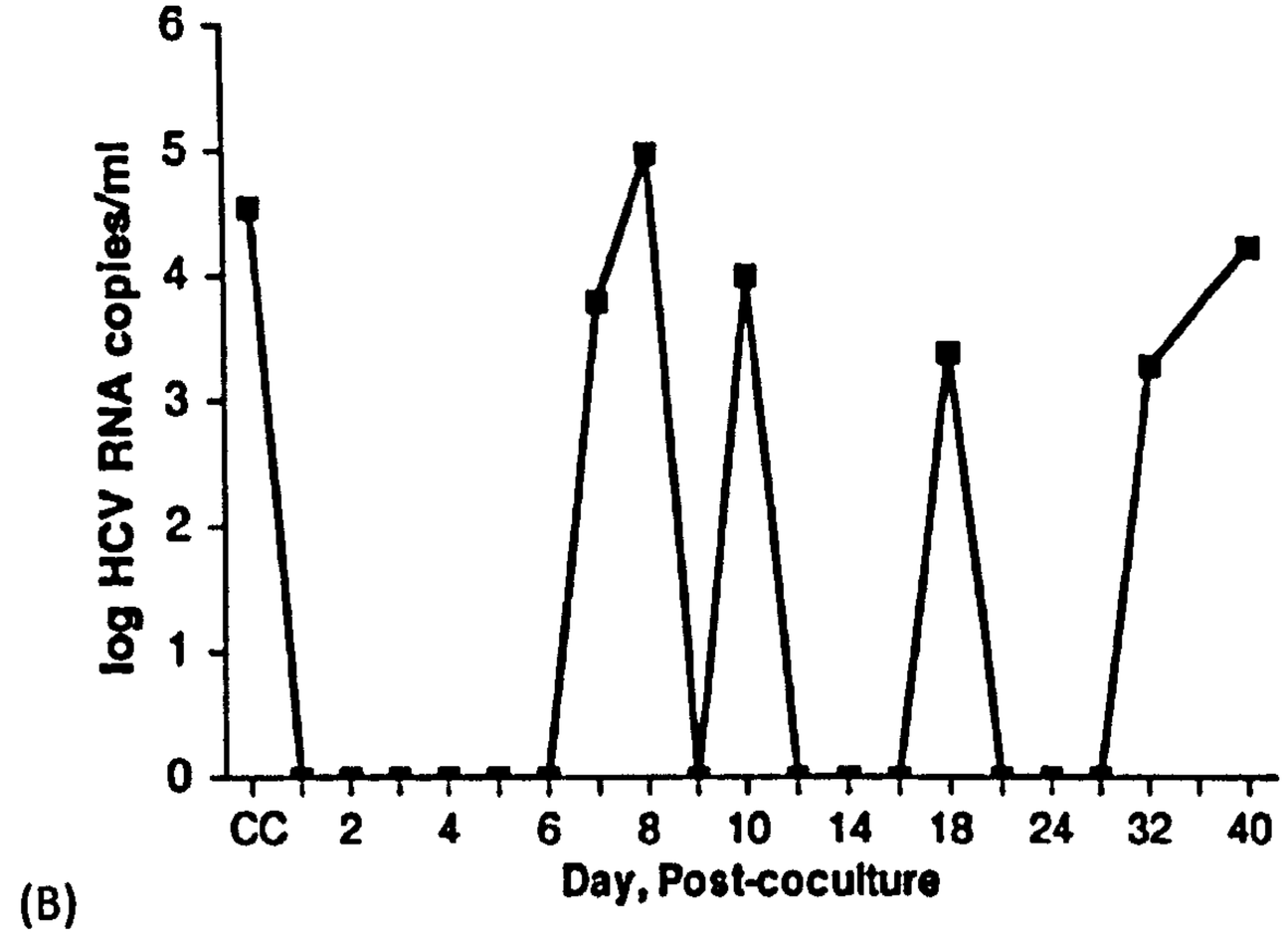


Figure 5.12: Fluctuating levels of HCV RNA (log10 scale on Y-axis) produced by fetal hepatocytes (A) transfected or (B) infected with an infectious genotype 1a replicon (from Lazaro et al.[82]).

could well be the reason for the differing levels of expression between hepatocyte batches, observed in the experiments described here.

An interesting observation, although it did not reach statistical significance in these results, was the apparent tendency for the replication-incompetent mutant to be expressed in primary hepatocytes (in mono- or co-culture), initially, at higher levels than the intact clone. A difference in baseline translational capacity or permissivity has not been reported in previous studies using the Huh7 cell line and, during the above experiments, there was no statistically significant difference between average baseline luciferase activities in primary hepatocytes following transfection with SGR-Luc-JFH1 or SGR-Luc-GND-JFH1 ($p = 0.409$). However, if there was a mechanism whereby dsRNA was subjected to an interferon- or apopotic- based response, it would be logical for cultures containing the replication-defective GND mutant, and therefore not producing dsRNA as a consequence of replication of the replicon, to demonstrate an advantage (over those transfected with SGR-Luc-JFH1) in their overall levels of replicon translation, while the baseline RNA levels remained similar. In this scenario, as or when an increase in RNA levels began to occur in cells transfected with replication-competent RNA, and cellular mechanisms began to remove RNA from the cytoplasm of all transfected hepatocytes, the temporary advantage of the replication-defective GND mutant would be quickly lost.

After 24 hours, some batches of primary hepatocytes clearly demonstrated increased expression of the replication-competent replicon, as compared with the replication-deficient mutant (and compared with the corresponding baseline levels of expression at 4 hours). Although experimental numbers in the present study precluded statistical analysis of the findings from "permissive" versus "non-permissive" batches,

it would be of great interest to carry out further experiments, with a greater number of different hepatocyte batches, and for durations of at least 10 days in both mono- and co-culture systems. Such experiments are essential in order to explore the consistency of results from "permissive" batches and record any reappearance of expression, as described by Lazaro *et al.* and shown in figure 5.12[82]. Simultaneous investigations could be made of the levels of viability, cell numbers and hepatocyte-specific function of these batches, over time, in order to obtain some basic comparative data from "permissive", versus "non-permissive", primary hepatocytes. Naturally, this would require the availability of large amounts of transfection reagents and liver samples and neither were available during the experimental period described here. Variation of permissivity for HCV replication is clearly seen in unselected (heterologous) Huh7 cell lines, as described in 5.1.2., but is somewhat complicated by the proliferation of the cell population. The relative lack of proliferation of primary hepatocytes offers a useful opportunity to explore the basis of permissivity for HCV replication further.

5.4.2.2. Translation and replication of JFH1 RNA within three-dimensional co-cultures of human primary hepatocytes

In a limited number of experiments, replicon-transfected human primary hepatocytes were grown in three-dimensional co-cultures with rat hepatic stellate cells (HSCs). It had previously been shown that HSCs actively organise hepatocytes into structured aggregates known as spheroids[72], in which a number of *in vivo*-like ultrastructural features are apparent, as described in Chapter 1. These spheroid co-cultures maintain higher levels of hepatocyte-specific function than monolayer cultures[70,72] and, furthermore, may be able to display cell-to-cell signalling and

responses, via the HSCs, including responses to infectious, inflammatory or fibrotic stimuli. As explained in Chapter 1, this model system therefore seems to offer an excellent experimental platform for investigating the requirements, and effects, of HCV replication, if it occurs in it. Furthermore, a previous study has shown that the efficiency of production of infectious HCV from Huh7 cells was enhanced when the cells were cultured in three-dimensional aggregates[162].

In the present studies, subgenomic RNA of JFH1 was transfected into primary hepatocytes which were subsequently co-cultured to form spheroids. The pattern of luciferase expression, reflecting translation and replication of the replicon, was similar to that observed in hepatocyte monocultures. Overall, there was no statistically significant difference between the average absolute levels of luciferase activity in monocultures or co-cultures at 4, 24 or 48 hours post-transfection. On average, however, co-cultures yielded a significantly greater increase in expression of the replication-competent RNA, compared to monocultures, at 24 hours post-transfection. Although these findings must be interpreted cautiously due to the very small numbers involved, they indicate either that some replication of JFH1 occurs in hepatocyte-HSC co-cultures, or that there was an excess of translation of luciferase over its degradation, during the first 24 hours. There are currently no published reports of replication or translation of either wild-type HCV or HCV replicons in hepatocyte-HSC co-cultures, therefore this result will be of immense interest if validated by future studies.

Furthermore, 100% (2/2) of the cell batches tested in co-culture displayed evidence of this transient increase in luciferase expression at 24 hours, whereas only 40% (2/5) of those batches tested in monoculture showed any such increase. Thus these small

numbers of experiments suggest that it is likely to be misleading to analyse the results from all hepatocyte batches together, as above. It is clear from the individual batch results in tables 5.2 and 5.3 that the hepatocytes from the first two batches (18 and 19) produced similar (declining) patterns of replicon expression with both SGR-Luc-JFH1 and its GND mutant; that is, there was no evidence of replication of the replicon, nor or of continued significant translation of the replicon, after 4 hours. In contrast, another two batches (20 and 26) of primary hepatocytes transfected with SGR-Luc-JFH1 and grown in 3D co-culture behaved quite differently, showing a considerable increase in luciferase activity between 4 and 24 hours post-transfection. Quite surprisingly, although the relative levels of luciferase activity were low, the luciferase doubling time (t_2) was broadly similar to that found for Huh7 cells. In the case of one batch (26), this increase was followed by a decline to below baseline after 48 hours (a corresponding measurement is not available for the other batch). This indicates that the replicon was being either replicated or translated (or both) within the cells in 3D co-culture for the first 24 h and that, thereafter, both the replicon and the luciferase were being eliminated.

Thus, the evidence suggests that, in certain primary hepatocyte populations, and for reasons that remain unclear, the replicon was eliminated and the luciferase was degraded almost immediately; whereas other batches showed some evidence of transiently increased translation and/or replication at 24 hours before a similar, rapid process of elimination or degradation ensued. This is an extremely interesting (if tentative) observation, and is consistent with the hypothesis, described in 5.4.2.1 and touched upon by Lazaro *et al.*[82], that permissiveness for expression and/or replication is strongly influenced by heterologous aspects of the host cell phenotype and are not consistent for all human primary hepatocytes.

The data from the present study provide conflicting evidence as to whether or not three-dimensional co-culture influenced the manifestation of this "phenotype" (in those cells where it appeared to be present). Essentially, one batch (21) produced inconsistent results between the hepatocytes in monoculture and those in three-dimensional co-culture; whereby the former demonstrated a decline in luciferase activity after 4 hours and the latter showed a transient increase at 24 hours. There was no such inconsistency between culture systems using batch 26. These apparently discrepant results raise further interesting questions regarding the physiological, host cell determinants of HCV translation and replication; but unfortunately, due to the limited amount of data, do little to allow such questions to be answered.

The potential inequalities in nutritional status between hepatocyte mono- and co-cultures, due to necessary differences in type and supply of cell culture media, further complicate the comparison of replicon expression between these two systems. If anything however, the hepatocytes in co-culture might have been expected to be less able to sustain metabolically-demanding procedures, such as translation and replicon processing, having had a reduced supply of both lipid and glucose post-transfection in comparison to monocultured cells and having been subject to nutrient competition from the HSCs. The fact that this hypothesis is *not* borne out by the relative measurements of replicon expression in each system thus provides a further, tentative, indication that co-culture with HSCs is advantageous in this context.

Interestingly, a very recently published study from Buck *et al.*[183] has reported on the infection of primary human hepatocytes, in a layered 3D co-culture with a mixture of

non-parenchymal liver cells, with serum-derived HCV. Their system appears to have produced more robust replication and infection than previous studies of primary hepatocytes in monoculture[75] and there was less variation between batches (33 of 36 were permissive to infection by high titres of serum-derived HCV). Unfortunately there was no comparison of the same batches in monoculture, to determine whether it was the culture conditions alone that increased the apparent permissiveness of the hepatocytes. It was also, perhaps, advantageous that these workers had the facility to use infectious virus and thus avoid the cellular damage caused by electroporation, although the use of serum-derived virus (with its numerous quasispecies) may be disadvantageous for future detailed analyses and comparison with the evidence provided by studies in cell-lines.

However, this encouraging evidence reasserts the need to gain more experimental data using the present experimental models to compare the permissiveness of cells from the same source patient and isolation procedure in monoculture and in co-culture. Although the current data set is too small for meaningful statistical analysis, it can be seen from tables 5.2 and 5.3 that 3D co-cultures produced higher (absolute) levels of luciferase expression than the 2D monoculture equivalents, from both intact replicon and the GND mutant, at baseline and (in 3 of 4 cases) after 24 hours. This is consistent with the finding, of Buck *et al.*, that three-dimensional culture enhances HCV virion production in primary hepatocytes and suggests that this may be due to increased levels of translation of HCV RNA. Subsequently, between 24 and 48 hours post-transfection, the rate of decline of luciferase activity in 3D co-cultures tended to be greater than that seen in 2D monocultures, suggesting that the former model may retain more effective anti-viral mechanisms than the latter.

Thus, taken together, these data (from both the present study and that of Buck *et al.*[183]) show that interrogation of the three-dimensional co-culture system may be extremely useful for determining the modulating factors of HCV expression in primary hepatocytes.

5.4.2.3. Summary of the expression and replication of the JFH1 clone within human primary hepatocytes

In summary, the subgenomic JFH1 replicon has been expressed, at low level, in human primary hepatocytes. Although there was evidence of *translation* of replicon at baseline and, in some hepatocyte batches, for 24 hours post-transfection, there was no statistical proof that *replication* occurred, consistently, within the first 48 hours of culture in the cells. Further experiments, incorporating additional controls, are required to determine the relative kinetics of RNA degradation, translation and replication, as well as the rate of luciferase clearance, in order to draw reliable conclusions. However, there was evidence of increased expression of the luciferase reporter gene in some individual batches of cells at 24 hours, suggesting either that some replication may have occurred, or that there was a temporary excess of translation over luciferase degradation. Thus there is evidence of major differences in permissivity for HCV translation and/or replication between (and perhaps, also, within) different batches of human primary hepatocytes. This is, potentially, a very important finding and further investigation of the factors which determine the permissivity (or otherwise) of different batches of cells is likely to be essential for understanding the *in vivo* pathogenesis of this virus.

Due to the limited opportunities to perform these experiments, only small numbers of data were obtained. It would be imperative to corroborate these findings by repeating these experiments, incorporating additional control steps to demonstrate that RNA, and the luciferase gene, can be efficiently transfected into and expressed by human primary hepatocytes under these culture conditions. It will be most important to perform more experiments with parallel monoculture and co-culture of transfected hepatocytes, in order to explore further any differences in the replicon's behaviour between these two models, and any variation in the differences from batch to batch of hepatocytes. Lastly, it would be highly desirable to transfect more cells on each occasion, if possible, in order to be able to follow the dynamics of replicon expression over a longer time period and thus determine whether expression is truly fully abrogated after around 48 hours or whether cyclical expression can be detected during more prolonged culture.

6. SUMMARY DISCUSSION

Infection with the hepatitis C virus is causing an increasing burden of hepatic disease, worldwide. Current drug treatments are prolonged, costly and often unsuccessful. As a relatively recently characterised virus, and one which is extremely difficult to study in non-human animals or cell-cultures, there are still many aspects of its pathogenesis which remain unclear. Newly developed replicating clones of HCV have now enabled more versatile and clinically relevant models of HCV infection to be developed. However, animal models remain scarce and/or imperfect, while almost all *in-vitro* research is performed using a neoplastic cell line. The opportunity to optimise two- and three-dimensional model systems, containing healthy, adult human primary liver cells, in which to investigate the replicative JFH1 clone of HCV, formed the basis of the experimental work described in this thesis.

There were a number of challenging aspects to this project. The relatively diverse nature of the aims necessitated synchronous development of several, very different, sub-projects. The methods for isolation of hepatocytes, and the protocols for their transfection, required considerable methodological development to ensure that the experimental procedures were sufficiently well-characterised and robust before they could be applied for use in pursuit of the overall aims of the project.

The rationale for, and experimental work leading to, a fully optimised protocol for isolating human primary hepatocytes under local conditions has been presented in chapter 3. This methodological development, although not broadly novel to the scientific community, was essential for almost all the subsequent work carried out during this project and presented in the later chapters.

The requirement for human liver tissue presented considerable practical difficulties. Although a number of (at least partial) methods for isolating hepatocytes from human liver tissue have been published, important details have often been omitted from such papers. In addition, the simple fact that the outcome of the procedure is highly dependent on the user, location and the tissue sample itself make local optimisation an essential and lengthy prerequisite for any subsequent experiments.

Obtaining source tissue samples was limited by the number of liver resection operations carried out, as described in chapter 3. Numerous factors led to the cancellation of operations (for example: patient fitness; availability of suitable post-operative care; or the arrival of more urgent cases). Once operations had begun, further problems often limited the chances of obtaining suitable tissue (for example: inoperability leading to termination of the procedure; unexpected distribution of tumour(s) within the resected specimen; destruction of the tissue (or its vasculature) by surgical techniques such as radio-ablation; or unintentional mishandling of the tissue by theatre staff). Thus, a total of only 27 specimens of tissue were obtained during this project, with which to optimise the methodology and investigate the research questions.

In addition to the sporadic, unpredictable and, above all, limited supply of tissue samples, the physiological variability of the specimens obtained meant that quantitative (and perhaps even qualitative) results were seldom truly comparable, despite every effort to minimise any avoidable variation. From a practical perspective, liver tissue was most usually conveyed to the laboratory after a very lengthy surgical procedure. This generally meant that although preparation for receiving the tissue would have started at the beginning of the day, the process of

extracting the hepatocytes by perfusion was usually begun late in the working day or evening. The entire procedure of perfusion and isolation took at least 3-4 hours before secondary experiments could begin. Media exchanges and/or first endpoint evaluations were usually required a further 2-4 hours later. Thus secondary experiments, to use the cells obtained from the tissue sample, were confined to an unpredictable and problematic schedule. This challenge was further exacerbated by the susceptibility of hepatocytes to rapid degeneration during any delay. In the absence of a highly effective means of cell or tissue preservation, these factors limited the opportunity for, and potential scope of, downstream experiments, despite the usually high total numbers of hepatocytes isolated when human tissue was available.

Both rat and human primary hepatocytes were successfully transfected *in vitro,* as described in chapter 4. Although transfection of rat hepatocytes using a cationic polymer resulted in only low level expression of a transfected plasmid, both rat and human hepatocytes were efficiently transfected by nucleofection. The validation of this second method presents numerous secondary opportunities to use human hepatocytes (whether in monoculture or co-culture) for a variety of future studies. It would be useful to perform prolonged studies of cell viability and differentiated hepatic function on transfected cells, in the future, to ensure that these characteristics remain maximised following nucleofection.

Nucleofection offered much greater efficacy and efficiency, in comparison to the cationic polymer, PEI, and also greatly reduced variation of those parameters between experiments, at least in rat hepatocytes. This is an extremely valuable advantage when using target cells which are subject to such unavoidably high levels

of biological variation at source. The ability to nucleofect cells immediately after isolation is helpful, as it enables transfected cells to be placed into co-culture with untransfected cells, such as HSCs. Although the nucleofection technique results in loss of viability in a significant proportion of cells, the fact that the cells remain in fluid suspension would also allow dead cells to be extracted prior to culture, by low-speed centrifugation, if required. Alternatively, there are now some different formulations of PEI which are conjugated with galactose, in order to improve specific interactions with hepatocytes via the asialoglycoprotein receptor[184,185], and thus have been reported to offer improved transfection efficacy in the primary hepatocytes of some species.

Finally, in chapter 5, the results of experiments to examine the capacity of the JFH1 clone to replicate within *in vitro* primary hepatocytes have been presented. The JFH1 replicon showed much lower levels of reporter gene expression in human primary hepatocytes, in comparison to Huh7 cells. This is to be expected, due to the physiological and physical differences between the cell types, and may suggest that a higher level of translational regulation and/or innate intra-cellular immunity persists in the primary cells.

Interestingly, there was a high level of variation, in both the amount and temporal profile, of reporter gene activity measured between different batches of hepatocytes, suggesting that the permissiveness of hepatocytes for expressing the replicon following electroporation is influenced by some physiological property (or properties) of the host cell. Further experiments to investigate the factor(s) influencing this variation will be extremely interesting and should examine possible correlations with the clinical and demographic details of the hepatocyte donors

(which was not possible during the present study and for which additional ethical approvals would be required), as well as assessments of the viability and function of the cells during culture. In addition, it may be interesting to examine whether the permissiveness of the hepatocyte batch correlates with the lipid content of the cells (both before and during 2D and 3D culture).

There was also evidence of a temporary rise in reporter gene expression, which may in turn indicate an increase in RNA translation (and possible replication), in certain batches of hepatocytes. This occurred both in monocultured hepatocytes and in those placed into three-dimensional co-culture with hepatic stellate cells. The latter model was more likely to show increased reporter gene expression at 24 hours, but also tended to show an increased reduction of expression at 48 hours. Due to the small number of experiments, it remains unclear whether co-culture may enhance the expression and/or replication of HCV in primary hepatocytes and this is potentially an extremely interesting area for further work. The possibility that three-dimensional co-culture might better preserve the intra-cellular anti-viral responses of hepatocytes is also an exciting, and important, area for future study.

Due to the limited availabilities of both human hepatocytes and transfection kits, at the time these experiments were carried out, sub-optimal numbers of experiments were performed and it would be desirable to carry out further work to validate these results. In particular, it would be important to repeat the experiments and incorporate two further controls. Firstly, an mRNA sequence bearing the luciferase marker gene only, in order to confirm that luciferase can be functionally expressed from a transfected RNA sequence in each batch of cells. Secondly, it would be helpful to transfect some cells in each batch with an mRNA sequence bearing the GFP

gene, in order to determine the numerical efficacy of RNA transfection for each batch of cells. Between them, these two additional controls would confirm that RNA can be successfully transfected into, and expressed by, each of the target cell batches. Lastly, the transfection of a luciferase-bearing DNA plasmid under the control of a switchable (e.g. *Tet*-on or *Tet*-off) promoter, would allow some measurement of the dynamics of luciferase degradation following translation and thus help to determine the relative contribution of synthesis and degradation to the overall levels of luciferase activity seen within each batch of hepatocytes.

Further work could then proceed to examine the expression of the JFH1 replicon in both mono- and co-cultured human primary hepatocytes. Based on the recent evidence published by Lazaro *et al.*[82], and given that human hepatocyte nucleofection kits are now freely commercially available, it would be prudent to extend the experimental period to examine the expression of the replicon over a period of at least 10 days. It would also be possible to transfect some cells with RNA generated from the pSGR-Luc-GFP-JFH1 plasmid, which encodes the fluorescent marker GFP in addition to the luciferase enzyme. Use of this plasmid would allow measured JFH1 expression (quantified by luciferase activity) to be normalised on the basis of overall transfection efficacy (quantified by GFP expression) for each batch, and thus allow easier and more robust analysis when summarising inter-experiment results.

The possibility that the dynamics of JFH1 survival and expression are different in co-cultured primary hepatocytes, as compared to mono-cultured cells, (suggested by the present study and by Buck *et al.*[183]) is intriguing. There are several possible reasons for such a discrepancy to occur. Firstly, it has been demonstrated previously

that hepatocytes which are maintained in organised three-dimensional spheroids by co-culture with HSCs retain higher and more durable levels of hepatocyte-specific physiological functions[70,72]. It would be logical to suppose that this relative increase in function, compared to two-dimensional mono-culture of primary hepatocytes from the same batch, extends to the overall translational capacity of the cells. Furthermore, it is possible that the HSC cell fraction, which develops an activated phenotype during *in vitro* expansion prior to co-culture, responds to the presence of newly isolated and transfected (and thus damaged) hepatocytes by creating a cytokine-led proliferative environment. A number of studies have shown that the expression of IRES-dependent genes is increased in dividing cells[173,186]; therefore proliferating hepatocytes would be expected to manifest a relative increase in transfected replicon expression. These hypotheses should be investigated in future studies to characterise the system.

Ideally, it might be possible to identify proliferating hepatocytes (for example, using the thymidine-analogue 5-bromo-2'-deoxyuridine (BrdU) and a fluorescent anti-BrdU antibody) and to distinguish the replicon-expressing cells (for example, using a replicon carrying the NS5A-GFP fusion protein, as shown in figure 5.2C). The occurrence and intensities of both markers could then be measured at various time points using fluorescence-assisted cell sorting, to differentiate between (and provide a relative measure of) replicon replication occurring without cell division and replication occurring as a consequence of cell division. If necessary, hepatocyte proliferation could be induced artificially, using hormonal additives such as hepatocyte growth factor in the culture medium, in order to facilitate this.

However, as HSCs have been shown to maintain hepatocyte-specific functions[70,72], it might also be expected that HSCs would maintain, and even stimulate, the intracellular interferon response in co-cultured hepatocytes, and thus result in faster clearance of replicon RNA and proteins (as *may* have happened in the present study). Closer examination of the differential dynamics of HCV replicon expression in the two culture models will therefore be of great interest, even if translation and/or replication is confirmed as not being sustained. RNA-interference studies, for example via co-transfection of the JFH1 replicons with anti-RIG1 or anti-IRF-3 RNAs, could elucidate the extent and level to which the interferon response occurs in each model. Future studies, perhaps using micro-arrays to identify any molecules associated with altered HSC-hepatocyte cell signalling during JFH1 expression, may then also be indicated.

Unfortunately, the lengthy and challenging nature of the methodological optimisation required during this work meant that it was not possible to achieve the final aim of the project (see section 1.4) within the time available. Although it was disappointing not to have been able to make any meaningful measurements of hepatocyte function and viability in the presence of JFH1, the development of both the mono- and co-culture systems for human primary hepatocytes has, at least, facilitated the conduct of such studies in the future.

It may also be noted, from chapter 1, that the ultimate aim of the project had been to use the full length, infectious JFH1 clone to infect primary human hepatocytes. Containment level 3 facilities would have been required for this work. Although a considerable amount of time was spent in preparing a containment level 3 facility and gaining the necessary approvals for this work, ultimately, full approvals from HSE

and the University Safety Committees were not confirmed in time to carry out meaningful numbers of replicated experiments with infectious virus. This was a disappointing, but ultimately unavoidable, outcome of many months (>18) of preparation. The Standard Operating Procedures and Risk Assessments developed as part of the current project and designed for use of the infectious clone are now in use by other researchers at the University and copies are appended in Appendix 8.3.

In summary, methods have been developed to allow HCV replicons to be transfected into primary human hepatocytes, for expression in two different culture systems. Both systems expressed the JFH1 replicon at much lower levels than Huh7 cells, as expected (discussed further in 5.4), but further experiments are required to determine whether human hepatocytes are truly less permissive for the clone; and, if so, why this might be. These studies have the potential to illuminate important mechanisms of innate resistance to HCV infection and how they may be modulated and, ultimately, enhanced. Furthermore, the viral mechanisms of immune evasion and extrinsic or intrinsic means for their down-regulation could also be investigated.

It is unlikely that this model can be used in place of Huh7 cell cultures for routine viral culture experiments. There is simply insufficient opportunity to obtain human liver tissue, and thence cells, for such volumes of work. It is also of note that the materials used (in particular, the commercial digestion buffer and nucleofection kits) are relatively expensive. However, there certainly appears to be sufficient evidence to justify the use of human primary hepatocytes for further investigation of JFH1 and other HCV clones. The hepatocyte mono-culture model will be useful for comparative work during characterisation of the co-culture model and may also be of use for the intermediate validation of results obtained through use of HCV replicons

and/or infectious clones in Huh7 cell monocultures. The hepatocyte-HSC co-culture model, which is functionally more representative of *in vivo* liver cells, is likely to offer a better platform for such validation in the future and also offers an important opportunity to investigate the interactions between HCV replication and innate immune and inflammatory responses. Although working with these models is challenging, they have enormous potential to provide unique insights into the pathogenesis of HCV.

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8. APPENDICES

8.1. Appendix 1 – Patient information sheet and consent form

The following pages show the patient information sheet and consent form for donation of liver samples to the present study.

Queen's Medical Centre Nottingham

University Hospital NHS Trust

PATIENT INFORMATION SHEET

Study Title: Enhancing the use of human liver cells within *in vitro* models of disease and drug metabolism.

Investigators: Mr I.J. Beckingham, Professor K. Shakesheff, Mr J.S. Hammond, Dr L.J. Dexter.

You are being asked to take part in a research study. Before you decide to contribute, it is important for you to understand:

1. Why the research is important?
2. What it will involve?
3. What are the benefits?

Please take time to read the following information and to discuss it with your surgeon before making a decision. If you are willing to take part, you will be asked to sign a consent form and given a copy to keep.

What is the purpose of this study?

The liver has many important functions within the body. Researchers use liver tissue to study these functions and to find better ways of treating disease.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

You are about to undergo surgery to remove a diseased segment of your liver. The gallbladder is also sometimes routinely removed during this operation. Once the surgery has taken place and the pathologist has examined the sample the left over tissue is usually destroyed.

What we are asking you to do is give your consent so that the remaining tissue can be used in research, and in doing so benefit patients of the future. The tissue would be treated as a gift; at no point will there be any financial benefit to you from its use.

It is important for you to understand your decision will not affect your treatment in anyway and that we do not remove any additional liver or gallbladder tissue for the research. In addition, none of the tissue used in the research will be used for the development of cell lines.

What do I have to do?

If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. The decision to take part in this research will not affect the surgery you are about to undergo.

What are the possible disadvantages and risks of taking part?

Taking part in the study will not affect the surgery you are about to undergo. The risks and benefits of liver surgery will have been discussed in detail with you in clinic prior to signing the consent form.

What are the possible benefits?

There will be no direct benefit to you, but taking part in this study means that you will help patients of the future, by aiding in the development of new treatments.

What if new Information becomes available?

The tissue that is removed will be utilised in research, but will not provide any additional information about your disease or its further treatment. You will not be informed of any tests performed on the tissue.

Will my taking part in this study be kept confidential?

All information resulting from you taking part in the study will be anonymous and known only to those conducting the research. The study records are entirely confidential and will not be available to anyone else. In all instances your confidentiality will be maintained in accordance with the 1998 Data Protection Act.

What will happen to the results of the research study?

The sample of tissue that you donate will be processed at the Queens Medical Centre and transported to The Department of Pharmaceutical Sciences, within the University of Nottingham and the University of Nottingham Medical School at Derby.

Results of the study will be published in a scientific journal. You will not be identified in any of these reports or publications.

Who is funding the research?

The research is being funded by the Engineering and Physical Sciences Research Council (EPSRC), the Fund for the Replacement of Animals in Medical Experiments (FRAME), the University of Nottingham and the Derby Hospitals NHS Foundation Trust.

Who has reviewed this study?

The study has been reviewed by the Nottingham Research Ethics Committee.

If you have any further questions about the study please contact Mr John Hammond, who can be accessed via Mr I.J. Beckingham's secretary (tel. 0115 9249924 ext. 36753).

Queen's Medical Centre Nottingham

University Hospital NHS Trust

Place patient label here

CONSENT FORM

Study Title: **Enhancing the use of human liver cells within in vitro models of diseases and drug metabolism.**

Please ask the patient to complete the following:

Have you read and understood the patient information sheet? ☐

Have you had an opportunity to ask questions and discuss the study? ☐

Have you received satisfactory answers to all your questions? ☐

Have you received enough information about the study? ☐

Do you understand that you are free to withdraw from the study

at any time? ☐

without giving a reason for withdrawing? ☐

without affecting your future medical care? ☐

I agree to take part in the study ☐

Patient name .....

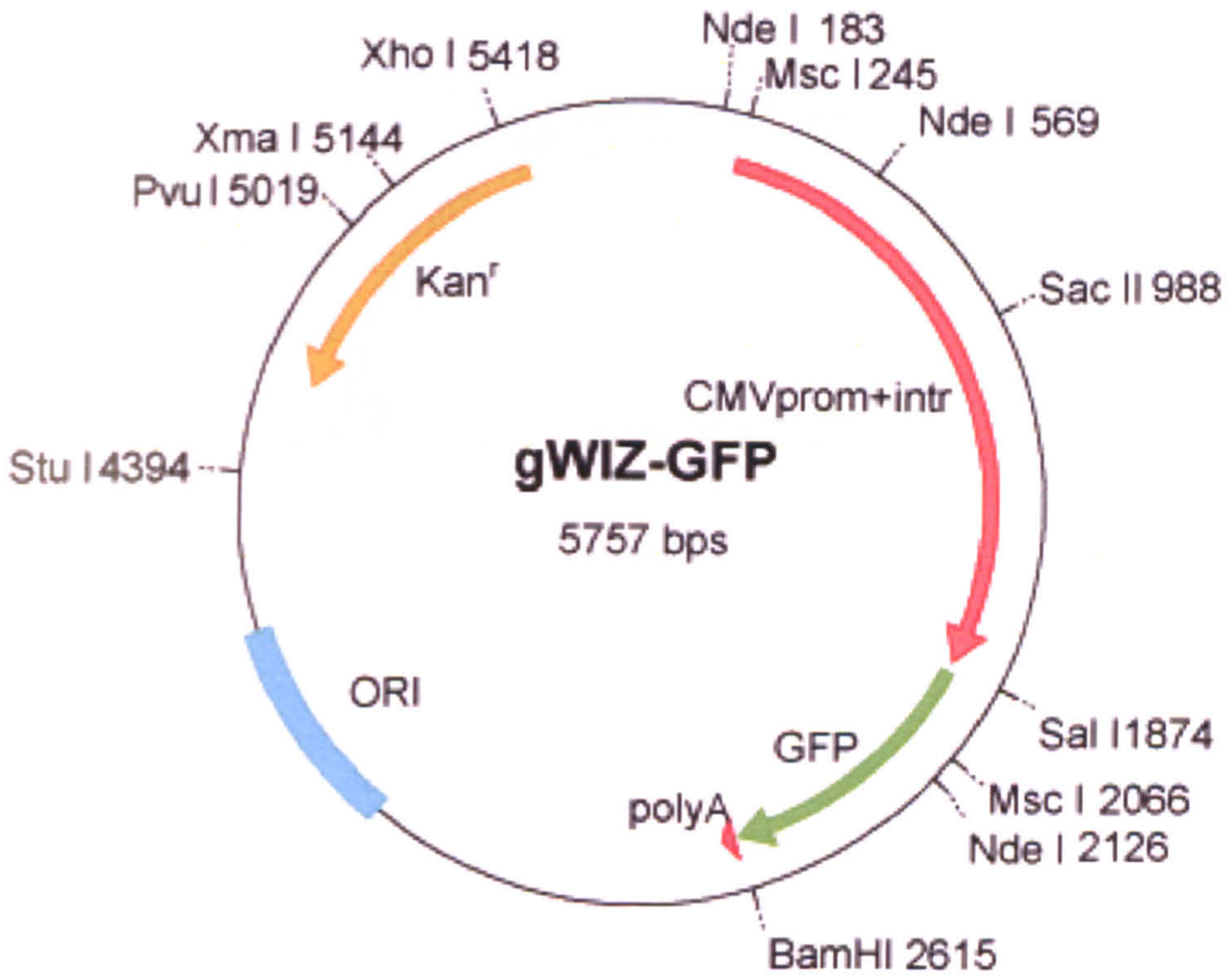
Signed ..... Date .....

Who explained this study to you? .....

Signed ..... Date .....

8.2 Appendix 2 – Plasmid maps and information

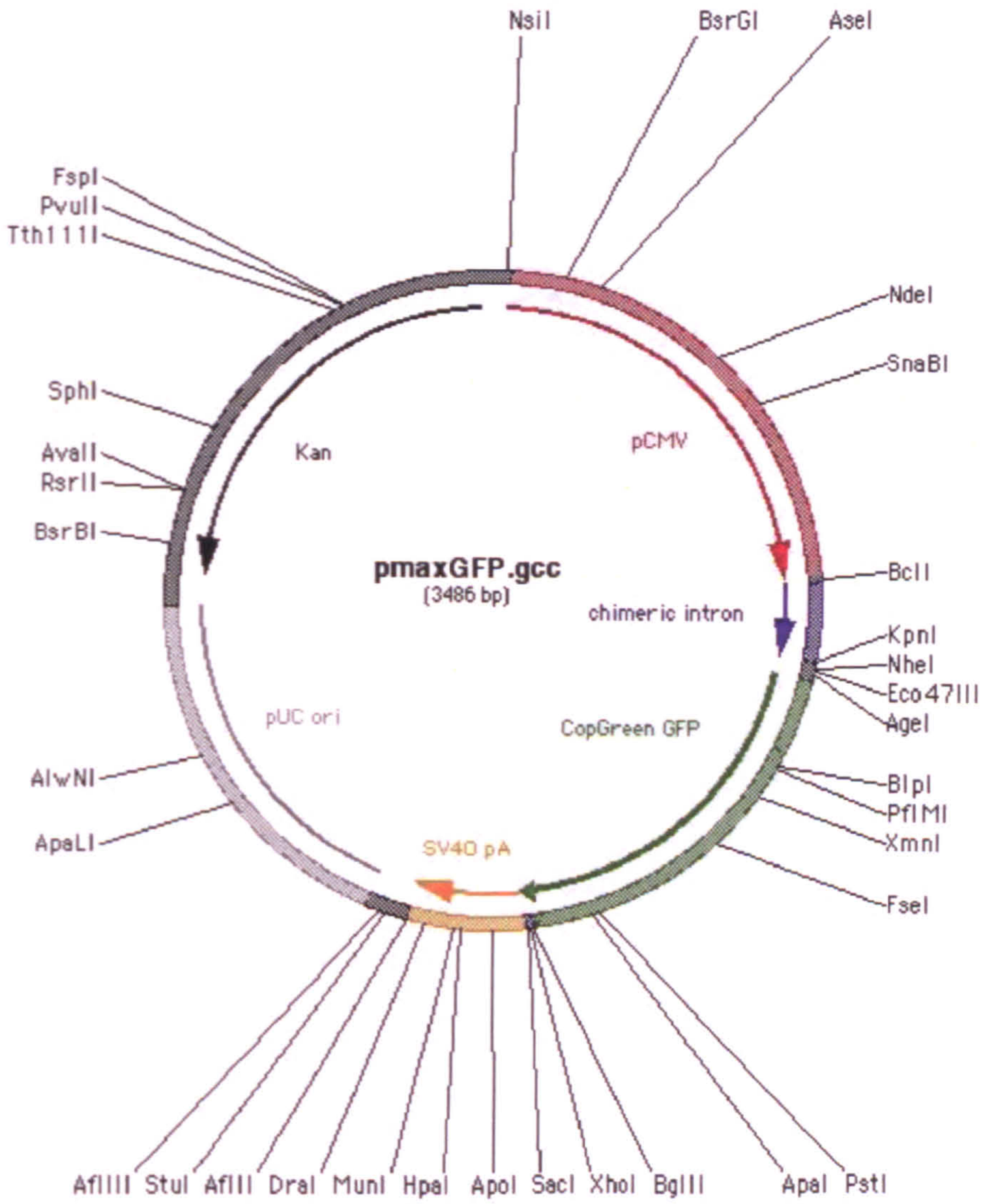
Plasmid map of gWizGFP, courtesy of Aldevron LLC:



pCMV + Intron	245-1864
GFP	1895-2614
PolyA	2626-2657
pUC Ori	
Kan Resistance	4637-5452

Sequence available at: http://www.aldevron.com/gWiz%20GFP.txt

Plasmid map of pMaxGFP, courtesy of Amaxa Biosystems:



Accessed from: http://digital.sabanciuniv.edu/tezler/etezfulltext/aksoylarhi.pdf

Plasmid maps of pSGR-Luc-JFH1 and pSGR-Luc-GND-JFH1 are unavailable.

Their construction is described by Targett-Adams *et al.*[166]

The sequence of the original JFH1 virus was described by Kato *et al.*[56]

Sequence data for the JFH1 clone is available via Genbank (accession no. AB047639).

8.3. Appendix 3 – Containment Level 3 Laboratory documents

The following pages contain the protocols and risk assessments for working with the full length JFH1 replicon, at containment level 3, which were designed and written as part of the work of the present study.

8.3.1. Huh7 nucleofection with full-length HCV replicons

Outline: This method allows transfection of full-length HCV replicons into Huh7 cells.

Method:

Preparation in CL3 suite (no risk of HCV infection):

- warm PBS, PBS 5% sucrose and Huh7 media
- aliquot media into culture plates and pre-warm in the incubator at 37°C
- label cuvettes as required
- transfer RNA aliquot to CL3 facility from -80°C freezer
- transfer Nucleofector device into MSC

Cell preparation in CL2 facility (no risk of HCV infection):

- trypsinise cells into a single cell suspension
- centrifuge required number of cells at 1300rpm (in D36)
- wash with PBS and centrifuge again
- transfer to CL3 suite

Nucleofection of Huh7 cells (potential very low risk of HCV infection after nucleofection):

- resuspend cells at 1m/100ul in PBS-sucrose
- transfer RNA to cuvettes and add 100ul cells
- nucleofect IMMEDIATELY on T14 and add IMMEDIATELY to prewarmed media in the plates
- distribute evenly and return to incubator at 37°C

Hazards:

Full-length replicons contain all the necessary genes to produce infectious HCV particles, once transfected into permissive cells such as Huh7s. Genome replication has only previously been reported from around 12hrs post-electroporation, and detectable levels of virus in cell culture media have only been reported from around 24hrs post-electroporation, thus the likelihood of encountering infectious virus during this procedure is very low. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell lines and cell culture media.

Risks of HCV infection:

Production of infectious virus is only possible after cells have been nucleofected. Particular care must therefore be taken when opening cuvettes and transferring the contents to culture plates. Any spillage of culture plate contents must be treated as category 3 risk. Risk of infection exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Good laboratory practice including use of PPE lowers the risk from such hazards. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Follow CL3 good laboratory practice, wear PPE and follow CL3 waste disposal routes to reduce risk.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Electroporation of full length JFH1 replicons into Huh7 cells

Brief Description: This method allows transfection of full-length HCV replicons, see attached protocol. Infectious virus is only known to be generated after minimum of 12hrs culture of transfected cells, therefore at the time of the procedure there is negligible risk of HCV infection.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV contained in cells or cell culture supernatant	2	1	2	4	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass.	2	1	1	2
Electrical hazard from nucleofection device if misused	3	1	1	3	Keep machine, cuvettes and environment clean and dry Do not introduce anything else into the cuvette holder Maintain yearly electrical testing	3	1	1	3

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, PBS or transfection buffer	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware and cuvettes	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes: Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

There is no electrical hazard from the nucleofection device through normal use. The electrical contacts are enclosed and a current will only be applied when the correct cuvette is detected. However, it is good practice to ensure that the device is subjected to regular electrical testing and is kept clean and dry.

Nucleofection buffers are physiological buffers of unknown (proprietary) composition, however no hazard is identified by the manufacturer.

8.3.2. Huh7 infection with HCV

Outline:

This method describes passage of infectious HCV derived from transfected cells by overlay infection onto naive Huh7 cultures. This may be useful to determine the tissue culture infectious dose 50% (TCID50) or to increase levels of excreted virus for further infections.

Method:

Preparation in CL3 suite (no risk of HCV infection if container remains sealed):

- pre-warm HCV-containing media to 37°C in water bath

Cell preparation in CL2 facility (no risk of HCV infection):

- transfer flasks of naive cells (with fresh media) to CL3 suite

Infection of Huh7 cells (risk of HCV infection in culture media):

- add appropriate amount of HCV-containing media to each flask
- distribute evenly and return to incubator at 37°C

Hazards:

After passage in Huh7 cells, levels of infectious HCV in the cell culture media may be high (at least equivalent to those found in vivo). Care must therefore be taken in handling and storing this media and any spillage of culture plate contents must be treated as category 3 risk. . Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell lines and cell culture media.

Risk of HCV infection:

Risk of infection from cells or culture media exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Good laboratory practice including use of PPE lowers the risk from such hazards. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite should be followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Huh7 infection with HCV

Brief Description: This method describes passage of infectious JFH1 HCV derived from transfected cells by overlay infection onto naive Huh7 cultures. This may be useful to determine the TCID50 or to increase levels of excreted virus for further infections. Infectious HCV virus may be present in cells and cell culture supernatants.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass.	2	1	1	2

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, PBS	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes: Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

8.3.3. Human primary hepatocyte nucleofection with full-length HCV replicons

Outline:

This method allows transfection of full-length HCV replicons into human primary hepatocytes.

Method:

Preparation in CL3 suite (no risk from organism):

- warm nucleofection buffer and NF media
- aliquot media into culture plates and pre-warm in the incubator at 37°C
- label cuvettes as required
- transfer RNA aliquot to CL3 facility from -80°C freezer
- transfer Nucleofector device into MSC

Cell preparation in CL2 facility (possible risk of blood-borne virus infection from unscreened donors):

- prepare a single cell suspension of human primary hepatocytes (separate protocol and risk assessment exists)
- centrifuge required number of cells at 50g and 4°C (D16)
- transfer to CL3 suite

Nucleofection of Huh7 cells (additional potential low risk of HCV infection after nucleofection):

- resuspend cells at 1m/100ul in NF buffer
- transfer RNA to cuvettes and add 100ul cells to each
- nucleofect IMMEDIATELY on Q25 (rat) or M23 (human)
- after 15mins incubation, add to prewarmed media in the plates
- distribute evenly and return to incubator at 37°C

Hazards:

Full-length replicons contain all the necessary genes to produce infectious HCV particles, once transfected into permissive cells such as Huh7s. In Huh7 cells, genome replication has only been reported from around 12hrs post-electroporation, and detectable levels of virus in cell culture media have only been reported from around 24hrs post-electroporation, thus the likelihood of encountering infectious virus during this procedure is very low. No data is yet available for the dynamics of replication and/or virus production in human primary hepatocytes. There is also a possible risk of other blood-borne virus infections from primary hepatocytes as the donors are not screened. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell culture media.

Risk of HCV infection:

Production of infectious virus is only possible after cells have been nucleofected. Particular care must therefore be taken when opening cuvettes and transferring the contents to culture plates and any spillage of culture plate contents must be treated as category 3 risk.

Risk of infection exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Good laboratory practice including use of PPE lowers the risk from such hazards. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite should be followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Electroporation of full length JFH1 replicon into human primary hepatocytes

Brief Description: This method allows transfection of full-length HCV replicons, see attached protocol. Infectious virus is only known to be generated after minimum of 12hrs culture of transfected cells, therefore at the time of the procedure there is negligible risk of HCV infection.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass.	2	1	1	2
Electrical hazard from nucleofection device if misused	3	1	1	3	Keep machine, cuvettes and environment clean and dry Do not introduce anything else into the cuvette holder Maintain yearly electrical testing	3	1	1	3

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, PBS or transfection buffer	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware and cuvettes	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

There is no electrical hazard from the nucleofection device through normal use. The electrical contacts are enclosed and a current will only be applied when the correct cuvette is detected. However, it is good practice to ensure that the device is subjected to regular electrical testing and is kept clean and dry.

Nucleofection buffers are physiological buffers of unknown (proprietary) composition; however no hazard is identified by the manufacturer.

All workers should attend Occupational Health prior to starting work with HCV or human primary cells.

8.3.4. Human primary hepatocyte infection with HCV

Outline:

This method describes infection of human primary hepatocytes with infectious HCV derived from transfected Huh7 cells.

Method 1 – infection of cells in suspension at T0:

Preparation in CL3 suite (risk from organism in culture media):

- aliquot HCV-containing media into culture plates and pre-warm in the incubator at 37°C

Cell preparation in CL2 facility (possible risk of blood-borne virus infection from unscreened donors):

- prepare single cell suspension, in hepatocyte plating media, of human primary hepatocytes (separate protocol and risk assessment exists)
- transfer to CL3 suite

Infection of primary hepatocytes in suspension (additional risk of HCV infection from culture media):

- add appropriate amount of cell suspension to each culture well
- distribute evenly and return to incubator at 37°C

Method 2 – infection of cells in culture at D1:

Preparation in CL3 suite (no risk from organism):

- aliquot media into culture plates and pre-warm in the incubator at 37°C

Cell preparation in CL2 facility (possible risk of blood-borne virus infection from unscreened donors):

- prepare single cell suspension(s) of human primary hepatocytes (separate protocol and risk assessment exists)
- add appropriate amount of cell suspension to each culture well
- distribute evenly and return to incubator at 37°C

Infection of cultures at D1 (additional risk of HCV infection from culture media):

- add HCV-containing media in appropriate amounts to each culture well
- return to incubator at 37°C

Hazards:

After passage in Huh7 cells, levels of infectious virus in the cell culture media may be high (at least equivalent to those found in vivo). Care must therefore be taken in handling and storing this media and any spillage of culture plate contents must be treated as category 3 risk.

There is also a possible risk of other blood-borne virus infections from primary hepatocytes as the donors are not screened. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell culture media.

Risk of HCV infection:

Risk of infection from cells or culture media exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Good laboratory practice including use of PPE lowers the risk from such hazards. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite should be followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Human primary hepatocyte infection with infectious HCV

Brief Description: This method describes passage of infectious JFH1 HCV derived from transfected Huh7 cells by overlay infection onto naïve primary hepatocyte cultures. Infectious HCV virus may be present in cells and cell culture supernatants.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass.	2	1	1	2

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, PBS or transfection buffer	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used cell culture plates or dishes	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

All workers should attend Occupational Health prior to starting work with HCV or human primary cells.

8.3.5. Maintaining cell cultures containing full-length HCV

Outline:
This method describes maintenance of cell cultures which have been exposed to full-length HCV constructs and therefore may contain or produce infectious HCV.

Method:
Cultures must be moved to and from the incubator and MSC or microscope on a non-porous tray. They must be properly covered.

To change media (RISK: cells and cell culture media may contain infectious HCV virus and, in the case of primary cells, other blood-borne viruses from unscreened donors):

- Pre-warm required media in water bath
- Assemble all required pipettes, containers and reagents into MSC
- Remove cultures to the MSC
- Aspirate media into Trigene to final concentration of 5%. Alternatively, if required, remove by pipette for storage (store in double-sealed container and swab exterior with a Trigene wipe prior to removal from the MSC) for assay later.
- Add fresh media to the cultures
- Return cultures to incubator

Hazards:
Levels of infectious virus in the cell culture media have the potential to be high (at least equivalent to those found *in vivo*). Care must therefore be taken in handling and storing this media and any spillage of culture plate contents must be treated as category 3 risk. There is also a possible risk of other blood-borne virus infections from primary hepatocytes as the donors are not screened. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell lines and cell culture media.

Risk of HCV infection:
Any spillage of culture plate contents must be treated as category 3 risk. Risk of infection from cells or culture media exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Good laboratory practice including use of PPE lowers the risk from such hazards. See risk assessment form for assessment of other hazards

Controls:
Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite should be followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Maintaining cultures containing full-length HCV

Brief Description: This method describes routine care of cultures which have been exposed to the full length JFH1 replicon. Infectious HCV virus may be present in cells and cell culture supernatants.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass.	2	1	1	2

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media and cell washings	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used cell culture plates or dishes, pipettes	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:
Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.
All workers should attend Occupational Health prior to starting work with HCV or human primary cells.

8.3.6. Luciferase activity measurement

Outline:
This method describes lysis of cultures to liberate luciferase enzyme in order to quantify HCV replication. However, this method is used only with cells containing the sub-genomic JFH1 replicon, therefore infectious HCV is not present at any stage.

Materials: Promega Luciferase assay kit E1500
Keep buffer at -20°C
Keep made-up substrate at -70°C

Lysis method for monolayer cultures:

- Make sufficient 1x lysis buffer (dilute 5x buffer with H_2O)
- Aspirate medium
- Wash cell layer with PBS
- Add 100µl 1x lysis buffer
- Scrape cells into eppendorf with pipette
- Spin at 13K for 1min (cell extract is reasonably stable once separated)

Lysis method for spheroid co-cultures:

- Make sufficient 1x lysis buffer (dilute 5x buffer with H_2O)
- Aspirate media and loose cell aggregates from the tissue culture wells and centrifuge at 250*g*.
- Aspirate the supernatant and retain cell pellet.
- Add working strength lysis buffer to the drained tissue culture plates (2 ml/well) and scrape into tube containing cell pellet. Pipette to mix.
- Centrifuge the suspensions at 250g to precipitate the cell fraction from the lysis solution (containing cell proteins).
- Swab the tubes with trigene wipes prior to removal from the CL3 suite
- cell lysates can be stored at -80°C for batch testing if required

Reading luciferase activity using the Turner TD-20e luminometer (D36)

- use scintillation vial inserts and caps from Hughes and Hughes (order no. 1116)
- turn on 120s before to allow bulb to warm
- integrate should be set at 10s
- delay should be set at 5s
- add 40µl cell extract to 100µl luciferase reagent and read immediately **one at a time!**
- stand vial in chamber, press start and wait until display reads "F"
- measurement is in arbitrary light units, display shows position of decimal point (dilute if necessary)

Hazards:

Cells for luciferase assay will not have been transfected or infected with full-length JFH1 therefore there is no risk that these culture may contain infectious HCV particles. Skin, eyes or mucous membrane may be irritated or damaged by lysis buffer concentrate (strong detergent). Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. There is also a possible risk of other blood-borne virus infections from primary hepatocytes as the donors are not screened.

Risks of HCV infection:

None. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite should be followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Luciferase activity measurement

Brief Description: This method describes lysis of cultures to liberate luciferase enzyme in order to quantify HCV replication. However, this method is used only with cells containing the sub-genomic JFH1 replicon, therefore infectious HCV is not present at any stage.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Skin, eyes or mucous membrane irritation or damage by lysis buffer concentrate (strong detergent)	1	2	2	4	PPE to be worn at all times	1	1	1	1
For primary cells, possible infection with blood-borne viruses (from unscreened donors) contained in cells or cell culture supernatant	2	1	2	4	Wear PPE. Use MSC. No use of sharps or glass.	2	1	1	2

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, PBS or lysis buffer	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

Eppendorfs containing lysate are removed from the suite for luminometric assay – swab with Trigene wipe and log in lobby book as per CL3 local rules.

All workers should attend Occupational Health prior to starting work with human primary cells.

8.3.7. Lysis of cells for quantification of viral RNA

Outline:
This method may be used for cells infected or transfected with full-length replicon or intact HCV virus. Cells and viruses are disrupted by a chaotropic agent, releasing naked RNA. Since RNA alone does not present an infectious risk, the lysate can be then be removed from the containment level 3 suite for storage (-70°C) or further processing, e.g. PCR.

Materials: Qiagen RNeasy Mini kit (or similar)

Method:

Notes and preparation:

- Up to $7x10^6$ cells can be processed per sample, in up to a 60mm diameter dish
- Ensure all reagents are at room temperature

The procedure should be carried out as quickly as possible. All steps should be carried out within the MSC. According to the kit manufacturer, no intact virus should be present after lysis, however for speed of preparation homogenisation should also be carried out in the MSC

To lyse cells (RISK: cells and cell culture media may contain infectious HCV virus and, in the case of primary cells, other blood-borne viruses from unscreened donors):

- Completely aspirate the cell culture medium
- Disrupt the cells by adding 350µl of buffer RLT
- Pipette the lysate into a microcentrifuge tube and vortex or pipette to mix

To homogenise the lysate (very low risk from organism:)

- Pass the lysate at least 5 times through a blunt 20G needle fitted to an RNase-free syringe

Once homogenised, to reduce viscosity and disrupt high molecular weight components such as genomic DNA, lysates can be stored at -70°C for several months.

To store lysate (no risk from organism):

- Swab exterior surfaces of microcentrifuge tube(s) with trigene wipe
- Change gloves and move samples out of MSC into -20°C freezer
- At a convenient point, leave the CL3 suite and move the samples to the -80°C freezer.

Hazards:
Using Huh7 cells, detectable levels of virus in cell culture media have only been reported from around 24hrs post-electroporation. However, after longer passage time, levels of infectious virus in the cell culture media may be high (at least equivalent to those found *in vivo*).

No data is yet available for the dynamics of replication and/or virus production in human primary hepatocytes. Care must therefore be taken in handling and storing this media and any spillage of culture plate contents must be treated as category 3 risk. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell lines and cell culture media. The lysis buffer contains a chaotropic agent and may cause irritation or damage to skin or mucous membranes if prolonged contact occurs.

Risks:

Any spillage of culture plate contents prior to the completion of lysis must be treated as category 3 risk. Risk of infection exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Chaotropic treatment is stated by the manufacturer to destroy infectious particles. Whether any live virus remains cannot be determined, however the titres can be assumed to then be very low. Further processing of extracts may therefore be carried out at containment level 2. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite lower the above risks if correctly followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Lysis of cells for quantification of viral RNA

Brief Description: Cells and viruses are disrupted by a chaotropic agent, releasing naked RNA. Chaotropic treatment is stated by the manufacturer to destroy infectious particles. Whether any live virus remains cannot be determined, however the titres can be assumed to then be very low. The lysate can be then be removed from the containment level 3 suite for storage (-70°C) or further processing, e.g. PCR. at containment level 2

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass. The microfuge must be placed and opened within the MSC, where used.	2	1	1	2
Skin, eye or mucous membrane irritation or damage by lysis buffer RLT (chaotropic agent)	1	2	2	4	Wear PPE Follow GLP	1	1	1	1

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, excess lysis buffer and cell washings	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

All workers should attend Occupational Health prior to starting work with HCV or human primary cells.

8.3.8. Lysis of cell-free culture media for quantification of viral RNA

Outline:
This method may be used for culture media from cells infected or transfected with full-length replicon or intact HCV virus. Virus is disrupted by a chaotropic agent, releasing naked RNA. Since RNA alone does not present an infectious risk, the lysate can be then be removed from the containment level 3 suite for storage (-70°C) or further processing, e.g. PCR.

Materials: Qiagen Viral RNA Mini kit (or similar)

Method:

Notes and preparation:

- Move the microcentrifuge into the MSC
- Ensure all samples are at room temperature
- Make fresh working AVL buffer solution (n x 0.56 = ml of buffer AVL required; add 10µl aliquoted carrier RNA per ml to obtain working buffer). Gently mix by inversion
- For each sample, prepare 560µl of working AVL buffer in a 1.5 ml microcentrifuge tube

The procedure should be carried out as quickly as possible. All steps should be carried out within the MSC. According to the kit manufacturer, no intact virus should be present after lysis, however for speed of preparation homogenisation should also be carried out in the MSC.

To lyse virus in media (RISK: cells and cell culture media may contain infectious HCV virus and, in the case of primary cells, other blood-borne viruses from unscreened donors):

- Completely aspirate the cell culture medium into a 1.5 ml microcentrifuge tube
- Centrifuge at 1500*g* to remove any cellular debris
- Add 140ul of sample supernatant to the working AVL mixture
- Vortex for 15s and incubate at room temperature for 10 minutes.

Viral particle lysis is complete after lysis for 10mins at room temperature and potentially infectious agents are inactivated. The lysate now presents negligible risk of infection and can be stored at -70°C for several months.

To store lysate:

- Swab exterior surfaces of microcentrifuge tube(s) with trigene wipe
- Change gloves and move samples out of MSC into -20°C freezer
- At a convenient point, leave the CL3 suite and move the samples to the -80°C freezer.

Hazards:
Full-length replicons contain all the necessary genes to produce infectious HCV particles, once transfected into permissive cells.

Using Huh7 cells, detectable levels of virus in cell culture media have only been reported from around 24hrs post-electroporation. However, after longer passage time, levels of infectious virus in the cell culture media may be high (at least equivalent to those found *in vivo*). No data is yet available for the dynamics of replication and/or virus production in human primary hepatocytes. Care must therefore be taken in handling and storing this media and any spillage of culture plate contents must be treated as category 3 risk.
Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. The lysis buffer contains a chaotropic agent and may cause irritation or damage to skin or mucous membranes if prolonged contact occurs. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell culture media.

Risks:
Any spillage of culture plate contents prior to the completion of lysis must be treated as category 3 risk. Risk of infection exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Chaotropic treatment is stated by the manufacturer to destroy infectious particles. Whether any live virus remains cannot be determined, however the titres can be assumed to then be very low. Further processing of extracts may therefore be carried out at containment level 2. See risk assessment form for assessment of other hazards

Controls:
Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite lower the above risks if correctly followed.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Lysis of cell-free culture media for quantification of viral RNA

Brief Description: Viruses present in the cell culture media are disrupted by a chaotropic agent, releasing naked RNA. Chaotropic treatment is stated by the manufacturer to destroy infectious particles. Whether any live virus remains cannot be determined, however the titres can be assumed to then be very low. Since RNA alone does not present an infectious risk, the lysate can be then be removed from the containment level 3 suite for storage (-70°C) or further processing, e.g. PCR at containment level 2.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass. The microfuge must be placed and opened within the MSC, where used.	2	1	1	2
Skin, eye or mucous membrane damage by lysis buffer AVL (chaotropic agent)	1	2	2	4	Wear PPE Follow GLP	1	1	1	1

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, excess lysis buffer and cell washings	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell culture media. Good laboratory practice including use of PPE lowers the risk from such hazards.

All workers should attend Occupational Health prior to starting work with HCV or human primary cells.

8.3.9. Disaggregating co-culture spheroids

Outline:

This method describes disaggregation of co-cultured human primary hepatocytes and stellate cells in the CL3 suite.

Method:

- Aspirate media and loose cell aggregates from the tissue culture wells and centrifuge at 250*g*.
- Aspirate the supernatant and retain cell pellet.
- Add pre-warmed Accutase to the drained tissue culture plates (2 ml/well) and incubate at 37°C for 5 minutes.
- After this time, scrape wells to remove cell deposits and add whole suspension back to the retained cell pellets obtained from the centrifugation stage.
- Incubate Accutase-treated cells at 37°C for a further 5 minutes.
- Agitate the cell suspensions by gentle pipetting at approximately 2 minute intervals during this period.
- Centrifuge the suspensions at 250g to precipitate the cell fraction from the enzyme solution.
- Resuspend the cell pellets as required for further assay

Hazards:

If cells have previously been transfected with full-length JFH1 replicon, or infected with replicon-derived virus, infectious virus may be present. Care must therefore be taken in handling and storing these cultures and media and any spillage of culture plate contents must be treated as category 3 risk. There is also a possible risk of other blood-borne virus infections from primary hepatocytes as the donors are not screened. Accutase enzyme may cause irritation to eyes, skin or mucous membranes if prolonged contact occurs. Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cell lines and cell culture media.

Risk of HCV infection:

Risk of infection from cells or culture media exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. Good laboratory practice including use of PPE lowers the risk from such hazards. See risk assessment form for assessment of other hazards

Controls:

Use of sharp implements is prohibited within the CL3 suite. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite must be followed. The centrifuge must be moved into the MSC for this experiment and swabbed with Trigene wipes prior to removal once complete.

Name of Assessor: Laura Dexter Group: Tissue Engineering Experiment Location: CL3 suite	Date of Assessment: 22/12/06 Date for Re-Assessment: 22/12/07

Protocol Title: Disaggregating co-culture spheroids

Brief Description: This method describes disaggregation of co-cultures in the CL3 suite. If cells have previously been transfected or infected with full-length JFH1 replicon infectious HCV virus may be present in cells and cell culture supernatants.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass. The microfuge must be placed and opened within the MSC, where used.	2	1	1	2
Possible irritation to eyes, skin or mucous membranes from Accutase enzyme if prolonged contact occurs	1	2	2	4	Wear PPE Follow GLP	1	1	1	1

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media, enzyme solutions and cell washings	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used plastic-ware	Double contained, sealed and autoclaved through CL3 waste stream.

Level of Supervision: None

Notes:

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells and cell culture media. Good laboratory practice including use of PPE renders the risk from such hazards negligible.

All workers should attend Occupational Health prior to starting work with HCV or human primary cells.

8.3.10. NS5A staining of cultures

Outline:

This method describes quantification of infected/transfected cells by antibody staining of the HCV non-structural protein 5a. This is translated from both the full-length and sub-genomic replicons.

Method:

Cultures (grown on plastic coverslips within culture wells) must be moved to and from the incubator and MSC or microscope on a non-porous tray. They must be properly covered.

- Aspirate media from cultures (*risk from organism at this stage*)
- Fix in 4% paraformaldehyde for at least 20mins at 4°C
- Wash x3 in PBS 1% FCS and block for 20mins with PBS 10% FCS
- Make primary antibody solution: sheep anti-NS5a 1/5000 in PBS 1% FCS
- Wash x3 in PBS 1% FCS and add 200µl working strength primary antibody per well for 2hrs at RT
- Make secondary antibody solution: donkey anti-sheep-TRITC 1/100 in PBS 1% FCS - *protect from light!*
- Wash x3 in PBS 1% FCS and add 200µl working strength secondary antibody per well for 1hr at RT - *protect from light!*
- Wash x3 in PBS
- Wash x1 in H_2O
- Mount coverslips with Citifluor and seal with nail polish - *protect from light!*

Once sealed, swab slides with trigene wipes prior to removal from the suite.

Hazards:

Full-length replicons contain all the necessary genes to produce infectious HCV particles, once transfected into permissive cells such as Huh7s. Genome replication has only been reported from around 12hrs post-electroporation, and detectable levels of virus in cell culture media have only been reported from around 24hrs post-electroporation. After this time, levels of infectious virus in the cell culture media may be high (at least equivalent to those found *in vivo*). Care must therefore be taken in handling this media. However, once fixed, cultures are considered to be hazard group 2.

Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells, cell culture media and serum (FCS). There is also a possible risk of other blood-borne virus infections from primary hepatocytes as the donors are not screened.

Risks of HCV infection:

Any spillage of culture plate contents must be treated as category 3 risk. Risk of infection exists mainly via percutaneous inoculation, but infection via eyes or mucous membranes can occur. See separate risk assessment for details of other risks.

Controls:

Use of sharp implements is prohibited within the CL3 suite. Glass slides must not be used. Normal rules of good laboratory practice, PPE and waste disposal within the CL3 suite lower the above risks if correctly followed.

Name of Assessor: Laura Dexter	Date of Assessment: 22/12/06
Group: Tissue Engineering	Date for Re-Assessment: 22/12/07
Experiment Location: CL3 suite	

Protocol Title: NS5A staining in CL3 suite

Brief Description: This method describes quantification of infected/transfected cells by antibody staining of the HCV non-structural protein 5a. This is translated from both the full-length and sub-genomic replicons. Infectious HCV virus may be present in cells and cell culture media. After fixation in paraformaldehyde, cultures are considered to be hazard group 2: however the entire procedure will be carried out within the MSC where possible. Once sealed, slides are swabbed with trigene wipes prior to removal from the suite. Glass slides/coverslips must not be used.

Hazard Assessment

Hazard	Matrix Value				Precaution	Matrix Value			
	H	R	C	RS		H	R	C	RS
Infection with HCV (and, in the case of primary cells, other blood-borne viruses from unscreened donors) contained in cells or cell culture supernatant	2	2	2	8	Work at CL3. Wear CL3 PPE. Use MSC. No use of sharps or glass.	2	1	1	2
4% paraformaldehyde solution is toxic, corrosive and a possible carcinogen.	2	2	2	8	Wear PPE Ensure good ventilation (use MSC)	2	1	1	2

Disposal Procedures

Chemical/Buffer/Reagent	Disposal Route
Cell culture media and cell washings	Aspirated into Trigene™ to final concentration of 5%. Leave overnight for disinfection before pouring down the sink in CL3 suite with copious amounts of water.
Used cell culture plates or dishes, coverslips, slides, pipettes etc.	Double contained, sealed and autoclaved through CL3 waste stream.
Used and surplus 4% paraformaldehyde solution	Double contained and disposed of through toxic waste route.

Level of Supervision: None

Notes: Note that there is always a possible low-level biological hazard (e.g. from unidentified viruses) when working with cells, cell culture media and serum. Good laboratory practice including use of PPE lowers the risk from such hazards.