An Investigation of Mechanisms Responsible for Modulated Biosynthetic Function in 3-Dimensional Cultures of a Human Hepatocyte Cell Line, for Potential use in a Bioartificial Liver Support System

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By
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Abstract

The thesis describes the performance of the HepG2 cell line, a proliferating liver cell line, potentially to be used as the cellular component of a bioartificial liver (BAL). HepG2 cells in 3-Dimensional (3-D) spheroidal culture demonstrate dramatically improved function, compared to monolayer culture. The main aims of the thesis were to investigate the phenomenon in this culture system, whereby function in 3-D culture is optimal between Days 8-10, and thereafter there is a decline in function in spite of continued proliferation and viability. For optimal use in a BAL, it is necessary to increase function per cell either at later time points of 3-D HepG2 culture when cell numbers are greater, and/or increase cell number at times of peak function i.e. Days 8-10 of 3-D culture.

The initial hypothesis was that the downregulation of activity observed from Day 11 occurred at the transcriptional level. Approaches to determine expression of liver-enriched transcription factors at times of peak and diminished function in 3-D culture and in monolayer culture were not conclusive.

To understand the mechanisms underlying the temporal change in function in 3-D culture, the hypothesis that hypoxia and/or other forms of stress were responsible for the drop in function observed was explored. Microarray data and Western blot analysis highlighted the expression of genes and proteins known to be upregulated by hypoxia at Day 15. Ambient oxygen concentration was increased to attempt to increase cell performance but was ineffective. Genes and proteins implicated in oxidative stress were expressed. Results from assays to measure oxidative stress in HepG2 culture demonstrated an increase at Day 15 compared with Day 8 spheroidal cultures. Attempts to alleviate this stress by supplementing the culture medium with additional anti-oxidants at later times of 3-D culture did not enhance cell performance.

Some stress-related proteins and genes were more strongly expressed in Day 8 cultures, as an adaptive response to increased metabolic activity during peak function, while others in Day 15 were turned off. These genes were investigated further at a functional level and results reflected the pattern observed.

3-D culture was manipulated with the addition of extracellular matrix (ECM) in

order to enhance cell performance. Cell proliferation was measured by total nuclei quantification, incorporation of BrdU as a measure of DNA synthesis and Ki-67 labelling, as a measure of the total growth fraction. Positively labelled cells were seen throughout the spheroid indicating that even at the centre of the spheroids; there was maintenance not only of viability, but also the capacity to proliferate, indicating other microenvironmental changes may be responsible for the diminished function observed at Day 15.

The thesis has highlighted the possible causes for the downregulation of function observed and modulation of the 3-D environment to overcome this, and emphasised the complex relationship between cell performance and the stress response, in order to improve a system which could provide the basis for the biological component of a BAL.

DEDICATION

I wish to dedicate my thesis to the loving memory of my dear father (Abbu) Faiqur Rahman Choudhury, and my endlessly supportive mother, Khudeza.

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Publications

L. H. Damelin, S. Coward, **S. F. Choudhury**, S. A. Chalmers, J. Cox, N. J. Robertson, G. Revial, M. Miles, R. Tootle, H. Hodgson and C. Selden. "Altered mitochondrial function and cholesterol synthesis influences protein synthesis in extended HepG2 spheroidal cultures." 2004. *Archives of Biochemistry and Biophysics,* 432 (2):167-77

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Abbreviations

AAT	Alpha-1-Antitrypsin
ABC	Avidin biotin complex
AFP	Alpha fetoprotein
AGP	Alpha-1-acid glycoprotein
ALF	Acute liver failure
APES	3-aminopropyltriethoxysilane
BAL	Bioartificial liver
BHT	Butylated hydroxytoluene
BrdU	5-bromo-deoxyuridine
BSA	Bovine serum albumin
C/EBP	CAAT enhancer binding protein
CDNB	1-chloro-2,4-dinitrobenzene
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
ECL	Enhanced chemiluminesence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid
EHS	Engelbreth holm sarcoma
ELISA	Enzyme linked immunosorbent assay
ELAD	Extracorporeal liver assisted device
ERK	Extracellular signal-regulated protein kinase
EROD	7-Ethoxyresorufin-O-deethylase
FCS	Foetal calf serum
FDA	Fluorescein diacetate
FHF	Fulminant hepatic failure
GCSF	Growth colony stimulating factor
GHS	Glutathione

HBSS	Hanks buffered salts solution
HG	High glucose
HIF	Hypoxia inducible factor
HNE	4- Hydroxynonenal
HNF	Hepatocyte nuclear factor
HO	Haemoxygenase
HRP	Horseradish peroxidase
HSC	Hepatic stellate cells
Ig	Immunoglobulin
IL	Interleukin
JNK/SAPK	c-Jun N-terminal Kinase/Stress Activated Protein Kinase
LIP	Liver enriched inhibitory protein
MAPK	Mitogen activated protein kinase
MARS	Molecular adsorbent recirculating system
MDA	Malondialdehyde
MOPS	Morpholinopropanesulphonic acid
MROD	7-Methoxyresorufin-O-dealkylase
NAC	N-AcetylCysteine
NPC	Non-parenchymal cells
OTC	Ornithine transcarbamylase
PBS	Phosphate buffered saline
PLL	Poly-L-Lysine
PERV	Porcine endogenous retrovirus
PI	Propidium iodide
RNase	Ribonuclease
RPA	Ribonuclease protection assay
RGD	Arginine-glycine-aspartic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SV40	Simian virus 40
TCA	Trichloroacetic acid

TAE	Tris acetic acid EDTA
TBE	Tris boric acid EDTA
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TGFβ	Transforming growth factor-beta
TNF	Tumour necrosis factor
TRH	Thyroid releasing hormone
VEGF	Vascular endothelial growth factor
3-D	Three-Dimensional
α-MEM	alpha Minimum Essential Medium

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Synopsis

Chapter 1 is a general introduction to the thesis, outlining the background to the research undertaken. The long term aim is to develop an extracorporeal liver support system temporarily replacing liver function, which would be a major advance for the treatment of liver failure. In particular, the thesis highlights the performance of the HepG2 cell line, a proliferating human liver cell line, potentially to be used as the cellular component of a bioartificial liver (BAL). HepG2 cells in 3-D spheroidal culture demonstrate dramatically improved function, compared to monolayer culture. Previous work carried out to investigate these effects is described, and the time course of performance vs. proliferation defined, which is especially important regarding potential use in a BAL.

Chapter 2 describes the general methods used throughout the thesis. More specific methods are described in their relevant chapters.

Chapter 3 and 4 attempt to understand the mechanisms underlying the temporal change in function in 3-D culture. Chapter 3 investigates the phenomenon with an initial hypothesis that the downregulation of activity observed from Day 11 onward despite uninterrupted cell proliferation, was at the transcriptional level. RNA protection analysis was performed to investigate expression of liver-enriched transcription factors at times of peak and diminished function in 3-D culture and in monolayer culture. Results of this were inconclusive, and a Microarray analysis of RNA expression was performed.

Chapter 4 explores the hypothesis that hypoxia and/or other forms of stress within the alginate beads might lead to downregulation of function after 10 days. The Microarray data was interrogated and analysis of the data highlighted the expression of genes known to be upregulated in the presence of hypoxia via Hypoxia Inducible Factor 1 (HIF-1), at a time of diminished function (Day 15) compared with optimal performance (Day 8). Western analysis was carried out

for HIF-1, as well as for a number of proteins also upregulated during hypoxia and a similar pattern of expression observed.

Further analysis of the Microarray database and Western blots were carried out to look for changes in genes and proteins, implicated in other stress responses such as oxidative stress, and assays to measure oxidative stress in HepG2 culture were carried out; results demonstrated an increase in lipid peroxidation and carbonylated proteins, as a measure of oxidative stress at Day 15 compared with Day 8 spheroid cultures.

Attempts to alleviate these stresses included increasing ambient oxygen concentration to increase cell performance, but was ineffective, and then supplementing the culture medium with additional anti-oxidants at later times of 3-D culture, which did not improve cell number or enhance protein synthesis per cell at Day 11 or Day 15 of 3-D culture.

However, some stress-related proteins and genes were more strongly expressed in optimally performing Day 8 cultures, and turned on as an adaptive response, as a consequence of increased metabolic activity. Some of these genes were investigated further at the protein and functional levels; catalase and GSH transferase showed parallel changes in specific activities and mRNA levels. Others are more strongly expressed in Day 15 cultures as performance diminishes. Thus the relationship between cell performance and the stress response is complex in this system.

Chapter 5 investigates further previous work aimed at increasing proliferation and improving cell function in 3-D HepG2 culture. This chapter manipulates 3-D culture with the addition of a biological differentiating agent, extracellular matrix (ECM) derived from human placenta and/or mouse sarcoma to increase cell proliferation at later times of 3-D culture. Cell proliferation was measured in three ways: nuclei quantification using crystal violet and haemocytometer counting, incorporation of a thymidine analogue BrdU into nuclei followed by immunocytochemical detection and quantification, and analysis of Ki-67 a proliferation associated antigen as a measure of the total growth fraction. BrdU and Ki-67 staining gave us an indication of the localisation and pattern of

proliferation in spheroidal culture. Similar percentage increases in cell number were observed, with all manipulations of ECM. Nevertheless, BrdU and Ki-67 labelled cells were seen throughout the spheroid indicating that even at the centre of the spheroids, there was maintenance not only of viability but also the capacity to proliferate. Therefore, the diminished function observed is not as a result of loss of viability, indicating other microenvironmental changes may be responsible.

Chapter 6 summarises the research undertaken with an overall discussion, and suggestions for future work.

Chapter 1

Introduction

Introduction

1.1 General Overview of the Liver

The liver is the largest solid organ in the body. Two blood vessels enter the liver, namely the hepatic portal vein with dissolved food substances from the small intestine, and the hepatic artery, with oxygenated blood from the lungs. Sinusoidal cells form a functional unit together with the parenchymal cells (hepatocytes) and hepatocytes are separated from each other by large capillary spaces called sinusoids. The plate structure of the liver and high permeability of the sinusoids allows each hepatocyte to be in close contact with the blood. The hepatic plates are arranged into functional units called liver lobules. The basal faces of adjoining hepatocytes are joined together by junctional complexes to form canaliculi, the first channel in the biliary system. Hepatocytes secrete bile into the canaliculi and at the ends of the canaliculi, bile flows into bile ducts. The liver is covered with a connective tissue capsule that branches and extends throughout the substance of the liver as septae and provides a scaffolding of support, and the highway along which afferent blood vessels, lymphatic vessels and bile ducts traverse the liver. Additionally, the sheets of connective tissue divide the parenchyma of the liver into lobules. The functions of the liver are varied and remarkable in that it maintains several biochemical pathways that permit the detoxification, or breakdown, of toxic products that accumulate because of the body's normal chemical functions and exposure to environmental poisons. The liver works intimately with nearly every fundamental process in the body, including homeostasis and the regulation of blood sugar levels. Metabolic functions of the liver are multiple and include synthesis of bile and certain proteins for blood plasma such as albumin, production of cholesterol and processing of haemoglobin (MacSween *et al.* 2002). It also has a remarkable capacity for regeneration, after damage (Rozga 2002).

1.1 1 Cell types in the liver

The parenchymal cells (hepatocytes) comprise approximately 60-70% of the liver volume. The other 30-40% is made up of non-parenchymal cells (NPC)s; the biliary epithelial cells lining the hepatic bile ducts and the hepatic sinusoid which harbours four different cells: endothelial cells, Kupffer cells, stellate cells (or also known as fat-storing cells, Ito cells or lipocytes) (Bouwens *et al.* 1992), and pit cells. Each cell type has its own specific morphology and functions, and no transitional stages exist between the cells. These cells have the potential to proliferate locally. Endothelial cells filter the fluids, exchanged between the sinusoid and the space of Disse through fenestrae that measure 175 nm in diameter and are grouped in sieve plates. Fenestrae occupy 6-8% of the cell surface. No intact basal lamina is present under these cells (Wisse *et al.* 1996). These changes mainly affect the passage of lipoproteins, which contain cholesterol and vitamin A among other components (Wilson *et al.* 1983).

The sinusoidal endothelial cells demonstrate endocytic capacity for ligands, including glycoproteins and components of the ECM such as collagen or fibronectin. They may also function as antigen-presenting cells and secrete eicosanoids, endothelin-1, nitric oxide and ECM components (Kmiec 2001).

Hepatic stellate cells (HSC) are pericytes, located in the space of Disse, with long, contractile processes, which probably influence liver (sinusoidal) blood flow. HSCs possess characteristic fat droplets and contain 80% of the body's vitamin A which is stored as long-chain fatty acid esters, and is the main source of retinoids, utilised by tissues throughout the body (Senoo 2004). Vitamin A deficiency transforms fat-storing cells into myofibroblast-like cells with enhanced ECM production. Activation of HSC is promoted by cytokines from Kupffer cells and platelets, of which transforming growth factor-beta (TGF-β) is of great importance. TGF-β stimulates the expression of ECM components, inhibits collagenase components, and also promotes the activation of fat-storing HSCs towards a myofibroblast phenotype (Nakamura *et al.* 2000).

Kupffer cells accumulate in periportal areas. They specifically endocytose endotoxin, which activates these macrophages (Xie *et al.* 2002). Lipopolysaccharide, together with interferon gamma, are very potent activators of Kupffer cells. Upon activation, these cells secrete oxygen radicals, tumor necrosis factor, interleukin (IL)-1, IL-6 that may damage hepatocytes, are active in the remodelling of ECM, and become cytotoxic against tumour cells (e.g., colon carcinoma cells). They play a major role in the response to inflammatory stimuli (Everett *et al.* 2003).

The biliary epithelial cells form the bile ducts and secrete bicarbonate rich solutions. The composition of bile is modified by these cells (Buanes *et al.* 1988;Tavoloni 1985). Bile acids are the most abundant biliary components, and play a major role in the maintenance of bile flow and are natural detergents that allow lipids to be digested in the intestinal lumen. They also secrete cytokines such as monocyte chemotactic protein-1 (MCP-1) which allows inflammatory cells to be recruited and promotes stellate cell proliferation and collagen synthesis (Lamireau *et al.* 2003).

Pit cells represent a population of large granular lymphocytes, and display a high level of spontaneous cytolytic activity against various tumour cells, identifying them as natural killer cells. The number and cytotoxicity of pit cells can be considerably enhanced with biological response modifiers, such as IL-12 (Wisse *et al.* 1996).

1.1.1.2 Parenchymal cells- Hepatocytes

Hepatocytes are the basic functional cell of the liver and constitute approximately 70% of the cellular content of the liver. They are the most important cell of the liver for metabolism. The liver has the unique ability to regulate its growth and size, and is under autocrine regulation releasing growth factors and cytokines. This regenerative property displayed by hepatocytes has been demonstrated following partial hepatectomy in response to decreased hepatic mass (Higgins and Anderson 1931).

1.1.1.2.1 Physiological functions

Hepatocytes are the most metabolically diverse cells in the body. They play critical roles in synthesizing molecules that are utilized to support homeostasis, such as maintaining blood glucose homeostasis. Hepatocytes also store many nutrients, carry out secretory and excretory functions, and play a role in the production and release of plasma proteins, as well as regulating energy balances.

1.1.1.2.2 Protein Metabolism

Hepatic protein synthesis accounts for approximately 15% of total body protein production including most of the plasma proteins. Albumin, the major plasma protein, is synthesized almost exclusively by the liver, with a normal plasma concentration of 40-50g/l. Its principal functions are to regulate plasma colloidal pressure and act as a major transport protein. Also, the liver synthesizes many of the clotting factors necessary for blood coagulation; prothrombin, fibrinogen, and α_1-Antitrypsin (MacSween *et al.* 2002).

Urea is synthesized by hepatocytes from ammonia generated by catabolism of amino acids derived either from digestion of proteins in the intestines or from endogenous tissue proteins, for excretion into urine, and deamination and transamination of amino acids, followed by conversion of the non-nitrogenous part of those molecules to glucose or lipids. Ammonia is very toxic and if not rapidly and efficiently removed from the circulation, will result in central nervous system disease (Mian and Lee 2002).

1.1.1.2.3 Carbohydrate metabolism

Glucose absorbed from the gut after a meal or glucose directly entering the blood stream as an intravenous infusion, reaches the liver and enters the hepatocytes by an active carrier-mediated process. Immediately it is converted to glucose-6-phosphate which is utilised by the liver for its various metabolic functions and for lipid synthesis (glycolysis). Hepatocytes contain dozens of enzymes that are alternatively turned on or off depending on whether blood levels

of glucose are rising or falling out of the normal range. Two important examples of these abilities are: excess glucose entering the blood after a meal is rapidly taken up by the liver, and glucose-6-phosphate which is converted to glycogen and stored in the liver (glycogenesis). Later, when blood concentrations of glucose begin to decline, the liver activates other pathways which lead to depolymerization of glycogen (glycogenolysis) and export of glucose back into the blood for transport to all other tissues. When hepatic glycogen reserves become exhausted, i.e. during fasting, hepatocytes synthesise glucose from amino acids (alanine and glutamine) and non-hexose carbohydrates (gluconeogenesis) (Banhegyi and Mandl 2001;Ferrer *et al.* 2003).

1.1.1.2.4 Lipid metabolism

Few aspects of lipid metabolism are unique to the liver, but many are carried out predominantly by the hepatocyte. Major examples of the role of the liver in fat metabolism include oxidizing triglycerides to produce energy. This is carried out in the smooth endoplasmic reticulum of the hepatocyte, which also synthesises triglycerides, cholesterol, phospholipids and bile acids. The liver breaks down more fatty acids than the hepatocytes need at any one time, and therefore exports large quantities of acetoacetate into blood where it can be readily metabolized by other tissues and used for ATP generation (Guidoux 1991). Some of this is packaged with lipoproteins and made available to the rest of the body. The remainder is excreted in bile as cholesterol or bile acids. The hepatocytes are the site of synthesis and secretion of serum lipoproteins (Bouma *et al.* 1988). They also play major roles in cholesterol and steroid metabolism and in the metabolism of the fat-soluble vitamins (vitamins A and D).

1.1.1.2.5 Detoxification

Hepatocytes are important in the detoxification of lipid-based drugs. The microsomes are responsible for the "conjugation" process involved in the inactivation of drugs (Chen *et al.* 2002). The major pathways for excretion of xenobiotics are via bile or urine, after they have been made hydrophilic. The

cytochrome P450 monooxygenases oxidise endogenous substrates, and drugs and carcinogens. P450 3A4 is of major importance, as it is the most abundant P450 in the human liver and is known to metabolize the majority of drugs whose biotransformation is known (Anzenbacher and Anzenbacherova 2001). P450 enzymes are located in the smooth endoplasmic reticulum and perform the Phase 1 reactions. Many compounds need to be conjugated in a Phase II reaction. The major conjugation reactions are glucuronidation of drugs such as morphine and acetaminophen (paracetamol), glutathione conjugation, amino acid conjugation, sulfation, acetylation and methylation (de Wildt *et al.* 1999). The Phase I intermediates can be highly reactive and are often more toxic than the parent compound.

1.2 Hepatic failure

Acute liver failure (ALF), also known as fulminant hepatic failure (FHF) is serious, often life threatening, and occurs when the whole liver begins to fail. It may be a complication of acute hepatitis due to viruses (hepatitis A, B), overdose or reaction to drugs (e.g. acetaminophen). It usually develops rapidly and demands immediate care (Gill and Sterling 2001).

1.2.1 Definitions and causes

FHF can be divided into hyperacute, acute, or subacute liver failure. In all types, liver failure is characterized by hepatic encephalopathy, jaundice, coagulopathy, and high mortality rates. Viral hepatitis, drugs, or toxins can precipitate ALF in patients without chronic liver disease. Metabolic stress such as bleeding or infections can precipitate acute-on-chronic liver failure in patients with chronic liver disease. The term, "acute liver failure" is, however, usually defined as patients developing hepatic encephalopathy, in the absence of pre-existing liver disease (MacSween *et al.* 2002). While the etiologies of FHF are multiple and varied, the prognosis is dependent on several factors, including the underlying cause of liver failure (Farmer *et al.* 2003). Cerebral oedema is the main cause of death in patients (Plevris *et al.* 1998).

Hepatitis viruses can be grouped together (hepatitis A-E) (Chen 2003). Biochemical changes associated with liver hepatitis include raised serum aminotransferase and alkaline phosphatase activity. Acute viral hepatitis is characterised morphologically by a combination of inflammatory cell infiltration, macrophage activity, hepatocellular damage i.e. hepatocyte swelling and regeneration, and necrosis of the liver. The most severely affected patients with ALF due to viral causes will survive only with liver transplantation (Plevris *et al.* 1998;Panteva *et al.* 2003). Paracetamol overdose remains the major cause of acute of liver failure in the UK (Plevris *et al.* 1998). The overall mortality rate is approximately 40% but is much higher in those with profound coagulopathy and renal failure.

1.3 Treatments for Hepatic Failure

Advances in intensive care and medical management have led to some improvements which treat the clinical manifestations of ALF, such as hepatic encephalopathy which is treated with withdrawal of dietary protein, lactulose and neomycin, but they are not wholly effective. Neomycin has the potential to increase the risk of renal failure (Plevris *et al.* 1998). Other complications include bacterial/fungal infections but the use of a wide spectrum of antibiotics such as aztrenam with vanomycin or piperaciliin with gentamycin have proved to be ineffective (Rolando *et al.* 1992).

1.3.1 Liver Transplantation

Unfortunately, without liver transplantation, the survival rate for patients with ALF is poor but the one year survival rate following liver transplantation has improved from ~ 30% in the 1960s and 1970s to more than 80% in the 1990s from cadaveric donors (Bramhall *et al.* 2001). Recent programmes have introduced living related liver transplantation as an alternative treatment due to the relative shortage of cadaveric donors, with subsequent increased survival rates (Karliova *et al.* 2002;Miwa *et al.* 1999). However, this potentially large group of donors is limited due to blood group mismatch, size incompatibilities, age or pre-existing disease, so providing a donor organ in only 30% of cases (Stockmann

and IJzermans 2002). Due to this overall shortage, an extracorporeal approach to the treatment of ALF is being investigated.

1.3.2 Extracorporeal/Artificial and Bioartificial Devices

A form of liver support that could aid patients with liver failure, either until they recover spontaneously or an organ becomes available, could be a major benefit. Essentially, there are two types of extracorporeal supportive therapy under development: bio-artificial devices, which use hepatocytes to perform the functions of the failing liver; and artificial devices which are described below.

Many devices have been developed which focus on detoxification alone as several substances such as bilirubin, bile acids, ammonia and lactate accumulate rapidly and cause multiple organ dysfunction and encephalopathy. Some of these endogenous toxins are water soluble, but most of them have a high binding affinity to albumin, a molecule overloaded with toxins in hepatic failure due to the reduced detoxification capacity of the liver. These devices do not possess the negative side effects of bioincompatibilty and lack of selectivity, but to date they have not improved the clinical status of these patients (Stange *et al.* 1999;Stockmann and IJzermans 2002). Earlier attempts to provide liver support included the use of detoxification procedures such as haemofiltration and haemoperfusion which (Ponting *et al.* 1992) will be discussed in detail later, and plasma exchange. However, these techniques were not significant in effectively removing albumin bound toxic molecules due to their affinity for albumin and their inability to permeate dialysis membranes (Ponting *et al.* 1992).

1.3.2.1 Haemodialysis

Haemodialysis requires a semi-permeable dialysis membrane through which fluids and small solutes may pass via diffusion, but may have an adverse effect on cardiac output and can lead to increased cerebral oedema.

Albumin dialysis is a new method that combines the efficacy of sorbents to remove albumin-bound molecules with the bioincompatibility of the modern dialysis membrane. The albumin acts as a specific molecular adsorbent and recirculates in a recycling system (i.e., molecular adsorbents recycling sytem, or

MARS). However, there is considerably little data regarding the use of MARS in ALF. One study described four such patients treated with MARS, where improvement of bilirubin and ammonia was observed but only one survived (Sen *et al.* 2002). A further recent study showed that a patient population with severe acute alcoholic hepatitis demonstrated improved bilirubin, renal function and encephalopathy. There was an improvement of a 3-month predicted mortality (pre-MARS: 76%, post MARS: 27%); and a 50% 3-month survival (Sen *et al.* 2003).

Although MARS is an exciting tool for the treatment of liver failure, most studies to date have been small, and the majority uncontrolled.

1.3.2.2 Haemofiltration

Haemofiltration and continuous haemodiafiltration are blood purification methods, as forms of renal replacement therapies, following liver failure. In this system, water and solutes are removed continuously and gradually, and in contrast to conventional haemodialysis, this allows for gradual correction of fluid balance and osmotic pressure. The membrane pore size of the haemofilter used is larger than that of the dialyzer used for conventional haemodialysis, allowing movement of substances with higher molecular weights (Oda *et al.* 2002).

1.3.2.3 Haemoperfusion

Haemoperfusion removes toxins and low-to-middle weight molecules i.e. bile acids, phenols, ammonia by using different adsorbents for a range of water soluble molecules, e.g. charcoal. Although charcoal is an effective adsorbent for a range of water-soluble molecules, it is not for ammonia or protein bound compounds. A randomised clinical trial has shown that charcoal haemoperfusion has little influence on survival (Liu *et al.* 2002).

1.3.3 Alternative treatments for hepatic failure- Bioartificial liver devices

Such is the complexity of liver function that it is generally agreed that such an artificial support would require a cellular component. Therefore the proposal to design a BAL is to aid patients until they either recover or receive a liver

transplant (Strain and Neuberger 2002). A BAL needs to accomplish a number of liver specific functions. The underlying principle is that a BAL device would circulate patients' plasma extracorporeally through a cartridge containing optimally functional hepatocytes.

Source of cells for a BAL

1.3.3.1 Animal cells

To use primary hepatocytes from other species, particularly porcine hepatocytes, would overcome the problem of obtaining normal human liver tissue. Primary porcine hepatocytes are indeed used in current BAL devices. It has been estimated that approximately 15 billion hepatocytes (10% of a normal liver) are required to sustain a patient in liver failure (Allen and Bhatia 2002).

Nevertheless, there would be the risk of xenozoonosis and immunological rejection. Porcine endogenous retrovirus (PERV) is regarded as a potential pathogen, as it can infect human cell lines *in vitro*, and human plasma can be detrimental to animal hepatocytes, due to activation of complement (Hoekstra and Chamuleau 2002). Until progress has been made on preventing animal proteins from being transmitted to the patient, animal cells are probably unsuitable for application in a BAL in the current climate.

1.3.3.2 Human cells

1.3.3.2.1 Primary hepatocytes

The primary human hepatocyte is the ideal candidate for the biological component of the BAL. However, primary hepatocytes need to be isolated from donor organs of which there is already a shortage. Primary hepatocytes rapidly de-differentiate and proliferate poorly *in vitro,* lose liver specific gene expression and become unstable (Bhandari *et al.* 2001). The large scale isolation, culture and sufficient numbers of cryopreservation of adult human hepatocytes, with long term stable highly differentiated function is not yet a reality.

1.3.3.2.2 Stem cells

An alternative source of human hepatocytes for a liver support system is stem cells (Selden and Hodgson 2004). *In vitro* cell transplantation or extracorporeal liver support therapies would benefir from utilizing stem cells if they provide a proliferating but readily differentiatable cell supply. Stem cells could therefore offer a potentially unlimited source of cells for hepatocyte replacement, and overcome the limitations imposed by existing techniques for immortalizing functional human hepatocytes and the risk of transmission of xeno-derived viruses and disease.

Stem cells, by definition, retain the ability for unlimited self-renewal while giving rise to progeny that differentiate along the lineage (Faris *et al.* 2001). It is now generally accepted that the liver contains cells with stem-like properties and that these cells can be activated to proliferate and differentiate into mature hepatic epithelial cells under certain pathophysiological circumstances in humans and animals (Zhang *et al.* 2003). For example, evidence from *in vitro* studies in rodents and humans suggested that stem cells give rise to liver cells, particularly in the presence of severe liver damage (Petersen *et al.* 1999;Alison *et al.* 2000).

There are a number of putative hepatocyte progenitors from a variety of sources. These are briefly described below.

Embryonic stem cells are derived from blastocysts and are multipotential for every cell type and therefore described as totipotent. Adult stem cells are mostly tissue specific and are located either in the bone marrow or in the liver itself. These are considered to be multipotent. Haemapoietic stem cells and mesenchymal stem cells are bone marrow derived. The foetal liver is also a source of haemapoietic stem cells in rodents and demonstrate proliferative capacity and differentiation (Cantz *et al.* 2003) even after transplantation, unlike the generally quiescent adult hepatocytes.

The oval cell population contains stem-like cells which can differentiate along hepatocytic, biliary, intestinal and pancreatic lineages in various rodent systems (Faris *et al.* 2001). These are known as hepatocyte progenitors and somewhat more committed than true stem cells.

Plasticity of stem cells has been demonstrated in the liver. For example when

liver stem/progenitor cells engraft in the portal space, they differentiate into bile duct cells, when engrafted into the parenchyma, they differentiate into hepatocytes (Alison *et al.* 1993). Recently, probably distinct stem cell populations potentially capable of developing into hepatocytes have been demonstrated in rodents (Alison *et al.* 1997) and humans, derived from the adult liver, where CD34+ and c-kit+ were cultured from human liver up to 8 days (Crosby *et al.* 2001). Selden *et al.* 2003 identified colonies from the non parenchymal cell population from a diseased liver which expressed stem cell markers OCT-4 and secreted albumin and AAT into the medium for several months. In peripheral blood of both mice and humans with treatment of growth colony stimulating factor (GCSF), mRNA expression was detected for markers AFP and CK19, indicating that they have the potential to develop into hepatocytes and/or biliary epithelial cells. In the adult liver, Evarts *et al.* 1996 demonstrated through pulse-chase experiments, the differentiation of oval cells (liver cell progenitors) into hepatocytes. However, identifying and particularly culturing liver cell progenitors in and from normal human liver has proved very difficult and it is easier to identify oval cells generated experimentally or *in vivo* pathophysiologically.

The mechanisms underlying the apparent conversion of stem cells into hepatocytes has been debated (Dahlke *et al.* 2004). Various reports suggested that stem cells could fuse with liver cells, which initially contain 2 or more additional sets of chromosomes. In some cases, this was followed by a reduction-division, which led to a normal chromosome set in the resulting cells (Wang et al. 2003;Vassilopoulos et al. 2003). Transdifferentiation is also a possible mechanism, but appears to be a rare event but analysis on a clonal level could provide further support for this hypothesis (Theise and Wilmut 2003). Currently research is ongoing to investigate the factors controlling the differentiation of stem cells into the desired cell type. Stable differentiation and/or commitment is necessary in order to repetitively culture the cell type of interest but so far approaches to manipulate stem cells from adult tissues have had only partial success, and so cell lines still remain the source for the biological component of the BAL.

1.3.3.2.3 Cell lines

Cultured hepatic cell lines, hepatoma/hepatoblastoma cell lines could eliminate the problem of human hepatocyte shortage, however there maybe a risk of tumourogenicity. Cultured as monolayers, none of these cell types retain sufficient functional capacity for use in BAL devices. Immortalisation of human cells *in vitro* requires that malignant transformation and de-differentiation associated with progressive tumorogenesis be excluded. The HH25 cell line for example was developed by co-culturing with rat liver epithelial cells and retains some differentiated functions such as albumin synthesis and cytochrome P450 function. Immortalised cell lines have been engineered to maintain differentiated function and proliferate *in vitro* (Roberts *et al.* 1994;Smalley *et al.* 1999). However, continuous replication and an increased rate of cell division and therefore metabolic activity, can render them genetically unstable, and can lead to the production of subclones with different metabolic capabilities. One example is the C3A hepatocyte cell line, a sub-clone of the HepG2 hepatoblastoma cell line.

Human hepatocytes that have been immortalised have been derived from foetal or mature liver tissue. Generally most human hepatocytes have been immortalised by introduction of a single M1 (non-dividing state or senescence) or M2 (second growth arrest) factor where cells enter a non-dividing senescence state.

The NKNT-3 cell line, created by introduction of Simian virus 40 (SV40)-T causing it to block the action of p53, allows cells to escape M1 and divide continually until the second growth arrest, M2 where the telomere ends become short, create chromosomal instabilities and rearrangements and eventually apoptosis (Hoekstra and Chamuleau 2002). SV40T Ag induced immortalisation of cells in general is often accompanied by de-differentiation and karyotypic instability, whereas hTERT-immortalised cells retain more differentiated functions and have less chromosomal damage. Our laboratory has designed human hepatic cell lines using a temperature-sensitive SV40T Ag. However, these cell lines only partially resemble *in vivo* normal human hepatocytes, due to progressive loss of cytochrome P450 activity, and lack of albumin secretion

(Smalley *et al.* 2001).

A final safety measure in immortalised cell lines is the addition of suicide genes, such as HSV thymidine kinase that provide negative selection with gangcyclovir . Immortalisation of human hepatocytes, which are metabolically active and expressing a full repertoire of liver specific functions have still not been achieved. The availability of *in vitro* expandable human hepatocytes would greatly advance liver directed cell therapies.

1.3.3.2.4 Foetal hepatocytes

Foetal hepatocytes have a higher proliferative capacity than mature hepatocytes, but depending on the age of the donor, they may not carry out all differentiated functions. There are also practical and ethical difficulties in utilising this system. However, considering the large quantities required for hepatocyte based therapies, using cells from the foetal human liver is an intriguing possibility, as these cells display a higher proliferative capacity then mature hepatocytes. It was established that hepatocytes from the foetal human liver are amenable to hTERT-mediated telomerase reconstitution without disrupting the ability to differentiate *in vivo* and in future potentially play a role in liver-directed cell therapies, but also relies on the availability of human tissue (Wege *et al.* 2003). Foetal hepatocytes have been harvested from the liver obtained from mid-trimester abortions, but the yield and the availability of hepatocytes were adversely affected by the condition of the foetus. Although, it was feasible to maintain these hepatocyte cultures for over 8 days, when hepatocytes were innoculated into a hollow fibre module to formulate a BAL support device, the cultures developed either cellular disintegration or bacterial infections (Anand *et al.* 2000).

The use of some of these sources of cell types as the cellular component of BAL devices and in clinical trials will be described further.

1.3.4. Extracorporeal BAL devices in clinical trials

The basic BAL bioreactor consists of a column containing a collection of hollow-fibre capillaries through which patient plasma is pumped. Hepatocytes are

innoculated into the capillary space, alone or attached to microcarrier beads. In the secondary circuit, plasma is separated, warmed, oxygenated, and then perfused through the lumen of the bioreactor capillaries, to allow free exchange of molecules between plasma and hepatocytes. The design must provide optimal *ex vivo* maintenance of hepatocytes, and given that conventional monolayer cultures are unable to maintain optimal function, hepatocytes would have to be induced to form cellular aggregates.

One example is the C3A hepatocyte cell line, a sub-clone of the HepG2 hepatoblastoma cell line, which is currently being tested in a BAL device, the ELAD system. ALF induced dogs with C3A cells in this system resulted in an 80% survival rate of treated animals compared with none of the control animals. A pilot controlled trial on humans with ALF showed no significant improved survival rate and no ammonia reducing activity (Sussman and Kelly 1993), and in addition, these cells attached as monolayers instead of 3-D culture on microcarriers. In a pilot controlled study in 1996, there was no significant difference observed between the survival rates of the extracorporeal liver assisted device (ELAD)-treated and the control groups (Ellis *et al.* 1996). Recent models of ELAD are modified versions with an oxygenator supplied to ensure adequate oxygen supply to the cells (Millis *et al.* 2002). Although 4 out of 5 patients survived in these trials, there was an increase in ammonia in the secondary circuit, and none of the patients who were treated experienced any neurological improvement. Strain *et al* 2002 suggested that more clinical trials need to be carried out, as the first clinical trial with this system consisted of 24 ALF patients. Currently, a larger multicenter trial of the ELAD is underway in the USA and UK under the direction of Vitagen (Moussy 2003;Sen *et al.* 2002). Vitagen announced their enrolment of a phase II trial of ELAD. The trial was to evaluate the safety and effectiveness of the ELAD system in bridging patients to either transplantation or recovery. Despite the severity of the patients disease progression, when controlled in this trial, initial outcomes comparing ELAD treated patients, showed 80% of ELAD treated patients, successfully bridged to transplantation or recovery, compared to 56% in the control group.

Demetriou and coworkers used porcine hepatocytes which were attached to microcarriers which are coated with collagen and innoculated into the extra fibre compartment of hollow fibre modules, and a charcoal perfusion column through which plasma passed, prior to entering the bioreactor (Rozga *et al.* 1993). However, there still remains the risk of zoonoses. Treatment of liver failure in pigs with the BAL utilising charcoal, but no cells, delayed the onset of intracranial hypertension to a significantly lesser degree, but survival time in those animals was similar to that seen in pigs subjected to a sham BAL containing neither cells nor charcoal (Khalili *et al.* 2001). This indicates that removal of small molecular weight toxins by means of high flux charcoal plasma perfusion is far less effective in treating experimental liver failure in pigs than the complete BAL, agreeing with their earlier results from studies in dogs (Rozga *et al.* 1993). Human patients with ALF were treated with a hybrid BAL consisting of an extracorporeal perfusion system containing porcine hepatocytes. The patient's blood was separated by plasmapheresis and the plasma was perfused through a charcoal sorbent column and a cartridge containing the porcine hepatocytes. The cartridge contains hollow fibers through which the patient's plasma circulates and 5×10^9 porcine hepatocytes attached to beads were innoculated into the extra-fibre compartment. The membrane separating the plasma from hepatocytes has 0.2-μm pores that allow the passage of molecules, such as immunoglobulins. However, ALF patients exposed to pig hepatocytes after repetitive treatments with a BAL device, developed strong IgG and IgM anti-pig Abs, mostly directed against the alpha Gal epitope (Baquerizo *et al.* 1999).

Gerlach and his colleagues designed a bioreactor system which essentially provides an optimised compartment for porcine hepatocytes to encourage them to reorganise into functional cellular aggregates by creating a three dimensional capillary space, to provide an efficient oxygen supply and allow the flow of plasma (Baquerizo *et al.* 1999;Gerlach *et al.* 1995). However, the Gerlach bioreactor is difficult to run outside the setting of the laboratory (Strain and Neuberger 2002).

The AMC BAL of Amsterdam requires at least 10 billion porcine hepatocytes and uses hollow oxygenation fibres. The bioreactor consists of a spirally wound, nonwoven polyester matrix in a cartridge, in which the hepatocytes are attached to the polyester matrix, and the design of this BAL is similar to one our laboratory would utilise. The unique feature of this BAL is that the plasma of the patient has direct contact with the hepatocytes, thereby reducing the diffusion distance and allowing mass exchange similar to the situation in the sinusoids of the intact liver parenchyma. A phase I study in Italy, showed that 6 of their first 7 patients were successfully bridged to orthotopic liver transplantation (Sosef *et al.* 2002). The function of this BAL was evaluated using anhepatic pigs, in other trials, but did not result in an improved coagulation state, i.e. prothrombin time, due to extensive consumption of clotting factors (Sosef *et al.* 2003).

The Sussman, Demetriou and Gerlach BAL devices are on clinical trial, but there are other BAL reactors which have not reached clinical trials, and although hepatocytes can function in BAL devices, detailed analysis of these studies are still awaiting reviews and appraisals. Progress has been made in this field, but it has been limited.

The successful development of the BAL depends on the hepatocyte component, the matrix support, and the bioreactor design. The bioreactor compartment must allow optimum cell culture, storage prior to use and cell integrity for use in the artificial support system. As well as the ability to culture hepatocytes within capillary membranes, allowing efficient gas exchange, substrate supply and waste removal to be carried out efficiently and practically. The cells may be seeded, cultured and grown within capillary membranes and perfused in the extra-capillary space, providing both mechanical and physiological protection from toxic blood or plasma.

In summary, none of the systems investigated have convincingly proved effective in replacing impaired liver function. Another important aspect is whether liver cells cultured in bioreactors can remain stable for a sufficient period of treatment. Selected metabolic or detoxifying functions of the bioreactor are difficult to

assess. Furthermore, since some applications combine biological units with other components, such as active charcoal, it is difficult to assess the role of hepatocytes in these settings.

Understanding the conditions necessary for a normal hepatocyte to express its full functional repertoire is important to establish fully functional cultures of liver cells, whether for experimental studies, work in drug development, or with the aim of creating a BAL. One major requirement is to maintain expression of normal differentiated function. To achieve this, a three-dimensional (3-D) environment for hepatocyte growth would resemble better the *in vivo* architecture of the liver to optimise the performance of liver cells growing *in vitro.*

1.4 A Three-Dimensional Environment for Hepatocytes

Hepatocytes require cell-cell and cell substrate interactions to survive and function. Furthermore, cell proliferation and differentiated function are often inversely dependent on one another and highly regulated in the micro-environment. All organs are organised three-dimensionally, so two-dimensional models, i.e. those based on monolayer cultures, cannot imitate their structures. Three-dimensional models can potentially mimic the *in vivo* physiology of the liver as cell shape and environment determines gene expression and the biological behaviour of the cells. If cultured cells remain cuboidal, differentiated function is maintained better than in monolayer culture (Berthiaume *et al.* 1996) which would be ideal to maintain optimal function for use in a BAL. There have been many approaches pursued to minimise this loss of differentiated function observed in monolayer culture, by co-culture with NPCs and various matrices. These will now be discussed with respect to their ability to induce 3-dimensional growth.

1.4.1 Collagen sandwich

Rat hepatocytes cultured in a collagen sandwich maintained normal morphology and a physiological rate of albumin secretion for at least 42 days, whereas hepatocytes cultured on a single layer of collagen gel ceased albumin secretion within a week (Dunn *et al.* 1989). The addition of ECM induces both cellular polarity and cell-ECM interactions (Hamilton *et al.* 2001). However, microcarriers such as collagen can also induce plasma clotting (Cunningham and Hodgson 1992).

1.4.2 Engelbreth Holm Swarm (EHS) mouse sarcoma-derived matrix

Engelbreth Holm Swarm (EHS) mouse sarcoma-derived matrix contains laminin and type IV collagen, growth factors etc. that can affect differentiated function. In hepatocytes cultured on thick EHS gel, production of plasma proteins and expression of cytochrome P450 was maintained (Lindblad *et al.* 1991). For cells cultured on plastic substratum and thin layer collagen, the ratio of albumin/beta-

actin mRNA was low and declined compared to cells cultured on EHS gel, where the ratio was high and serially increased during the culture period (Berthiaume *et al.* 1996;Gomez-Lechon *et al.* 1998;Nagaki *et al.* 1995). However, EHS matrix is of animal tumour origin and therefore inappropriate for a human BAL, but useful as proof of principle studies in the absence of human ECM which is difficult to obtain.

1.4.3 Alginate

Alginate is commercially available as alginic acid as a sodium salt, which is a linear polysaccharide. Alginate, isolated mainly from marine brown seaweed, was discovered by the British chemist Stanford in 1815. Alginic acid polymers are present in three types, depending on the part of the seaweed they are extracted from: 1) consisting entirely of D-mannuronic acid, 2) consisting entirely of L-guluronic acid, 3) alternating L-guluronic acid and mannuronic acid. Alginate being the skeletal component of the seaweed is both strong and flexible. The chain arrangement provides strength for the molecule and is responsible for fibre forming properties. Only sodium and potassium alginate salts are soluble in water. By pouring the sodium salt of alginic acid into calcium ions, the calcium salt of alginate is formed and the solution changes into a semi-solid state with an exchange of calcium ions for sodium ions. The gel formed is 99-99.5% water and 0.5-1.0% calcium alginate, if a 1% gel is prepared. The "hydrogel" is thermostable over 0-100°C. Maintaining sodium:calcium <=25:1 avoids gel destabilisation. The higher the guluronic content, the more rigid the gel.

1.4.3.1 Advantages of using alginate encapsulation

Microencapsulated cells or tissue in alginate, as artificial organs, are under study for treatment of a variety of other diseases, including Parkinson's disease, hypocalcaemia and diabetes (Xue *et al* 2001;Picariello *et al* 2001;Darquy and Reach 1985;Clayton *et al* 1993). Industrial applications include encapsulating yeast in ethanol production (Navratil *et al* 2002;Najafpour *et al* 2004). The use of an encapsulation system protects the cells or tissue within. Compared to other possible encapsulation techniques such as macrocapsules and intravascular

devices, microcapsules have several advantages. The large surface area of small beads results in enhanced survival of tissue due to better nutrition and oxygen supply, and in addition microcapsules can be implanted with minimal invasive surgery. It is, however, important to recognize the need for working with wellcharacterized alginate in order to obtain gel beads with reproducible properties. For medical applications it is also of crucial importance that the alginates are documented according to regulatory requirements and contain low levels of impurities.

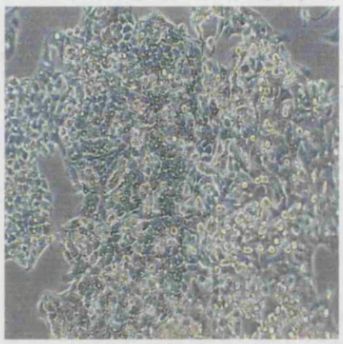
Use of other different three-dimensional support systems, have included porous glass and silicon. When comparing alginate to porous glass and silicon, cells encapsulated in alginate performed better. Protein secretion was higher in cells encapsulated in alginate, although silicon supported greater protein secretion than did porous glass (Selden *et al.* 1998). Cultured hepatocytes can be induced to form spheroids by microgravity and elliptical shaking (Berger *et al.* 1995;Khaoustov *et al.* 1999;Khaoustov *et al.* 2001). However, once out of these inductive conditions, the spheroids disaggregate, contrary to that seen with spheroids cultured in alginate.

1.4.3.2 Cell choice for encapsulation in 3-D culture by alginate

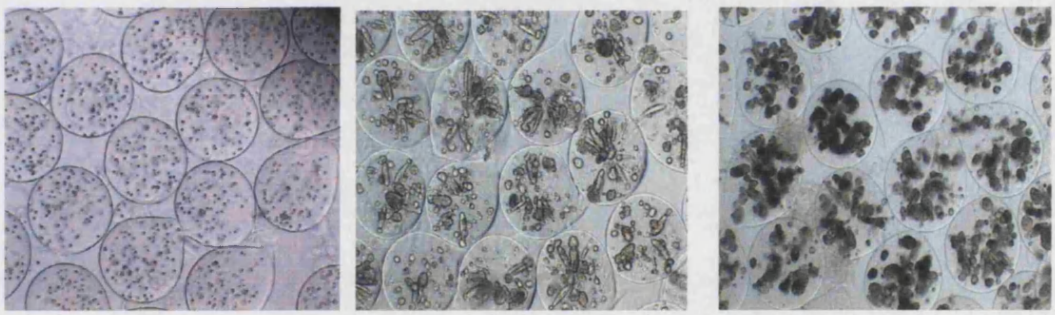
The sources of potential cells for a BAL have been previously described. Two human hepatocyte cell lines were previously assessed for function, morphology and proliferation: HepG2 cells and HHY41 cells in monolayer and in 3-D culture (Khalil *et al.* 2001). HepG2 cells in 3-D culture were chosen for further investigation. In this thesis the HepG2 cell line derived from a human hepatoblastoma free of known hepatropic viral agents is investigated. Minces of liver biopsies were initially overlaid on feeder cultures of irradiated mouse cell layers. After several months of passage of individual colonies, with restricted growth, a proliferating cell line that became feeder independent was obtained (Javitt 1990). HepG2 cells are a mature cell line, capable of carrying out many differentiated liver functions e.g. protein synthesis of albumin and cytochrome P450 function. In general, protein synthesis is better preserved than cytochrome P450 detoxificatory pathways. HepG2 cells in monolayer express normal low-

density lipoprotein (LDL) receptors and internalise very low density lipoproteins (VLDL), synthesise bile acids and secrete them into the medium. Electron microscopic appearance of the monolayer also supports the idea of HepG2 being a mature cell line, as there are, for example, tight junctions bordering on bile canaliculi with microvilli indicating a capacity for active secretory processes, which suggests HepG2 cells may be an appropriate cell line for use in a BAL (Kono *et al.* 1995;Kono and Roberts 1996). Roberts *et al* 1994 developed the HHY41 cell line by culturing human hepatocytes derived from normal liver tissue between collagen gel layers in medium enriched with growth factors for four weeks. The cells were not treated with carcinogens or virus transfection. The cells retained the characteristics of differentiated hepatocytes for example cytochrome P450 function and protein secretion and they proliferated freely *in vitro* (Roberts *et al.*1994). Of the two cell lines described above, cytochrome P450 assessed by 7-Ethoxyresorfin-O-deethylase (EROD) and 7-Methoxyresorufin-O-dealkylase (MROD) activity was greater in HepG2 cells than HHY41 cells, although the same pattern of enzyme activities were observed in both cell lines. HHY41 cells in alginate expressed all five urea cycle enzymes and synthesized urea. Monolayer HepG2 cells do not demonstrate the five enzymes of the urea cycle. However, in 3-D spheroids, they expressed 4 urea cycle enzymes at a rate comparable to that of rat liver, but ornithine transcarbamylase (OTC) was undetectable. Since OTC is the rate-limiting enzyme in the urea cycle, its lack in HepG2 results in no overall urea synthesis. Currently others in the group are working on overexpressing OTC in HepG2 cells by transfection and cloning. HepG2 cells were considered the cell line of choice, as a subclone of HepG2 had previously been approved for clinical usage in a BAL.

1.4.4 Improved liver-specific function demonstrated by HepG2 cells in 3-D culture - Data from previous work in the laboratory as a basis for the aims of this thesis



HepG2 cells in monolayer (seeded at 0.5 x 10^6 /ml) reaching 70-80% confluency at Day 8 of culture



Day 0 Day 8 Day 15

3-D culture in alginate

Fig.1 Phase contrast microscopy using an inverted microscope (Eclipse TE200, Nikon, Maidstone, UK) allowed HepG2 cells to be visualised throughout culture (x10)

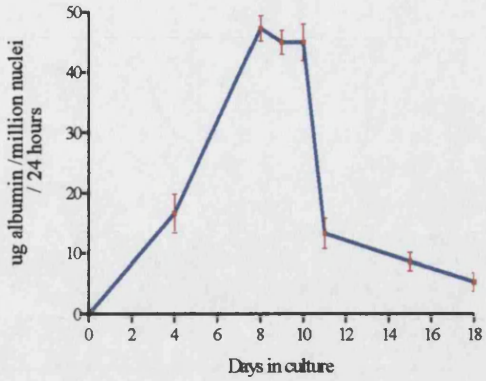


Fig. 2a Albumin secretion by HepG2 cells in 3-D culture

Mean values ± S.D, n=6 for each time point

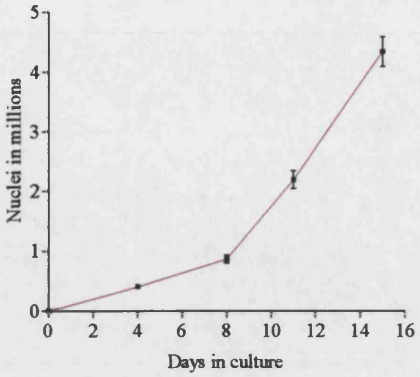


Fig. 2b Cell proliferation of HepG2 cells in 3-D culture

Mean values ± S.D, n=6 for each time point

In HepG2 cells, peak function was observed at Day 8. At Day 8, a 2.5-9.0 fold increase in protein secretion per cell was observed for albumin, fibrinogen, prothrombin, alpha-1-antitrypsin (AAT) and alpha-1-acid glycoprotein (AGP) as compared to secretion from monolayer cultures. Khalil *et al* 2001 showed a slower rate of proliferation of HHY41 cells than HepG2 cells.

However, HepG2 cells in 3-D culture demonstrate better function per cell in terms of protein secretion for albumin, fibrinogen and AAT.

Morphologically, HepG2 cells encapsulated in alginate, compared to monolayer culture, form a 3-D configuration and this is demonstrated in Fig.1.

The same pattern of secretion was seen in both cell lines, in which peak function was achieved between Days 8 and 10, with subsequent decreases in protein secretion, inspite of continued viability and proliferation (Fig 2a and 2b). This has been demonstrated in this thesis and by Khalil *et al* 2001.

In order to use a feasible amount of cells in a BAL to replace liver function, it is necessary to maintain or improve cell function at later time points when cell numbers increase. In this thesis, function will primarily be defined as albumin secretion per HepG2 cell.

HepG2 cell proliferation in spheroids is initially slow and after a lag phase up to Day 8, double at a rate of every 4.5 days (Fig.2b). Therefore, for example to replace one-third of the liver with our cells working optimally at Day 8 (Fig.2a and 2b) it would require approximately 17.5 litres of cells. This volume would be required to replace albumin production and is calculated on the basis of ~2 x 10^{11} cells in the liver, (~7 x 10^{10} = 1/3 of cells in the liver) producing ~12 g albumin/24h. At Day 8 (peak function) in our culture system, we have achieved a cell density of 4 x 10^6 /ml. Therefore to replace one-third of the function of the liver: 7 x 10^{10} /4 x 10^6 = 1.75 x 10^4 mls = 17.5 litres. Using cells which had been cultured longer, e.g. Day 15 spheroids where cell numbers are much greater, a much smaller volume of cells to replace liver function would be required e.g. 1-2 litres, which would be more practical for therapeutic purposes (Roberts *et al.* 1994) provided per cell function could be maintained at the Day 8 peak function. Part of the aim of this thesis is to explore ways of improving function. Whilst it is known that albumin could be administered exogenously, it serves here simply as an example of measurable liver specific function.

1.5 Causes for downregulation of function

Various hypotheses were investigated previously as possible causes for this down regulation of function, including measuring protein content within different compartments of the spheroids and the alginate matrix, to see if protein diffusion was impeded. Alginate was crosslinked with stronger divalent cations than calcium chloride i.e. barium and strontium to see if function could be maintained. Other alginate sources were also used. However, no significant differences, with regard to proliferation and function were observed.

Having ruled out simple physical explanations, the first part of this thesis serves to explore changes in transcriptional levels within the different cultures to see whether key genes are being switched on and off during periods of peak and diminished function.

1.6 OVERALL AIMS

The overall aim of the thesis was to:
To characterise and improve the methods to enhance the performance of HepG2 cells in alginate spheroidal culture:

1) By determining both gene and protein expression during enhanced performance at Day 8 of spheroidal culture compared to monolayer culture, and diminished function at Day 15 of spheroidal culture with peak function, Day 8 spheroidal culture.

2) Manipulating the 3-D culture system in the light of these results.

1.7 INITIAL HYPOTHESES

My initial hypothesis was that A) different transcription factors are present at times of peak function, 3-D culture (at Day 8) compared to monolayer culture and times of diminished function in 3-D culture (at Day 15) compared to peak function (Day 8).

and B) decreased levels of liver-enriched transcription factors in Day 15 3-D cultures are contributing to the diminished amount of liver specific proteins such as albumin and AAT compared to peak function at Day 8.

Chapter 2

General Methods

Chapter 2- General Methods

2.1 General Cell culture- Culture and maintenance of HepG2 cells

2.1.1 Materials

HepG2 cells were cultured in alpha Minimal Essential Medium (α-MEM) with ribonucleosides and deoxyribonucleosides (Invitrogen) supplemented with the following ingredients:

Table 1 Culture medium components for HepG2 cells

Component	Stock concentration	Final concentration	Store
Foetal Calf Serum (FCS)	100%	10%	- 20°C
Penicillin/Streptomycin	5000U/ml	50U/ml	- 20°C
Glutamine	200mM	2 mM	- 20°C
Fungizone	270.6mM	1.35mM	- 20°C
	All the above are supplied by Invitrogen (Scotland)		
Linoleic acid albumin	258.6mM	0.14mM	+ 4°C
Hydrocortisone	2.8mM	0.12μM	-20°C
Thyroid releasing Hormone (TRH)	2.8 mM	0.12 μM	-20°C
	All the above are supplied by Sigma (Dorset, UK)		
Insulin (Novo Nordisk, UK)	0.6mM	0.18μM	+ 4°C
Sodium selenite (Merck Ltd, UK)	0.58mM	0.12μM	+ 4°C
*D -Glucose Solution (Sigma)	2.50M	22mM	+ 4°C

* Extra Glucose is only added to medium for 3-Dimensional culture of cells, not when cells are in conventional monolayer

- HepG2 cell line from ECACC (Wiltshire UK)
- Tissue culture flasks and medium from Nunc (Life Technologies, Invitrogen, Scotland)
- Hanks Buffered Salt Solution (HBSS), without calcium or magnesium (Life Technologies, Invitrogen, Scotland)
- Trypsin Ethylenediaminetetra-acetic acid (EDTA) (in citrate saline/glucose) (Life Technologies, Invitrogen, Scotland)
- 0.2% Trypan Blue in 1X phosphate buffered saline (PBS).

2.1.2 HepG2 culture

HepG2 cells were seeded in monolayer in 175cm^2 flasks at a density of 0.5 million/ml to reach a confluency of 70-80% a week later and a final yield of 35-40 million cells. Cells were cultured in supplemented ∝-MEM (without high glucose (HG)) and medium changed every 48 hours. The cells were maintained in incubators at 37°C in a humidified atmosphere of 95% air: 5% CO_2.

2.1.2.1 Trypsinising

The cells were passaged/trypsinised when they reached 70-80% confluency and were a single sheet of rounded uniform cells.

After removal of the medium, the cells as monolayers were washed three times with warm HBSS (Ca^{2+}/Mg^{2+} free). Warm (37°C) trypsin (10mls for 175cm^2 and 5mls for 80cm^2 flasks) was added through a 0.2μm filter (Minisart, Sartorius) and incubated at 37°C for 30-90 seconds. After firmly tapping the sides of the flasks, the cells detached as a monolayer sheet from the surface and were poured rapidly into a Nunc tube with warm complete ∝-MEM (25mls for 175cm^2 flask, 12mls for 80cm^2 flask) to neutralise the trypsin. The cells were centrifuged at 300g at room temperature for 4 minutes (Heraeus, UK). The supernatant was discarded and the pellet was resuspended in warm ∝-MEM complete (5mls for 175cm^2 and 2mls for 80cm^2) with gentle pipetting. Cells were gently syringed with a sterile 21G needle 4-5 times to disaggregate cell clumps and form a single cell suspension.

2.1.2.2 Cell yield and viability

Cell density and viability was determined by trypan blue exclusion. Trypan blue stains dead or injured cells due to breakdown of the cell membrane; intact membranes will exclude trypan blue. In the following order 160µl of HBSS, 20µl of Trypan Blue and 20µl of the cell suspension were added. Yield and viability were estimated after 2 minutes in a haemocytometer. The cells were reseeded at an appropriate cell density into fresh flasks or frozen in 5% Dimethyl Sulphoxide (DMSO) in 50% FCS in liquid nitrogen for future use.

2.1.3 Preparation for alginate encapsulated HepG2 cells

2.1.3.1 Materials

Alginic acid sodium salt from *Macrocystis pyrifera,* kelp. Cat No. A2033 (medium viscosity, Sigma Chemical Co).

- 0.15M NaCl pH 7.4
- 0.102M $CaCl_2$ in 0.15M NaCl pH 7.4
- Dulbeccos Minimal Essential Medium (DMEM) with glutamax, sodium pyruvate and 10% FCS and Penicillin/Streptomycin (Invitrogen 31966-021)
- α- MEM complete with HG
- 60-70% confluent flask of HepG2 cells.

2.1.3.2 Apparatus

- 23G co-axial cannula (constructed at the Medical Engineering Unit, Hammersmith Hospital)
- Electronic syringe pump and syringe piston (Harvard Apparatus Co.)
- Medical air gas cylinder.

2.1.3.3 Alginate preparation

The 2% w/v alginate preparation was made with alginic sodium salt (light sensitive) added very gradually to 0.15M NaCl pH 7.4, and left to stir overnight and autoclaved on the day of use at 121°C for 10 minutes.

2.1.3.4 Encapsulation of HepG2 cells

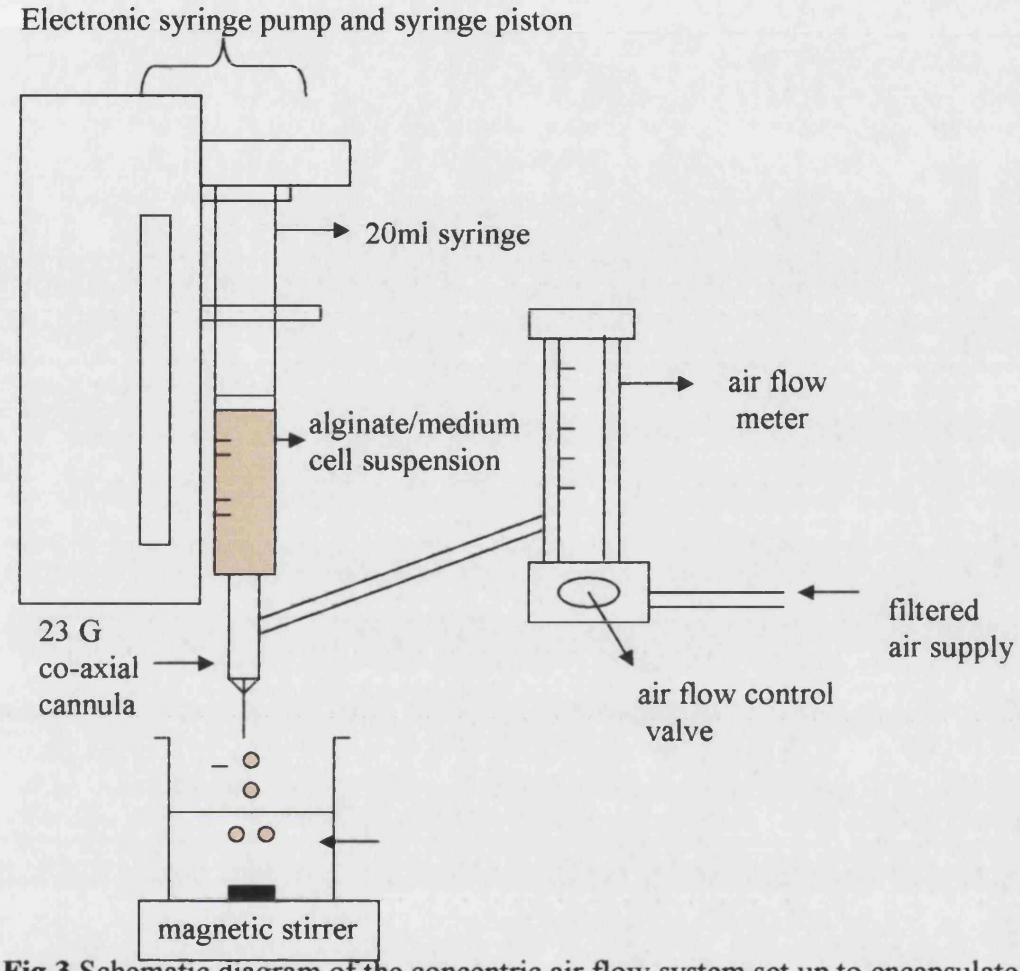


Fig.3 Schematic diagram of the concentric air flow system set up to encapsulate HepG2 cells and promote 3-Dimensional culture

The 2% w/v alginate preparation was mixed with HepG2 cells at a required cell density of 0.5 x 10^6/ml and α-MEM with HG to achieve a final 1% alginate concentration. The suspension was mixed very gently and loaded onto a syringe of appropriate volume, fitted with a specifically designed 23G co-axial cannula. The cannula system was connected to an air supply, with the syringe piston controlled by an adjustable valve (units + cc/min). The solution was pumped through at 1.5ml/minute. The syringe diameter was also set on the pump depending on the syringe used. The system was set up under sterile conditions in a tissue culture laminar flow hood.

Micro droplets of alginate-encapsulated cells were formed by a concentric air

flow created around the cannula. The alginate beads solidify on contact with calcium chloride cationic divalent solution (0.102M in 0.15M NaCl). Beads were collected for a timed period into a container with a 200μm nylon mesh bottom immersed in the calcium chloride solution.

After polymerisation in the calcium chloride solution for 15 minutes the beads were washed in DMEM solution and transferred into a T175cm^2 tissue culture flask.

The beads were resuspended in the flask and aliquotted into 6 well culture plates with 100μm mesh filters in each well on which the beads were supported. Each well contained a volume of 0.25mls of beads in 8mls of α-MEM medium with HG. The culture medium was replenished every 48 hours. In this way beads received medium from above and below.

2.1.4 Harvesting of Alginate-Encapsulated cells

2.1.4.1 Materials

- 4mM EDTA (disodium salt) (Merck Ltd) in 0.15 M NaCl pH 7.4
- 0.15M NaCl pH 7.4

2.1.4.2 Method

The day before harvesting, cell strainers containing 0.25ml of beads were placed into a fresh well of a 6 well plate with 5mls of α-MEM HG medium. Exactly 24 hours later, the conditioned medium was removed from the wells and frozen at -20°C for further analysis. The beads were transferred from the cell strainers into a 6 well plate. Warm EDTA solution (chelating agent) was added to each well (1.9 mls per 0.25mls of beads in each well) and incubated for 30 minutes at 37°C. EDTA chelates calcium cations depolymerising the alginate allowing the cell spheroids to break out of the alginate beads. Cell spheroids were broken up with multiple, vigorous syringing, using a 23G needle, avoiding foaming and the cell suspension was spun at 10000g for 5 minutes at room temperature (Eppendorf 5402, Germany). Any excess EDTA/alginate was removed by washing with 0.15M NaCl, followed by centrifugation. The pellet containing nuclei was resuspended in the appropriate volume of crystal violet solution (1%) depending

on the harvesting time point.

Harvesting Time Point (Day)	Volume of crystal violet solution (ml)
4	0.4
8	0.8
11	1.0
15	1.5

Table 2 Typical volumes of crystal violet solution added at harvesting time points

2.1.4.3 Determining cell yield

2.1.4.3.1 Materials

- 0.1M citric acid containing 1 % w/v crystal violet and 0.2 % Triton X-100 pH 3-4.

2.1.4.3.2 Method

After the addition of crystal violet solution, the samples were incubated at 37°C for 1 hour and then left overnight at 4°C. Nuclei were counted using a haemocytometer; each sample was counted in triplicate.

2.1.5 For cell viability

2.1.5.1 Materials

- Propidium iodide (PI) –1mg/ml-(store in dark at 4°C)
- Fluorescein diacetate (FDA) 1mg/ml made up in DMSO (kept at room temperature)
- 1X PBS warm

Simultaneous staining with FDA and PI is used for the identification of living and dead cells. PI passes through dead cell membranes and intercalates with DNA to form a bright red fluorescent complex. FDA passes though living cell membranes and is hydrolysed by intracellular esterase to convert non-fluorescent FDA to fluorescein. Fluorescein accummulates inside the cell and fluoresces green; FDA stains the cytoplasm and fluoresces green showing viable cells (Clarke *et al.* 2001).

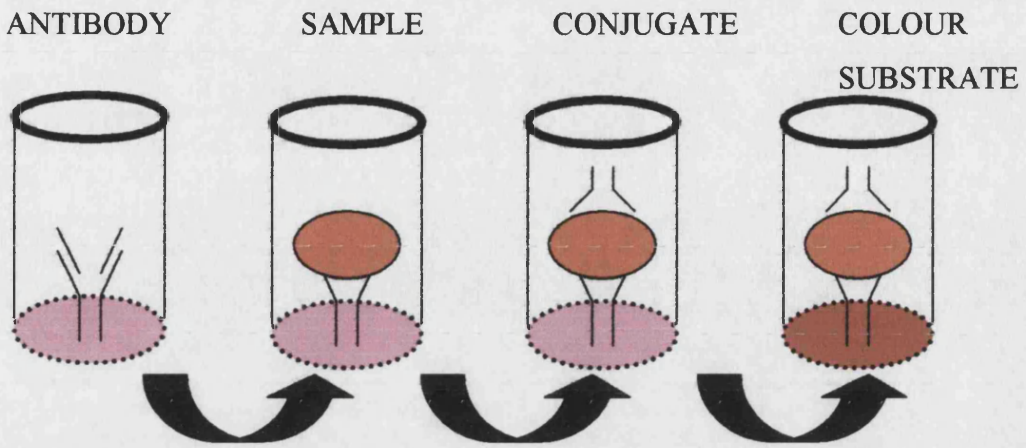
2.1.5.2 Method

Approximately 1 ml medium containing beads were placed in a microfuge tube, beads were washed twice with PBS and resuspended in 1ml of PBS. 10µl of PI and 20µl of FDA was added, mixed gently and allowed to stand for a few minutes. The beads were washed again twice with PBS and finally resuspended in 0.5ml of PBS. The beads were viewed under a fluorescent microscope using a filter with an excitation filter of 510-560nm and an emission filter of 590nm for PI stained cells, and for FDA stained cells, using an excitation filter of 465-495nm and an emission filter of 515-555nm.

2.2 Measuring presence of liver specific secreted proteins

The enzyme linked immunosorbent assay (ELISA) uses a colorimetric detection system to quantify specific protein content. Antigen is captured by an unlabelled antibody and detected with a secondary antibody conjugated to an enzyme to give a visual signal, which can be quantified. Examples of detection systems include horseradish peroxidase (HRP) and alkaline phosphatase which are the most widely employed enzyme conjugates in enzyme immunoassay. The substrate for peroxidase is hydrogen peroxide. In HRP assays, cleavage of H_2O_2 is coupled to the oxidation of a hydrogen donor which changes colour during the reaction.

InDirect Sandwich ELISA



EVERY STEP IS FOLLOWED BY INCUBATIONS AND WASHINGS

The indirect sandwich ELISA was used to determine amounts of secreted liver-specific proteins, albumin, AGP, fibrinogen, prothrombin, AAT and alpha fetoprotein (AFP). Medium was conditioned for 24 hours and kept at -20°C until further analysis.

2.2.1 Materials

- Nunc immuno 96-well plates (Maxisorp) (Invitrogen, Scotland)
- Wash Buffer- 1X PBS containing 0.05% Tween 20
- Coating Buffer
  - Sodium carbonate (Na_2CO_3) 0.318g
  - Sodium hydrogen carbonate ($NaHCO_3$) 0.586g

  } In 200ml at 4°C
- Working OPD solution
  - OPD 4mg (1,2- phenylenediamine dihydrochloride, DAKO) dissolved in 12mls dH_2O. 6µl of hydrogen peroxide (30% solution) were added immediately before use. The solution was protected from light
- Sulphuric acid (2M) - Stop solution
- Primary antibodies and secondary horse-radish peroxidase (HRP) conjugated antibodies (DAKO Ltd, High Wycombe).

Protein	Primary Antibody	Secondary Antibody	Standards
Albumin	Rabbit polyclonal anti-human albumin (10mg/L) Cat No. A0001 (1/1400)	(HRP) conjugated rabbit polyclonal anti-human albumin (1/8000) Cat No. P356	Human serum albumin (Sigma Ltd) Range: 25-200ng/ml
A1GP	Rabbit polyclonal anti-human A1GP (10mg/L) Cat No. A0011 (1/1900)	HRP conjugated rabbit polyclonal anti-human A1GP (1/1000) Cat No. PE324	Human A1GP (Sigma Ltd) Range: 6.25-200ng/ml
A1-AT	Rabbit polyclonal anti-human A1AT (10mg/L) Cat No. A0012 1/900	HRP conjugated rabbit polyclonal anti-human A1AT (1/1000) Cat No. PE876	Citrated human plasma Range:12.5-200ng/ml
Fibrinogen	Rabbit polyclonal anti-human fibrinogen (10mg/L) Cat No. A0080 1:400	HRP conjugated rabbit polyclonal anti-human fibrinogen (1/4000) Cat No. P445	Citrated human plasma Range 6.25-200ng/ml
Prothrombin	Rabbit polyclonal anti-human prothrombin (10mg/L) Cat No. A325 (1/1000)	HRP conjugated rabbit polyclonal anti-human prothrombin (1/1000) Cat No. P445	Citrated human plasma Range 12.5-200ng/ml
Al-FP	Rabbit polyclonal anti-human A1 FP (10mg/L) Cat No. A008 (1/200)	HRP conjugated rabbit polyclonal anti-human A1FP (1/7500) Cat No. P445	Human AFP (DAKO) Range 12.5-200ng/ml

Table 3 Antibodies and standard curve ranges used to measure liver specific proteins

2.2.2 Method

For each ELISA, the appropriate primary antibody was diluted in coating buffer; wells were coated with 100µl. The plate was wrapped in cling film to prevent evaporation and left overnight at 4°C or at 37°C for 2 hours.

Plates were washed 3 times with wash buffer PBS/ 0.05% Tween 20. Appropriate standards in 100µl were added in triplicate to the plate, the plate was sealed and incubated for 1 hour at 37°C. The plate was then washed 3 times with wash buffer.

An appropriate dilution of the secondary HRP conjugated antibody in PBS/Tween 20 (wash buffer) was made (Table 3); 100µl was added and incubated for 1 hour at 37° C. The plate was washed 5 times with PBS/0.05% Tween 20.

To the plate 100µl of OPD solution was added at timed intervals and left covered in foil until a sufficient colour change (orange) had taken place (usually between 1 to 4 minutes depending on the protein being assayed). The reaction was stopped by addition of 50µl of 2M sulphuric acid at the same time intervals. Within 1 hour, absorbances were read at 492nm using a micro-plate reader (Anthos HTII, Anthos Labtec Instruments, Salzburg, Austria) and analysed by Biolise Software. Results were expressed as ng protein per ml.

2.3 Preparation of total RNA from HepG2 cells in monolayers and cultured in alginate at Day 8 and Day 15 of culture and human liver.

High purity and integrity of RNA isolated from cultured cells or tissue is essential for effective use in applications such as nuclease protection assays, Northern blotting, cDNA synthesis for microarray and reverse transcription PCR. The RNAgents Total RNA Isolation System from Promega (Cat No. Z5110) was used and subsequently the Qiagen RNeasy mini kit (Cat No. 74104) to rapidly extract pure and intact RNA. These commercially available kits avoid the need to utilise time-consuming and tedious methods such as CsCl step-gradient ultracentrifugation, ethanol or LiCl precipitations, or methods involving the repeated use of toxic substances such as phenol and/or chloroform. These

methods are able to recover smaller RNAs which do not sediment efficiently during centrifugation through CsCl. The use of LiCl can result in the loss of RNA smaller than 5.8S and the carryover of lithium salts can inhibit cDNA synthesis reactions.

The essential steps required for the successful isolation of intact RNA includes efficient disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and elimination of contaminating DNA and protein.

The RNAgents Total RNA Isolation System from Promega includes a denaturing solution which contains two potent inhibitors of RNase activity, guanidine thiocynate with N-lauryl sarcosine and β-mercaptoethanol which act to disrupt the nucleoprotein complexes allowing RNA to be released free of protein. Contaminants are then separated by acid extraction using phenol: chloroform: isoamyl alcohol.

The RNeasy mini kit was used to clean up RNA previously isolated by the Total RNAgents method. This method was not utilised for total RNA prepared for the Ribonuclease protection assay but was included as a step for the preparation of total RNA for cDNA synthesis in microarray analysis.

Before this purification step, samples were treated with DNase digestion to denature any contaminating genomic DNA in the samples.

Lysis buffer (containing β-mercaptoethanol) and ethanol are added to the samples to provide conditions that promote selective binding of RNA to the RNeasy membrane. The kit combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. The sample is applied to an RNeasy spin column where the total RNA binds to the membrane, and contaminants are washed away. High quality RNA is eluted in RNase free water.

Total RNA was extracted from samples of monolayer HepG2 cells, and 3-D HepG2 spheroids cultured in alginate (n=3).

All steps were carried out in an RNase free environment, surfaces were clean, solutions were treated with DEPC, glassware was baked at 200°C overnight and sterile tips with filters were used.

2.3.1 Materials

- RNAgents Total RNA Isolation System from Promega (Cat No. Z5110)
- DEPC (Diethyl Pyrocarbonate, Sigma, UK)
- RNase Away (Molecular BioProducts Cat no. 7003)
- HBSS on ice
- 4mM EDTA in 0.15M NaCl pH 7.4
- 1X PBS (treated with 0.1% DEPC)
- Ethanol
- 0.1% DEPC treated water

Baked apparatus at 200°C

- Pestle and Mortar
- Homogenisers
- Glassware.

2.3.2 Total RNA preparation from monolayer HepG2 cells

A 70% confluent T175 flask of HepG2 cells in monolayer yields ~2.5 x 10^7 cells. Two flasks were used. Briefly, the flask was washed twice with ice cold sterile PBS (treated with 0.1% DEPC previously). Denaturing solution (3mls) was added to each flask and the flasks were rocked by hand until the solution became viscous. The entire solution was transferred to Oakridge tubes on ice (also previously treated with 0.1% DEPC). To the tube 1/10 volume 3M Sodium acetate pH 5.2 was added and mixed by inversion 4-5 times to precipitate the RNA. An equal volume of phenol: chloroform: isoamyl alcohol was added, mixed by inversion 4-5 times and vortexed vigorously for 15 seconds. The solution chilled on ice for 15 minutes. The phenol mix separates the solution into phases. The top aqueous phase contains the pure RNA. DNA remains in the

organic phase and protein at the interface. The tubes were centrifuged at 10000g for 20 minutes at 4°C (MSE Europa). The top aqueous layer was removed avoiding the interface and transferred to a clean tube. An equal volume of isopropanol was added and incubated at -20°C for 1-2 hours to precipitate the RNA. The solution was centrifuged at 10000g for 15 minutes at 4°C (MSE Europa). The pellet was resuspended in an appropriate amount of denaturing solution and vortexed until dissolved. An equal volume of isopropanol was added, mixed, vortexed well and cooled for a 1 hour at -20°C. The solution was centrifuged at 10000g for 15 minutes at 4°C (MSE Europa). The pellet was washed with 75% ethanol (diluted with 0.1% DEPC treated water) and broken up. The pellet was centrifuged at 6500g for 10 minutes at 4°C (MSE Europa). The pellet was left to air dry for 1-2 minutes and resuspended in RNase free water. The total RNA in solution was quantitated and analysed for purity by measuring its optical density at 260nm and 280nm. RNA was aliquotted and stored at -80°C.

2.3.3 Total RNA preparation from 3-D HepG2 cultures

Cell yield from spheroids was determined as described previously. Sufficient beads for ~5 x 10^7 cells were transferred into Nunc tubes. The beads were allowed to settle and medium removed. Beads were washed twice with cold HBSS on ice. For every 0.25ml of beads, 1.9ml of 4mM EDTA in 0.15M NaCl pH 7.4 (treated with 0.1% DEPC) and 1% v/v β-mercaptoethanol was added fresh immediately to the EDTA solution, just before incubation with the EDTA. The tubes were inverted until the alginate dissolved. The tubes were centrifuged at 200g for 4 minutes at 4°C (Heraeus, UK) and the supernatant removed. Cold PBS (0.1 % DEPC treated-5ml) was added to the tubes and centrifuged at 200g for 4 minutes at 4°C (Heraeus, UK). The supernatant was removed and an appropriate amount of denaturing solution was added. The remainder of the RNA isolation was identical to that described for monolayer.

2.3.4 Total RNA preparation from human liver tissue

Normal human liver tissue was obtained under the approval of the Royal Free Local Research Ethics Committee, Reference 38-200 entitled "Culture if human

liver cells obtained at surgery" (See Appendix A for further details). Liver tissue was placed immediately in liquid nitrogen. The pestle and mortar was cooled with liquid nitrogen and the tissue was transferred to the cold pestle and mortar under nitrogen. The tissue was homogenised in liquid nitrogen and the fine powder transferred into Oakridge tubes. An appropriate amount of denaturing solution was added and the RNA isolation procedure was continued as described for cells in monolayer and 3-D culture.

2.4 Protein lysate preparation for Western blot analysis

2.4.1 Materials

- *Lysis Buffer* - 10mM Tris pH 7.4
  1% Glycerol
  1mM Sodium orthovanadate

- *2X Electrophoresis buffer* - 125mM Tris pH 6.8
  4% sodium dodecyl sulphate (SDS)
  10% Glycerol
  0.006% Bromophenol blue
- 1X PBS (cold)
- Cell scrapers
- HBSS on ice
- 4mM EDTA in 0.15M NaCl pH7.4
- 2% β-mercaptoethanol.

Apparatus

- Sonicator (Soniprep 150, Sanyo, Leicestershire, England)

2.4.2 Method

2.4.2.1 From 3-D cultures

For Day 8 and Day 15 3-D cultures, sufficient alginate-HepG2 beads were taken from each condition so that an equal number of cells were present. Typically, ~ 50 x 10^6 cells from each culture condition.

Beads were transferred to polypropylene conical tubes. The beads were allowed to settle and the medium carefully removed with a sterile pipette. The beads were washed with cold HBSS and 1X PBS. PBS was removed and EDTA solution added (1.9mls/0.25ml beads). The tubes were mixed manually until the alginate dissolved and released the HepG2 spheroids. The samples were spun at 200g for 10 minutes at 4°C (Heraeus, UK). While the samples were spinning, the lysis buffer was brought to boiling point. After the samples had been centrifuged, the supernatant was removed, and 3-4mls of boiling lysis buffer added to the remaining pellet. The samples are mixed and vortexed well to break up the pellet. The samples were briefly microwaved for 5-10 seconds uncapped. The cells were lysed by sonication, briefly for 30 seconds, avoiding foaming.

The samples were vortexed and a small amount ~100µl was used to determine total protein using the Lowry method. The remaining samples were diluted in 2X electrophoresis buffer to obtain a final concentration of 1mg/ml. Samples were stored at -80°C until further analysis.

2.4.2.2 From Monolayer Cultures

HepG2 cells cultured as monolayers in T175cm^2 flasks (~50 x 10^6) were washed twice with cold 1X PBS. To each flask 3mls of boiling lysis buffer was added. The flasks were rocked until the solution became viscous. The solution was pipetted into polypropylene conical tubes, centrifuged as for 3-D cultures and treated as described earlier.

2.4.3 Total protein determination

The mini Lowry method was carried out for the determination of total protein (Schacterle and Pollack 1973).

2.4.3.1 Materials

- 96 well plate
- Copper sulphate reagent- 10% Na_2CO_3, 0.2% Potassium Sodium Tartrate, 0.05% $CuSO_4.5H_2O$, 2% NaOH

25g of Na_2CO_3, 5g of NaOH, 0.5g of potassium sodium tartrate were made up to approximately 200mls ddH_2O. 195mg of $CuSO_4.5H_20$ was dissolved in a few mls of ddH_2O. The copper sulphate solution was slowly added to the alkaline tartrate solution, and the final volume was adjusted to 250ml. This solution was stored in a polypropylene bottle, protected from light, at room temperature and was stable for a month.

- Folin and Ciocalteau Phenol reagent (F/C Stock, Sigma Aldrich Cat No. F-9252) stored at 4°C and diluted 1:16 with water prior to use
- Protein standard curve was constructed with known protein amounts of bovine serum albumin (BSA), prepared by doubling dilution (ranging from 0 - 32µg/well) in 0.3 M NaOH / 0.1% SDS or sample buffer

2.4.3.2 Method

50µl of sample or standard was added in triplicate to wells of a 96 well plate. 50µl of copper sulphate solution was added to the wells in the same order at 10 second intervals. The plate was incubated at room temperature for exactly 10 minutes from first addition of the copper sulphate solution on an orbital mixer. Colour detection was started by the addition of 200µl of diluted F/C reagent at 10-second intervals. The plate was briefly mixed in an orbital shaker and incubated at 55°C for 5 minutes to complete the colour reaction. The plate was removed and left to cool in a tray of ice. The absorbance was measured at 620nm in a microplate reader (Anthos HTIII), connected to Biolise Software to plot a standard curve and calculate the amount of protein per well in µg for each sample.

2.5 Western blotting for detection of specific proteins

This method is used to identify a specific protein in a complex mixture and simultaneously determine its molecular weight. The procedure requires: -

1) Size separation of the denatured proteins in the mixture by SDS-Polyacrylamide Gel Electrophoresis (PAGE). The anionic detergent SDS was used with β-mercaptoethanol and heat to dissociate the proteins before they were loaded onto the gel. Denatured proteins bind SDS in a sequence independent manner which is proportional to the molecular weight. A negative charge is conferred to each protein, allowing separation under electrophoresis.

2) Transfer of the separated proteins to a membrane while retaining their relative position.

3) Detecting the protein under investigation by using antibodies raised against the target protein and confirmation of its size relative to standard proteins of known size. The proteins were visualised by an enzyme-linked chemiluminescent reaction onto autoradiographic film.

2.5.1 SDS-PAGE Electrophoresis

2.5.1.1 Materials

- Pre-cast 4-20% Tris-Glycine Gradient gel (Invitrogen, Scotland)
- Novex Mini Gel Apparatus (Invitrogen, Scotland)
- 10X (Tris/Glycine/SDS) Running Buffer (National Diagnostics,UK)
- Molecular weight standard -Rainbow coloured marker ranging from 250kDa-10kDa (Amersham Life Sciences, Buckinghamshire).

2.5.1.2 Method

Samples (in electrophoresis buffer) were heated to 100°C for 4 minutes in a boiling water bath and loaded onto a pre-cast 4-20% Tris-Glycine with 1X Tris/Glycine SDS Running buffer, with 10µl of the rainbow marker and the samples were run at 125V, 35mA for 2 hours until the bromophenol blue dye was within 1cm of the gel base.

2.5.2 Protein detection

2.5.2.1 Materials

- The following antibodies were obtained from Becton Dickinson (UK) except for actin (Autogen, Bioclear UK)

Protein	Catalog No	MW (kDa)	Control	Dilution	Stock Conc^{n} µg/µl	Final Conc^{n} µg/µl
HIF-1α	H72320	120	HeLa+	1:250	250	1
MEKK3	M79820	71	SW-13	1:250	250	1
MKK3b	M85420	37	HeLa	1:250	250	1
JNKK1/ MKK4	M66420	44	Rat cerebrum	1:500	250	0.5
P38δ/ SAPK4	P72020	42	A431	1:500	250	0.5
MAPKp49/JNK1 SAPK1	M54920	49	PC12	1:250	250	1
JNK Phospho-Specific	S37220-050	43/56	Hela + Anisomycin	1:250	250	1
STI1/Hop-p60	S65720	62	A431	1:250	250	1
HO-1	H59320	32	SW-13	1:250	250	1
HO-2	H77220	36	SW-13	1:250	250	1
Cox-2	C22420	70	Macrophage	1:250	250	1
Actin	Sc-1616	43	-	1:500	0.2	0.0004

Table 4. Commonly used antibodies with working dilutions

- PVDF membrane, 3MM Whatman Filter Paper Sandwich (Invitrogen LC2002)
- Transfer buffer- (200ml) Tris Base (12mM)
  Glycine (96mM)
  Methanol (20%)
- Wash buffer - (800ml) 10mM Tris pH 7.5
  100mM NaCl
  0.1% Tween 20
- Goat Anti-Mouse IgG antibody (Becton Dickinson # M15345)
- Rabbit Anti-Goat IgG antibody (Dako #P0449)
- Enhanced Chemiluminescence plus (ECL+) detection kit (Amersham Pharmacia Cat No. RPN 2132)
- Kodax BioMax MR film (Sigma Aldrich).

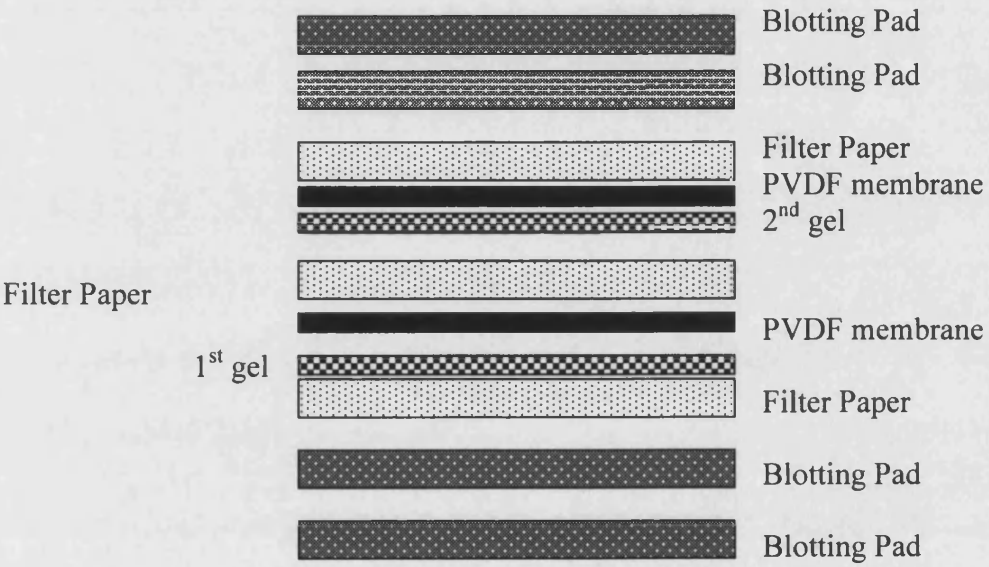
2.5.2.2 Eletrophoretic transfer of proteins to membrane

During Western blotting, proteins separated by SDS-PAGE were transferred from a gel to a solid matrix support. This was probed with antibodies that were specific to a particular antigenic epitope displayed by the target protein, allowing for the identification of specific proteins in complex mixtures without the need for immunoprecipitation.

2.5.2.2.1 Method

The filter paper was placed in transfer buffer, and the PVDF membrane was briefly soaked in 100% methanol, and rinsed twice for 5 minutes with distilled water, and equilibrated in transfer buffer.

The blotting pads were soaked in 700mls of transfer buffer to saturate. The gel was removed from its plastic cassette and placed in the apparatus for transferring as follows;



The module was closed and filled at the top with transfer buffer until covered. The outer buffer chamber was filled with deionised water, which ideally should be approximately 2cm from the top of the lower buffer chamber. The gels were transferred for 2 hours at 25V.

The apparatus was disassembled. Gels were placed in coomassie blue solution for 45 minutes at room temperature with gentle agitation and destained overnight to determine efficient transfer of proteins. The PVDF membranes with the transferred proteins were quickly placed in blocking buffer made up of 5% non-fat dried milk in wash buffer for 1 hour at room temperature with gentle agitation. For detection of phosphorylated proteins, it is generally not recommended to use milk as a blocking agent in conjunction with anti phosphotyrosine antibodies, as

milk contains an abundance of tyrosine phosphorylated proteins such as phospho-casein. However, there is more background if just milk alone was used, so a blocking buffer made of 1% non-fat dried milk, 1% BSA, 50mM sodium fluoride in 1X TBS with 0.05% Tween 20 was used. The primary and secondary antibody for phosphorylated proteins was also made up at the appropriate dilutions in this blocking buffer. The primary antibody for all other proteins were made in solutions in the appropriate dilutions in 5% non-fat dried milk in wash buffer with 0.05% Tween 20 and the membranes were incubated at 4°C overnight with gentle agitation. The membranes were washed with several changes every 5 to 10 minutes with wash buffer (0.01% Tween 20) for 1 hour at room temperature. The membrane was incubated in goat anti-mouse Ig (HRP conjugated) or rabbit anti-Goat Ig (HRP conjugated) in 5% non-fat dried milk in wash buffer with 0.05% Tween 20 in a 1:2000 dilution for 1 hour with gentle agitation. The membranes were washed with changes every 5-10 minutes in wash buffer (0.01% Tween 20). The antibody reacts strongly and specifically to mouse IgG in a number of immunochemical and immunological assays. After washing, the membranes were transferred to a dark room, excess wash buffer was drained and the signal developed by immersion in active ECL+ reagent at 40:1 mix of component A and B. Typically for 2 membranes of ~8.5cm by 7.5cm each, 2ml solution A and 50µl of Solution B were mixed together, protected from light and ~1ml of this solution used for each membrane and incubated for 5 minutes at room temperature. The excess was drained, and the membranes were wrapped in Saran wrap and exposed to autoradiographic film.

The ECL+ detection kit (Amersham Pharmacia) is a very sensitive method for the detection of immobilised specific antigens conjugated to HRP labelled antibodies. Initial exposures were of 10 seconds and extended as necessary. Exposed films were developed in a bench top developer (Compact X4, X-ograph Imaging Systems, Malmesbury, Wiltshire).

2.6 Powerblot-Protein Array

To complement the DNA Microarray, a protein array was carried out. Approximately 8mg of protein lysate was prepared from Day 8 3-D HepG2 cells, Day 15 3-D HepG2 cells and monolayer HepG2 cells as described in section 2.4. The samples were analysed by Western blot by Becton Dickinson (USA) (the Powerblot), using every antibody in their catalogue.

This technique on a larger scale can be described as proteomics, which is a complementary technology to DNA Microarrays to analyse patterns of gene expression at the protein level. Genes may be present but not necessarily transcribed. Proteins are usually the functional molecules. The PowerBlot data is provided as a file with all the proteins detected, in order of confidence, 1 through 5, with 5 being the highest confidence. The confidence level is based on fold change, reproducibility, and signal intensity. Briefly, level 5 includes proteins with changes greater than 2-fold in triplicate from good quality signals, level 4, changes 1.50-1.99-fold in triplicate from good quality signals and so on. In the results I have summarised the proteins in functional groups in confidence level 4 and 5, as these results are more rigorous.

2.7 Statisitics

Statistical analysis was performed using the two-sample student's t-test assuming unequal variances of the data ranges. Two-tailed tests were used to determine the significant differences between means and the levels of significance were assessed at the following probabilities:

- $p \leq 0.05$ = *
- $p \leq 0.005$ = **
- $p \leq 0.0005$ = ***

Chapter 3

The Ribonuclease Protection Assay to investigate transcriptional levels in 3-D cultures as a cause for the diminished function observed

Chapter 3- The Ribonuclease Protection Assay to investigate transcriptional levels in 3-D cultures as a cause for the diminished function observed

3.1 Causes for downregulation of function

Various hypotheses were investigated previously as possible causes for this down regulation of function as described in the introduction.

Having ruled out simple physical explanations, the first part of this thesis serves to explore changes in transcriptional levels within the different cultures to see whether key genes are being switched on and off during periods of peak and diminished function.

The initial hypotheses as described in chapter 1, was that a) different transcription factors are present at times of peak function, 3-D culture (at Day 8) compared to monolayer culture and times of diminished function in 3-D culture (at Day 15) compared to peak function (Day 8) and b) decreased levels of liver-enriched transcription factors in Day 15 3-D cultures are contributing to the diminished amount of liver specific proteins such as albumin and AAT compared to peak function at Day 8.

3.2 Transcriptional control of liver specific function

Gene regulation is the basis for the phenomenon of differentiation by which different cells become structurally and biochemically specialised for different functions. Actions and properties of each cell are determined by the proteins they contain which is determined by its corresponding mRNA, frequency of mRNA translation and its stability. Although gene function is conducted via its protein product, there are several observations that the amount of protein produced is directly dependent on the amount of mRNA that encodes it (Cereghini 1996;Edwards 2003;Keene 2003).

Performance per cell diminishes at Day 15 compared to Day 8, and is generalised rather than limited to specific function, therefore the level of control of expression is more likely to be at the transcriptional level.

3.2.1 General overview of gene control

In eukaryotes there are a number of stages between initial synthesis of primary RNA transcript and production of mRNA. The initial transcript is modified at its 5' end by addition of a cap structure containing a modified guanosine residue and the cap is an unusual 5'-5' triphosphate linkage. The 5' end is further modified by methylation of N7 of the terminal guanine. This "cap" serves several functions including protecting the 5' end from phosphatases and nucleases, thus enhancing the efficiency of mRNA translation. RNA splicing can be used by cells to regulate gene expression to create different protein products from a single gene. Alternative splicing of the primary transcripts produced from complex transcription units is often regulated to yield different mRNAs (Cho and Campbell 2000;Proudfoot 2000). An example of this method of gene control comes from the fibronectin gene. Fibronectin is an ECM glycoprotein involved in cell adhesion and migration (Yamada *et al.* 2003). The multiple exons in the fibronectin primary transcript are spliced to produce two isoforms of fibronectin, one secreted by hepatocytes and the other by fibroblasts and other cell types. Splicing of fibronectin pre-mRNA in fibroblasts yields an mRNA containing two exons, which encodes protein domains that interact with cell-surface receptors making fibroblast fibronectin adherent to cell surfaces. However, splicing of fibronectin pre-mRNA in hepatocytes excludes those two exons, which make the fibronectin protein adherent so that the fibronectin secreted by hepatocytes is not strongly adherent to cell surfaces, but is free to circulate in the serum (Misteli 2000).

3.2.1.1 Transcription

Control of the transcription process occurs at DNA sequences usually upstream of the transcription start site. Two types of regulatory DNA sequences exist, promoters and enhancers (Cereghini 1996).

3.2.1.2 RNA synthesis

The TATA box in the promoter of the gene, determines precisely where RNA synthesis begins (located 20-30 base pairs upstream of the start site). Promoters also contain a CAAT box and GC box usually – 40 and –110 bases respectively from the transcription start site. Enhancers increase the rate of transcription of specific genes and serve as recognition sites for transcription activator proteins (Zohar *et al.* 2001).

The basic transcriptional mechanism begins with synthesis of mRNA in the nucleus. Transcription factors (DNA binding proteins) and coactivators bind to enhancer sequences. They are joined by general transcription factors and RNA polymerase II to assemble a transcription initiation complex at the promoter.

RNA polymerase is positioned at a promoter by transcription factors and binds to double-stranded DNA. RNA polymerase can initiate transcription of DNA into RNA, unwinding the duplex, separating the base pairs of DNA. A second ribonucleotide pairs with the template and joins to the first base by a phosphodiester bond. Polymerase copies the DNA, separates the DNA base pairs ahead and forms base pairs behind. RNA is elongated as the polymerase transcribes the gene. Only one DNA strand is transcribed into RNA (Misteli 2000). A general class of general initiation factors (TFIIB, TFIID, TFIIE, TFIIF and TFIIH) function intimately with RNA polymerase II and are required for selective binding of polymerase to its promoters, formation of the "open" complex, and synthesis of the first few phosphodiester bonds of nascent transcripts (Krumm *et al.* 1995). *In vitro* studies have shown how additional constitutive factors such as DNA topoisomerase I and other adaptor proteins are important for transcription initiation and activation (Umehara and Horikoshi 2003). Protein-protein interactions are important and many DNA binding proteins involved in transcriptional activation do not interact with the basic transcriptional machinery but provide the structural components necessary to bring distal activators in association with general factors (Vellanoweth *et al.* 1994).

3.2.2 Liver-specific transcription

Present knowledge of molecular signals that govern liver development and differentiation has come from analysis of experimental systems including the developing embryo, cell and tissue culture, knockout mice and transplantation of hepatic precursor cells. Growth factors and families of transcription factors including hepatocyte nuclear factors (HNF) 1, 3 and 4 and CCAAT/enhancer binding protein (liver-enriched transcription factors), abundant in the liver but not exclusive to the liver, are important elements required for cell differentiation (Hayashi *et al.* 1999).

The tissue specific expression of genes is based on the presence of *cis*-acting sequences in the promoter and enhancer regions that interact with sequence specific DNA binding domains in nuclear transcription factors, which can enhance or depress transcription. The promoter sequence of each gene is unique and contains a number of recognisable motifs that allow regulation of expression of that gene by a group of transcription factor molecules. These DNA binding proteins bind to their complementary promoter sequences (Cereghini 1996). The determination of the tissue distribution of these factors and analysis of their relations has led to the hypothesis that the association of liver-enriched transcription factors and ubiquitous transcription factors such as AP-1 (Activator Protein), Oct-1 and CoupTFI (ovalbumin upstream promoter transcription factor) is important and perhaps even sufficient for the maintenance of liver-specific gene transcription and maintenance of a fully differentiated state (Darlington 1999;Hayashi *et al.* 1999;Zaret 1996). There are two transcription factor complexes, nuclear factor NFκB and Stat3 that are activated as part of the initial response of the remnant liver following partial hepatectomy (Darlington 1999).

Cultured cells can show a decrease or a complete loss of expression of tissue-specific function, however present knowledge has been gained from a combination of cultured primary cells, cell lines and studies during development. All of these have their limitations, but each has provided some insights. For example HNF-3β, a member of the hepatocyte nuclear 3 family is required for survival of the embryo so HNF-3β is not readily amenable to analysis by knockout strategies (Hayashi *et al.* 1999).

3.2.3 Transcriptional regulation – liver enriched transcription factors (LETFs)

3.2.3.1 The homeoproteins

The homeoproteins are a family of proteins that share a common domain of 60 amino acid residues, the homeodomain, conserved throughout evolution from yeast to human. These homeodomains act as transcription factors.

Hepatocyte nuclear factor 1 (HNF-1) is a homeodomain containing a transcriptional regulator that is essential for the liver-specific expression of albumin, AFP, AAT and transthyretin and the hepatitis B virus large surface protein (Hayashi *et al.* 1999). The HNF-1 family includes HNF-1α and HNF-1β (also called variant HNF-1). It recognises a pseudopalindromic sequence (consensus g/aGTTAATNATTAACc/a) that is present in the promoter regions of many liver specific genes (Jacob *et al.* 1997). The functions of different transactivation domains localised at their C-terminal regions show that HNF-1α has a higher potency of transactivation than HNF-1β. HNF-1α is expressed in well differentiated hepatoma cell lines such as H4II, Fao and HepG2, but is absent in the de-differentiated variants H5 and C2 (Babajko *et al.* 1993;Bailly *et al.* 1998). On the other hand HNF-1β retains its expression in some de-differentiated variants and somatic cell hybrids. An HNF-1α binding site has been identified in the HNF-4 promoter (Kuo *et al.* 1992). In H5 cells, which lack liver specific expression, transfection of HNF-4 activates the HNF-1 gene. Although the H5 variant cells lack HNF-4 and HNF-1, other transcription factors CAAT Enhancer Binding Protein (C/EBP)α and HNF-3 are present in cells lacking liver specific gene expression which suggests that liver specific genes are regulated by combinations of transcription factors rather than a single factor (Darlington 1999;Hayashi *et al.* 1999). Studies have also shown that HNF-1 and HNF-4 mRNA levels are influenced by ECM and that ECM could stimulate cell differentiation by activating HNF-4 gene expression. HNF-1 can also act synergistically with transcription factors of different functions e.g. C/EBP. One example is the activation of human albumin (Nagaki *et al.* 1995).

The HNF-3 family contains to date three proteins, HNF-3α, HNF-3β, HNF-3γ which have been identified. These proteins share a high homology in the winged

helix/fork head DNA binding domain and in two short similar regions in their C-terminal and N-terminal regions that show transactivity. The principal contact surface is provided by the α-helix, which is flanked by two long loops that contact the DNA backbone. The consensus recognition sequence is A(a/t)TRTT(g/t)RYTY (R:purines; Y:pyrimidines) (Darlington 1999;Vallet *et al.* 1995). In combination with other liver-enriched transcription factors such as HNF-1, HNF-3 binds and transactivates numerous liver specific genes such as transthyretin, albumin, and AAT. The HNF-3 factors have been implicated in the nucleosomal organisation of the albumin gene and also found to regulate the HNF-4 promoter (Hayashi *et al.* 1999). A study analysing the role of HNF-3 transacting factors on the expression of liver specific genes in differentiated hepatocytic cells produced a truncated protein that inhibits the access of native HNF-3 factors to DNA. The data showed that overproduction of this truncated protein decreased the expression of albumin, transferrin and transthyretin, whereas a decrease in truncated protein production restored the expression of some of these genes in differentiated hepatoma cultured cells (Benvenga and Robbins 1998;Darlington 1999;Hayashi *et al.* 1999). The concentration of HNF-3α increases when hepatocyte derived cell lines are cultured on a thick ECM gel substratum as opposed to monolayer culture on plastic. This substratum induces differentiated cell morphology and liver specific gene transcription in primary cultures. HNF-3β is not often amenable to analysis by knockout strategies, as its function is required for the survival of the embryo (Darlington 1999).

HNF-4 is expressed in the liver, kidney and intestines in the adult and is constitutively transcribed at low levels. HNF-4, first identified by its interaction within the transthyretin and AAT promoters, is part of the nuclear hormone receptor family that is expressed in hepatic diverticulum at the onset of liver development. The consensus site for HNF-4 is GGGTCAAAGGTCA. It activates a diverse set of liver genes responsible for a variety of functions. For example, transthyretin whose normal function is to transport thyroid hormones and other hydrophobic substances in the blood (Boobis *et al.* 1996), and AAT, a serine protease inhibitor found in plasma, and are both regulated by HNF-4 (Hu and Perlmutter 2002). Genes also activated by HNF-4 include OTC, the rate-

limiting enzyme in the urea cycle and the cytochrome P450s (Peterson and Renton 1986). These genes are activated by HNF-4 in early liver development (Bulla 1997). No specific ligand for HNF-4 has been identified. Its C-terminal ligand binding domain is involved in transactivation, ligand binding and protein dimerisation. In genetic analysis of hepatocyte differentiation, HNF-4 can transactivate endogenous HNF-1α and liver genes such as AAT; it can also induce re-differentiation of a de-differentiated hepatoma cell line, H5, by stable transfection of exogenous HNF-4 (Bulla 1997). HNF-4 interacts with regulatory elements in promoters and enhancers of genes whose products are involved in cholesterol and amino acid metabolism, gluconeogenesis and blood coagulation. HNF-4 is phosphorylated at tyrosine, serine and threonine residues and various inhibitors and stimulators of protein kinase pathways modify the HNF-4 mediated transcriptional activation. Members of the CoupTF family can act as negative regulators of the transactivation exerted by HNF-4. The effects of HNF-4 are also important for the maintenance of differentiated hepatic epithelial cell morphology by inducing re-expression of E-cadherin (Zaret 1996).

HNF-6 has been described relatively recently. It contains two different DNA binding domains. One corresponds to a novel type of homeodomain and the other is homologous to the *Drosophila cut* domain. HNF-6 is expressed in tissues that originate from the endoderm. HNF-6 binding sites are found in the promoter regions of HNF-3β, HNF-4, transthyretin, and AFP. HNF-6 expression disappears transiently from the liver between embryonic Days 12.5 and 15, but reappears in the liver after Day 15 and this parallels the expression of HNF-3β transcripts (Liu *et al.* 1991;Zaret 1996).

3.2.3.2 CCAAT/Enhancer Binding Protein(C/EBP) Family

C/EBP was originally isolated from rat as a protein binding to CCAAT and contains a basic leucine zipper domain involved in DNA recognition (Darlington 1999;Zaret 1996). The consensus binding site for C/EBP is the sequence ATTGCGCAAT. C/EBPα mRNA transcripts have been identified in a wide range of cells but the protein itself is detected only in differentiated hepatocytes and adipocytes. C/EBP stimulates the transcription of genes such as albumin and

transthyretin. This transcription factor is required for the conversion of preadipocytes into fully differentiated adipocytes (Gonzalez and Lee 1996;Zaret 1996). Constitutive antisense C/EBPα mRNA impairs the expression of C/EBPα itself and adipocyte specific genes. Proliferating hepatocytes in the hepatectomised liver show a reduction of C/EBPα mRNA within the first three hours of surgery, corresponding to transition from G0 to the G1 phase of the cell cycle, which returns to normality by 72 hours, through a gradual increase after the S phase. Induction of C/EBPα expression in HepG2 cells results in reversible arrest of proliferation and delays tumorigenesis in immunodeficient mice (Zaret 1996).

C/EBPβ is critical for liver regeneration and positively regulates gluconeogenesis through activation of the phosphophenolpyruvate carboxykinase gene. Several signalling pathways can be stimulated post-translationally by C/EBPβ. Calcium-dependent kinases can mediate phosphorylation and increase its transactivation potential (Darlington 1999). Among the isoforms of the C/EBPβ gene is the liver enriched inhibitory protein (LIP) from which the amino-terminal transactivation domain is missing (Grimm and Rosen 2003). One mechanism by which LIP is generated is through use of alternate AUG start sites (Xiong *et al.* 2001). LIP dimerises with CEBPβ to reduce its transcriptional activation.

Finally, in the C/EBP family is C/EBPδ, which mediates that regulation of acute phase response; it has low activity until activated by inflammatory stimuli (Liu *et al.* 1991;Patel *et al.* 1995).

In the liver, these families of transcription factors do not work in isolation but as a regulatory network in particular with respect to cytochrome P450 genes. Several different liver-enriched transcription factors including HNF-1α, HNF-3, HNF-4 and C/EBPβ and more ubiquitous transcription factors, Sp1, GABP α/β and NF2d9 are responsible for governing the transcription of cytochrome P450 genes (Celis *et al.* 2000;Rana *et al.* 1995).

3.3 Techniques to measure gene regulation

Various experimental approaches could be taken to assess gene expression and investigate presence of and/or increases or decreases in levels of liver enriched transcription factors. These may contribute to the upregulation of function, and be achieved by comparing Day 8, 3-D spheroids (peak function) with monolayer HepG2 cells (less function). Investigation of the liver enriched transcription factors which may contribute to diminished function can be achieved by comparing Day 15 spheroids to Day 8 spheroids. These techniques include using the ribonuclease protection assay, which was used in this study.

3.3.1 Ribonuclease Protection Assay

The Ribonuclease Protection Assay (RPA) is an extremely sensitive method for the detection and quantitation of mRNA species in a complex mixture of total cellular RNA (Prediger 2001). A labelled probe is synthesized complementary to part of the target RNA to be analysed using a PCR primer that contains the T7 promoter sequence. After hybridisation of the probe with target RNA, the mixture is treated with RNase to degrade single-stranded, unhybridised probe. Labelled probe hybridising to its complementary target RNA is protected from nuclease digestion and can then be separated on a polyacrylamide gel and visualised by autoradiography (for radioactively-labelled probes). The advantages of RPAs are that low abundance mRNAs can be detected and quantified. There is no loss of signal, which may occur by northern blotting, which require transfer to a solid support, and as the probes in the RPA are shorter than the RNA species being detected, the target RNA can be less intact, without affecting the RPA. The RPA also allows simultaneous detection of multiple targets in a single sample (Henttu 2001;Tymms 1995).

3.3.1.1 Methods

Total RNA was prepared from HepG2 cells in monolayer and cultured in alginate at Day 8 and Day 15 of culture and human liver as previously described in Section *2.3.*

3.3.2 Checking RNA integrity

3.3.2.1 Materials

- Agarose (Molecular Biology Grade)
- 0.1% DEPC H_2O
- 10X 3- (*N* -morpholino)propanesulfonic acid (MOPS)
- Formaldehyde
- Formamide
- 0.24 - 9.5 kB RNA ladder (Invitrogen Cat No. 15620-016)
- Bromophenol Blue (0.25% /15% Ficoll)
- Ethidium Bromide (10mg / ml)
- 10X Running Buffer

  17g MOPS

  1.64g NaAc

  8ml 0.5M EDTA pH 8.0

  5.7 ml 5M NaOH

  Make up to 400ml with DEPC H_2O. Autoclave and protect from light. Straw-coloured buffer works well, but darker buffer doesn't.
- Sample buffer

  10X Running Buffer - 100µl

  Formaldehyde - 178µl

3.3.2.2 Method

Before running the samples, the sample buffer was checked to ensure that the formamide used was sufficiently deionised. To check the sample buffer, 5µl of H_2O, 12.5µl of deionised formamide, 7µl of sample buffer and 5µl of Bromophenol blue/Ficoll mix was added together. The mixture should be a

denim/sky blue colour. If the mixture was green/blue it indicated that the formamide had become ionised.

For a 150ml gel, 1.1% agarose gel was prepared in 16.5ml of 10X Running Buffer, with 118.95ml of DEPC H_2O. The agarose was dissolved by heating, and once cooled, (less than 60°C), 29.1ml of formaldehyde was added, and the gel poured.

For approximately 5µl of RNA solution, 12.5µl of formamide solution was added and mixed thoroughly. The solution was heated for 5 minutes at 50°C. 7µl of sample buffer (formaldehyde and 10X Running Buffer) was added and mixed thoroughly. The solution was heated for 8 minutes at 65°C and a further 5µl of bromophenol blue/Ficoll mix was added and stored on ice ready to load onto the agarose gel.

The samples were loaded with 4µl of the RNA ladder, and run in 1X Running Buffer at 100V for 3 hours changing the running buffer every 30 minutes until the bromophenol blue dye had migrated two-thirds the length of the gel. The gel was removed and in the dark stained in 800mls of 1X Running Buffer with 100µl of 10mg/ml ethidium bromide for 20 minutes, and destained by washing in 1X Running Buffer alone, changing the Running Buffer until the bands were visible. The gel was photographed by short-wave UV transillumination (300nm) on a gel documentation system and the densities of the 28S and 18S bands measured to obtain a ratio (Epi Chemi II Darkroom, Ultra Violet products, Cambridge).

3.3.3 Preparation of template DNA

3.3.3.1 Methods

RNA probes were generated by *in vitro* transcription with T7 polymerase and DNA template. DNA templates were produced by polymerase chain reaction (PCR). PCR primers were designed with the 19 base T7 promoter region TAATACGACTCACTATAGG from the primer selection site on the 'Human Genome Mapping Project Resource Centre' (http://alces.med.umn.edu/rawprimer.html). Probes were designed such that the size of the protected fragment is at least 10% different from any other full-length

probe or protected fragment.

Table 5 Design of primers for liver-enriched transcription factors

Transcription Factor	Size (bp)	Primer Sequence
HNF-1	424	5'- CAGCTCCTCTGTCTCGAGCG-3' (F) 5'- TAATACGACTCACTATAGGTCCCAGGGT AGACGCGGTA-3' (R)
HNF-4	292	5'-ATCCAGGGAAGATCAAGCGG-3' (F) 5'-TAATACGACTCACTATAGGTTGTGTTGGC AACGATGACG-3' (R)
C/EBPα	139	5'- AGGAACACGAAGCACGATCAGT-3' (F) 5'-TAATACGACTCACTATAGGAATGGTGGTTT AGCAGAGACGGTA-3' (R)
C/EBPβ	181	5'- GACAAGCACAGCGACGAGTACA-3' (F) 5'-TAATACGACTCACTATAGGCAGCTGCTTG AACAAGTTCCG-3 (R)
Human Albumin	368	5'- CCCTGTGCAGAAGACTATCTATCCG-3' (F) 5'-TAATACGACTCACTATAGGGGCAAAGCAG GTCTCCTTATCG-3' (R)

3.3.3.2 First strand cDNA synthesis from human liver mRNA

3.3.3.2.1 Materials

- Oligo (dt), 500ug/ml (Sigma Genosys)
- Recombinant RNasin RNase Inhibitor (Promega)
- SUPERSCRIPT II RNase reverse transcriptase kit (Cat No. 18064-014) (Invitrogen, Scotland)
- 0.1M Dithioreitol (DTT)
- 0.1M dNTP mix.

3.3.3.2.2 Method

5µl of Oligo (dT), 5µg of total RNA and nuclease free sterile water were made up to a volume of 100µl. All components were mixed thoroughly and heated at 70°C

for 10 minutes and immediately placed on ice. The contents of the tube were collected by pulse centrifugation (Eppendorf 5402, Germany). 20µl of 5X First Strand Buffer, 10µl of 0.1M DTT, 5µl of 10mM dNTP mix and 2µl of RNasin Inhibitor were added to the tube and mixed gently. The mixture was incubated at 42°C for 2 minutes. 5µl (1000 units) of SUPERSCRIPT II was added, mixed gently with a pipette and incubated for 1 hour at 45°C. The reaction was inactivated by heating at 70°C for 15 minutes.

3.3.3.3 Polymerase Chain Reaction to generate DNA templates
3.3.3.3.1 Materials

- PCR primer pairs (Sigma Genosys)
- Advantage-GC cDNA Polymerase Mix (Cat No. 8433-1, Clontech Labarotories)
- 10mM dNTP mix.

3.3.3.3.2 Method

PCR Reaction	X1	X6
PCR-grade H_2O	14	84
5X GC cDNA PCR	10	60
Reaction Buffer GC-melt (5M)	10	60
dNTP mix (10mM)	5	30
Advantage-GC cDNA Polymerase mix	1	6
Forward Primer (10pmol / µl)	2.5	
Reverse Primer (10pmol / µl)	2.5	
cDNA template	5	

The reagents were made up to a final volume of 50µl with 2 negative controls replacing template DNA with water and using primers for human albumin which is abundant in the liver. The components were mixed thoroughly and centrifuged

for 1 minute at 10000g (Eppendorf 5402, Germany) to collect the contents of the tube.

3.3.4 PCR cycling conditions

Reactions were carried out in a Perkin-Elmer DNA Thermal Cycler Model 480

Initial denaturation	94°C for 5 minutes	
Denaturation	94°C for 20 seconds	} For 30 cycles
Annealing	55°C for 20 seconds	
Extension	68°C for 50 seconds	
Final extension	68°C for 7 minutes	
Cooling	4°C for 10 minutes.	

The samples were stored at 4°C until ready for further analysis by agarose gel electrophoresis.

3.3.4.1 Gel Electrophoresis

3.3.4.1.1 Materials

- Agarose (Molecular Biology Grade, Invitrogen)
- 1x Tris Acetic acid, EDTA (TAE)
- Ethidium bromide (10mg/ml)
- Hyperladder IV Cat no. 109F (Bioline Reseach)
- Loading Buffer
- 6X TAE
- 30% glycerol
- 0.25% Bromophenol Blue.

3.3.4.1.2 Method

100ml, of a 2% agarose gel was prepared in 1X TAE with 5µl of ethidium bromide and placed in a gel tank with 1X TAE as running buffer with ethidium bromide (50µl of ethidium bromide in 1 litre of TAE).

1µl of loading buffer was added to 5µl of the PCR reaction, and loaded into the

wells of the agarose gel, using 5µl of Hyperladder DNA marker, ranging from 100-1000bp. The gel was electrophoresed at 100V and stopped when the bands had migrated two-thirds the length of the gel. The bands were visualised under a UV transilluminator (Ultraviolet Products, Cambridge).

3.3.5 Clean up of double stranded DNA in PCR template
3.3.5.1 Materials

- Wizard PCR Preps DNA Purification System (Promega, Madison, UK)
- Low melting point agarose gel (Sigma Aldrich, UK).

3.3.5.2 Methods
Samples were separated on a 1% low melting point agarose gel (poured in a cold room) (5µl of 10mg/ml ethidium bromide in 100ml of 1x TAE buffer), until they had migrated two-thirds of the gel. Bands were excised and cleaned up using a purification system.

Briefly, the agarose section with the desired fragment was transferred to a microfuge tube and heated in a waterbath at 70°C until it had dissolved. 1ml of resin, which binds DNA, was added to the melted agarose. The sample was mixed without vortexing. The resin/DNA mix was transferred to a syringe barrel of a column and pushed into the minicolumn with the plunger. The plunger was removed and 2mls of 80% isopropanol was added to the syringe to wash away salts. The column was removed from the syringe and transferred to a centrifuge tube. The tube was centrifuged for 2 minutes at 10000g at room temperature (Eppendorf 5402, Germany) to dry the resin. The column was transferred to another tube and 50µl of dH_2O was added. After approximately 1 minute the tube was centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany) to elute the DNA. A small sample was run out on a 1% agarose gel to ensure there was efficient recovery of the preparation.

3.3.6 Radiolabelling of cDNA probes

MAXIscript, *in vitro* transcription can be used to incorporate virtually any labelled nucleotide into RNA. Traditionally 32P labelled UTP is used. The high promoter specificity of the T7 RNA polymerases allows the transcription of one strand of the template with virtually no cross-talk from the promoter on the opposite strand. As a positive control pTRI RNA 18S antisense control template was used, containing an 80bp insert of a highly conserved region of the human ribosomal RNA gene and was used as an internal standard or reference. 18s rRNA makes up 20% of total cellular RNA.

3.3.6.1 Materials

- Maxiscript In Vitro Transcription Kit, Cat No. 1312 (Ambion, UK)
- 18S rRNA cat no 7339 (Ambion, UK)
- ^{32}P UTP (18.5 MBq) Cat no. PB20383 (Amersham, Buckinghamshire).

3.3.6.2 Method

4 RNA probe samples and 2 internal controls rRNA (18S)

	Transcription factor samples	Control
Nuclease-free dH_2O	3.25µl	10.6µl
DNA template	2.0µl	/
10x Transcription Buffer	1.0µl	2.0µl
10mM GTP	0.5µl	1.0µl
10mM CTP	0.5µl	1.0µl
10mM ATP	0.5µl	1.0µl
^{32}P UTP (800 Ci /mmol, 10mCi/ml)	1.25µl	0.4µl
T7 RNA polymerase	1.0µl	2.0µl
PTRI 18S (rRNA internal control)	/	2.0µl
Total volume	10µl	20µl

All the contents above were mixed thoroughly by pipetting, and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany), and were incubated at 37°C for 2 hours. During this incubation period, the denaturing acrylamide gel was prepared for probe purification as follows:

Component	Volume (ml)
20% acrylamide (in 7M Urea + 1x tris boric acid EDTA (TBE) in DEPC H_2O)	17.5
7M urea and TBE	52.5
25% ammonium persulphate	0.153
TEMED	0.066

After 2 hours incubation, 1µl of sample was taken from each of the samples and put into corresponding tubes with 49µl of RNase free dH_2O (1/50 dilution). 0.5µl of DNase was added to the transcription factor samples and 1.0µl to the control samples. The samples were thoroughly mixed, vortexed and incubated at 37°C for 30 minutes. An equal volume of gel loading buffer was added to the samples (9.5µl to the test samples and 20µl to the control samples). The samples were mixed and heated for 5 minutes at 95°C. The wells of the gel were thoroughly washed with the running buffer made of 1X TBE in DEPC treated water to rinse out any urea, prior to sample loading on a gel prepared for probe purification. The gel was run at 300V until the leading dye band (bromophenol blue) had just run off the bottom of the gel.

While the gel was running, 5µl of the 1/50 diluted samples were pipetted onto 2 glass microfibre filter papers. One of the filter papers with the sample represented total counts and was left to dry on the bench and the other was placed on a vacuum manifold, washed twice with cold 10% trichloroacetic acid (TCA), once with methanol, and once with acetone, and represented the precipitated RNA i.e. the proportion of the UTP incorporated into RNA. These samples were left to dry. Each sample on a filter was placed in a vial with scintillation fluid and counted in a β counter (Beckman Coulter, CA, USA); specific activity could be

determined from % radioactivity incorporation, total cpm and mass of probe from the samples.

The gel was transferred to 3MM Whatman filter paper and covered with Saran wrap. Flourescent stickers were placed on 3 corners of the gel and the gel was exposed to photographic film (Kodax Biomax Cat No. 8294985) for approximately 2 minutes. The appropriate bands were cut out of the film and used as a template to cut the correct bands out of the gel; these were placed in corresponding sterile microfuge tubes to which 300µl of elution buffer was added. The samples were immersed in the buffer and incubated overnight at 37°C.

3.3.7 Hybridisation of sample RNA and radiolabelled probe

After incubation of samples in elution buffer, 2µl of each test sample and 5µl of the control sample were spotted on filter paper to calculate cpm/µl. A total volume containing 1x 10^4 cpm of each of the labelled probes was mixed with 10µg of total RNA from monolayer HepG2 cells, Day 8, 3-D HepG2 cultures and Day 15 3-D HepG2 cultures containing the same cell numbers, and human liver. In addition, to show comparative expected band sizes, the probes were mixed with human liver alone including the 18S control and with yeast RNA alone.

Two controls were set up for each probe to verify the efficiency of the hybridisation procedure. The tubes contained the same amount of labelled probe used for the experimental probes and yeast RNA equivalent to the highest amount of sample RNA (0.9µl). The volumes were adjusted with H_2O and the probes and sample RNA were co-precipitated with ammonium acetate (0.5M, 20µl) and 2.5 volumes of neat ethanol (0.5ml). Samples were incubated at -20°C for 30 minutes and the RNAs were pelleted by centrifugation at 10000g at 4°C, for 30 minutes (Eppendorf 5402, Germany). The supernatant was removed taking care not to dislodge the pellet, and tubes were centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany) to remove any residual liquid. Pellets were dried for 5 minutes on the bench. The samples were resuspended in 10µl of hybridisation buffer, vortexed to thoroughly mix for 5-10 seconds and then incubated at 95°C for 4 minutes to denature the RNA in solubilisation buffer. The samples were

incubated at 42°C overnight to hybridise probe to its complement in the sample RNA.

3.3.8 RNase digestion of hybridised probe and sample RNA

Following hybridisation, RNase digestion buffer was made up with RNase A/RNase T1 mix at a dilution of 1/50, (higher than the recommended 1/100 dilution suggested with the kit) and 150µl of the RNase mixture was added to each sample RNA and to one of the control tubes with yeast RNA prepared for each probe in the experiment. Theses tubes serve as positive controls for the function of the RNases and to check that the probe is being protected in the absence of homologous sequence. The samples were vortexed and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany). RNase digestion buffer without RNase was added to the remaining yeast RNA controls. These tubes served as a control for probe integrity and should show a single band migrating of the expected size. All the samples were thoroughly mixed and vortexed and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany) to collect all the contents at the bottom of the tube. The samples were incubated at 37°C for 30 minutes. During this incubation, RNase will digest all unhybridised RNA and probe molecules leaving only the double stranded region in the RNA-RNA hybrids protected from nuclease digestion. Following the incubation 225µl of RNase Inactivation/Precipitation Solution was added with 150µl of ethanol to aid the precipitation of these very small fragments. The tubes were vortexed and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany). Samples were left at -20°C for 30 minutes and centrifuged at 10000g at 4°C for 30 minutes (Eppendorf 5402, Germany) to pellet the precipitated products of the RNase digestion. The supernatant was carefully removed as the pellet does not adhere to the tube. Final traces were removed by re-centrifuging the tubes for about 5 seconds and then leaving them on the bench for 5 minutes.

3.3.9 Separation and detection of protected fragments

The pellets were resuspended in 4µl of gel loading buffer, vortexed and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany).

All the negative controls (no target/no RNase) were further diluted 1/10 as these were expected to have more counts than other samples. Samples were then incubated at 95°C for 5 minutes to completely solubilise the RNA, vortexed and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany). The tubes are stored briefly on ice before loading onto a gel, prepared as follows:-

Component	Volume (ml)
7M urea and TBE	84
20% acrylamide and TBE	36
25% ammonium persulphate	0.262
TEMED	0.112

Prior to loading, the wells of the gel were rinsed with running buffer. The gel was run at 300V until the leading dye band had migrated to the bottom of the gel, this typically took about 3 hours. The gel was soaked in 10% acetic acid for 40 minutes to fix and then transferred to filter paper, covered with Saran wrap and dried on a gel dryer for 1 hour, with heat at -80°C. The gel was then exposed to radiographic film (Kodax Biomax Cat No. 1435726) for an appropriate time with an intensifying screen to detect labelled probes.

Results

3.3.10 Investigating expression of liver-specific transcription factors as a possible mechanism for down regulation of function at the mRNA level

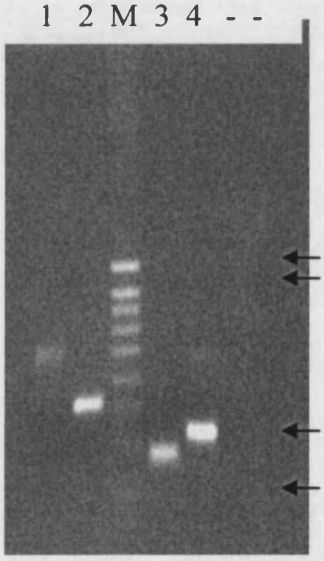
3.3.10.1 RNA extraction

Sample	Dilution	A_{260}	A_{280}	$A_{260:280}$	µg RNA	Total volume (µl)	µg/µl
Human Liver 1	1:50	0.176	0.106	1.70	176	500	0.352
Human Liver 2	1:50	0.228	0.138	1.65	228	500	0.456
Human Liver 3	1:50	0.247	0.140	1.76	247	500	0.494
Day 8	1:50	1.076	0.60	1.80	1076	500	2.151
Day 15	1:50	0.474	0.236	2.01	474	500	0.948
Monolayer	1:50	1.151	0.692	1.68	1151	500	2.302

Table 6 Absorbances and yields of RNA extracted from different samples used in the RPA

3.3.10.2 Reverse transcription and PCR reaction

cDNA was made from human liver RNA generated by reverse transcription. The cDNA was amplified by PCR using specific primers.



The molecular weight markers are 1000, 800, 700, 600, 500, 400, 300, 200, 100bp

Fig. 4 PCR products of (1) HNF-1, **424bp** (2) HNF-4, **292bp** (3) C/EBPα, **139bp** (4) C/EBPβ **181bp** and (-) negative control with water and albumin primers.

The PCR products were run on a 1% agarose gel prior to *in vitro* transcription (Fig.4), to check they were of the expected size.

3.3.10.3 Detection of Radiolabelled cDNA probes

The PCR products were transcribed with T7 RNA polymerase using ^{32}P UTP to generate radiolabelled RNA probes. The probes were purified on a denaturing acrylamide gel to remove any unincorporated nucleotides (Fig.5). This shows that the radioactively labelled probes have been purified as shown by the strongest band at the top of each lane, representing a transcription factor.

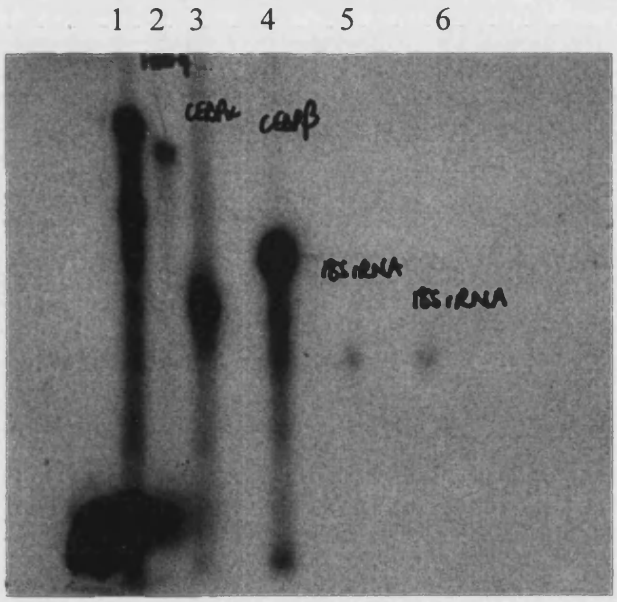


Fig. 5 ^{32}P radioalabelled probes for transcription factors. The strongest band at the top of the "smear" is the product for each transcription factor.

1, HNF-1

2, HNF-4

3, C/EBPα

4, C/EBPβ

5, 18S rRNA (internal control)

6, 18S rRNA (internal control).

3.3.10. 4 Expression of mRNA levels

Each transcription factor and the 18S rRNA control was incubated with human liver alone to facilitate identification of the bands obtained with mixed probes in the monolayer HepG2 cells, Day 8 and Day 15 HepG2 cells in 3-D culture reactions (Fig.6).

In addition the following controls were run :-

1) negative controls - yeast RNA was incubated with each individual probe without the addition of RNase to indicate the integrity of the probe. (Band present).
2) positive control - yeast RNA was incubated with each individual probe and digested with RNase. (No protection and band absent).
3) 18S rRNA in all the samples with each target RNA and all the probes- internal control and therefore signal should be the same in each sample.

For comparison between samples the area density for the 18S spot in each sample was divided by that in the liver sample and the area density of the transcription factor bands in the same lanes divided by these values.

The results in Fig.6 below show that there was insufficient digestion of the positive controls with yeast RNA and RNase, and therefore the results could not be interpreted as protected probes could not be distinguished from undigested probes.

3.3.10.4.1 Detection of mRNA levels

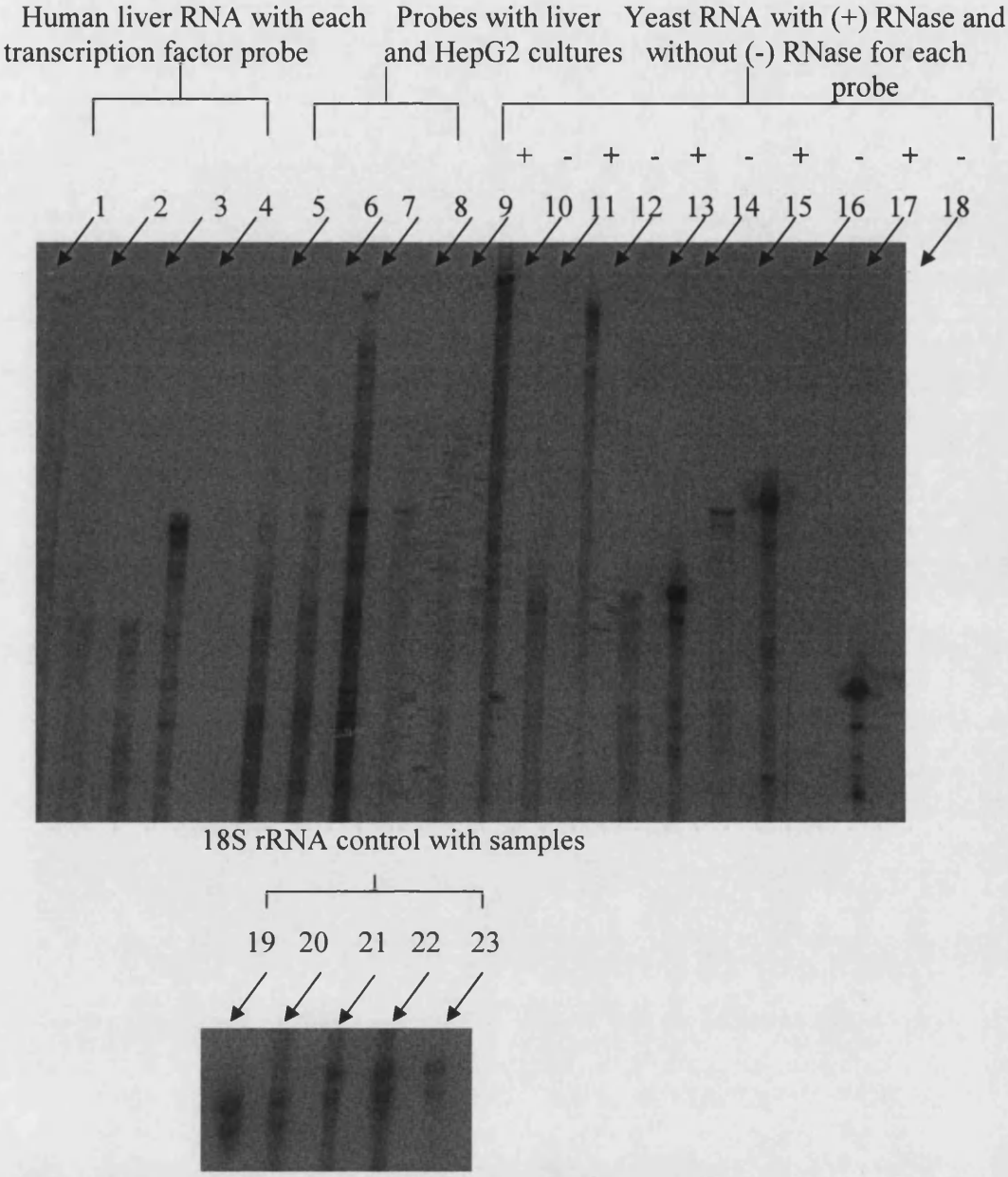


Fig. 6 RPA detection of transcription factor mRNA levels

1-4 (1) HNF-1, (2) HNF-4, (3) C/EBPα, (4) C/EBPβ RNA probes with liver RNA

5-8 Transcription factor probes with (5) Day 8, (6) Day 15, 3-D HepG2 cells (7) Monolayer HepG2 cells (8) Human Liver

9-18 Yeast RNA with (9,10) HNF-1, (11,12) HNF-4, (13,14) C/EBPα, (15,16) C/EBPβ, (17,18) 18S rRNA control with (+) and (-) RNase

19-23 Internal control 18S rRNA control with Liver, (19); and all transcription factors with (20) Day 8, 3-D HepG2 culture (21) Day 15 3-D HepG2 culture, (22) Monolayer HepG2 cells (23) Human Liver.

3.3.10.4.2 Detection of mRNA levels after re-exposure to the gel for a longer period, increased detection of protected fragments

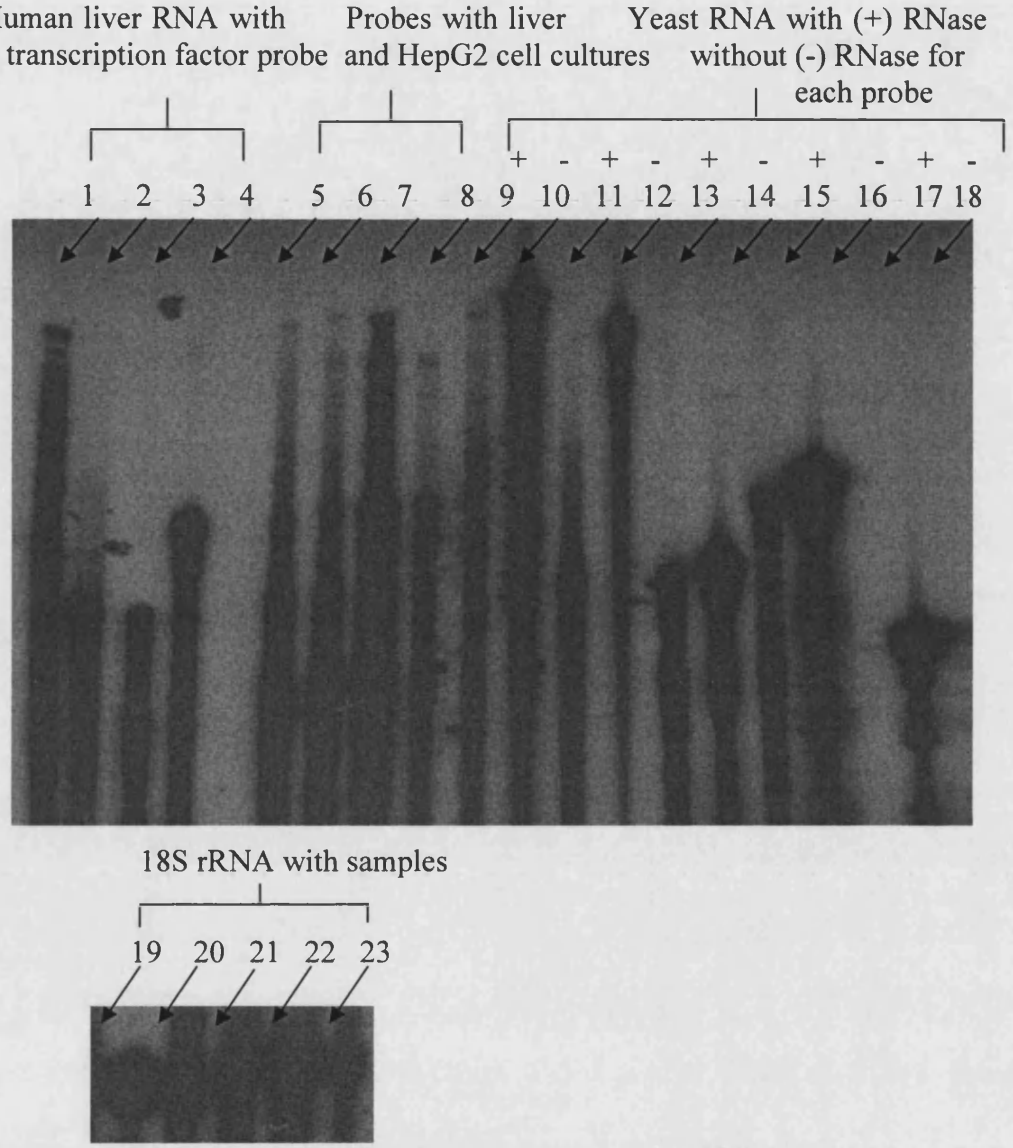


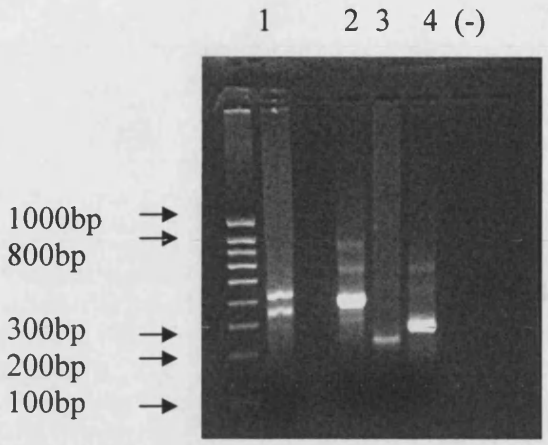
Fig. 7 RPA detection for transcription factor mRNA levels- long exposure
1-4 (1) HNF-1, (2) HNF-4, (3) CEBPα, (4) CEBPβ RNA probes with liver RNA
5-8 Transcription factor probes with (5) Day 8, 3-D HepG2 culture, (6) Day 15, 3-D HepG2 culture (7) Monolayer HepG2 cells (8) Human Liver
9-18 Yeast RNA with (9,10) HNF-1, (11,12) HNF-4, (13,14) C/EBPα, (15,16) C/EBPβ, (17,18) 18S rRNA control with (+) and without (-) RNase
19-23 Internal control 18S rRNA control with Liver, (19); and all transcription factors with (20) Day 8, 3-D HepG2 culture (21) Day 15, 3-D HepG2 culture, (22) Monolayer HepG2 cells (23) Human Liver.

3.3.10.5 Generation of modified probes to overcome the problem with insufficient digestion of the positive controls

There was insufficient digestion of the positive controls with yeast RNA and RNase. This was exacerbated with longer exposure (Fig. 7) and interferes with interpretation of the results because it is not possible to distinguish protected probes from undigested probes in this situation. Longer probes with non-matching sequence at the 3' end were therefore generated (see below).

The original PCR products were gel purified with Wizard PCR Preps DNA Purification System (Promega, Madison, UK). Each PCR product was incubated with 1mM dGTP, 1x Terminal deoxynucleotidyl Transferase (TdT) buffer, 30 units of rTdT and incubated for 30 minutes at 37°C. Incubation with TdT catalyses the addition of G' multiple deoxyguanidines to the 3' OH ends of the DNA (tailing the PCR products).

The template was amplified with the C-Prim Primer (5'-GGAATTCGCGGCCGCCCCCCCCCCCCCCC-3') and the reverse primer with the T7 promoter region with the same PCR conditions used to generate the PCR products for the initial RNase protection assays. When these probes are generated and used with the target RNA in the RPA, assuming the same problem of insufficient digestion, there should be 2 bands for each probe (corresponding to undigested and protected probes) and the bottom band should be the protected fragment. In the yeast RNA controls and incubation with probes and addition of RNase, if incomplete digestion occurred only a single larger band should be present as the bottom band is due to protection of the probe.



The molecular weight markers are 1000, 800, 700, 600, 500, 400, 300, 200, 100bp

Fig. 8 Tailing of transcription factor with dGTPs, with PCR products of the expected size with the addition of 20-30bps. (1) HNF-1 (2) HNF-4 (3) CEBPα (4) CEBPβ and (-) negative control with water and albumin primers.

The probes were purified on a denaturing acrylamide gel to remove any unincorporated nucleotides as shown in Fig.5. However HNF-4 did not transcribe efficiently so was not used as a probe (results not shown).

The RPA was carried out with the modified probes and the gel had to be exposed a second and longer period of time (Fig.9). The protected fragment corresponding to the expected size for HNF-1 and C/EBPα was not present in the target RNA samples with all the transcription factors. Although some of the controls with the yeast RNA and RNase showed some protection, the signals were not as strong as the experimental samples so this value was deducted as background when the final quantitation was made.

3.3.10.5.1 Gel exposed for 2 weeks

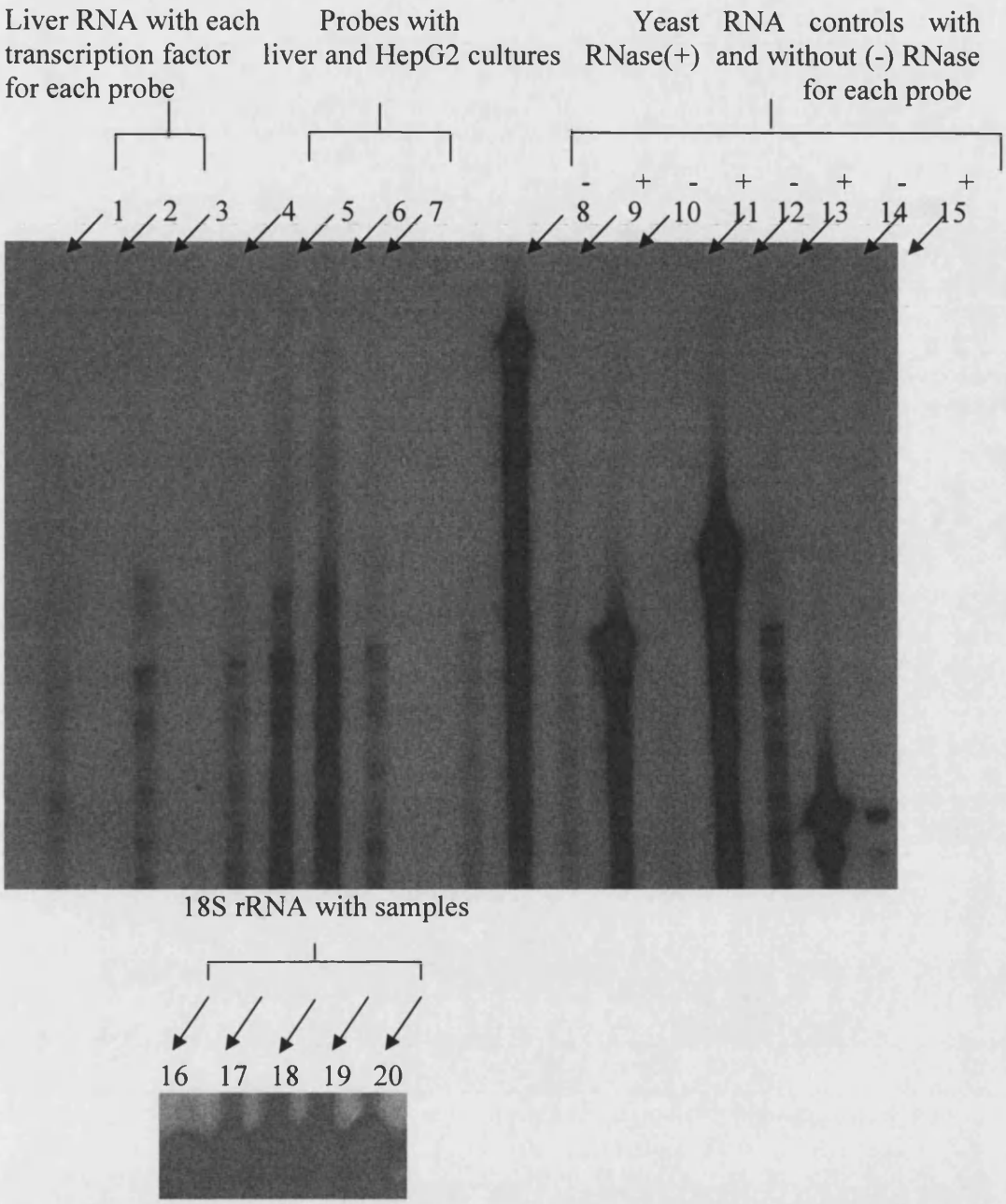


Fig. 9 RPA detection for transcription factor mRNA levels on gel exposed for 2 weeks

1-3 (1) HNF-1, (2) CEBPα, (3) CEBPβ RNA probes with liver RNA

4-7 Transcription factor probes with (5) Day 8, (6) Day 15, 3-D HepG2 culture (7) Monolayer HepG2 cells (8) Human Liver

8-15 Yeast RNA with HNF-1, (8,9) CEBPα, (10,11) CEBPβ, (12,13) 18S rRNA (14,15) without (-) and with (+) RNase

16-20 Internal control 18S rRNA control with Liver, (19); and all transcription factors with (20) Day 8, 3-D culture (21) Day 15 3-D culture, (22) Monolayer HepG2 cells (23) Human Liver.

Unfortunately the band obtained with the C/EBPα probe co-migrates with the background undigested bands from other probes so that it was not possible to interpret these bands. The protected fragment for HNF-1 could not be observed in these samples suggesting that the RNA if present was only present at very low levels.

This method proved to be unhelpful in determining the contribution of transcription factors to the upregulation and downregulation of function observed in alginate culture. The transcription factors were present at very low levels and the gel had to be exposed for a longer period in order to detect the protected fragments corresponding to the transcription factors in the RNA samples from the different culture conditions, so the negative samples showed some protection. A Microarray analysis was therefore carried out to look at the expression of genes at the mRNA level comparing peak function at Day 8 with monolayer HepG2 cells, and diminished function at Day 15 with peak function at Day 8, 3-D culture. The database was initially interrogated for the expression of liver enriched transcription factors and to obtain more information on gene expression in HepG2 cultures.

3.4 Microarray analysis of RNA from monolayer HepG2 cells, Day 8 and 15 HepG2 cells in 3-D culture

Microarray technology offers vast amounts of information on the expression of genes by probing the mRNA content of cells and determining which genes are expressed under a given condition. It is possible to measure the levels of thousands of different mRNAs in a single hybridisation step. Microarrays are usually made by the deposition of DNA spots on a solid support allowing the use of fluorescence dyes for detection and miniaturising the procedure. Either oligonucleotides or cDNAs are used. In this system chips were used, which were made from oligonucleotides.

3.4.1 Overview of methods used for RNA preparation for Microarray analysis

HepG2 cells in monolayer culture and 3-D culture

↓

RNA Isolation

↓

DNase treat RNA

↓

RNAeasy clean up → Check purity on ethidium bromide stained formaldehyde agarose gels

↓

Concentrate → Measure amount / purity of RNA

↓

1st strand cDNA synthesis

↓

2nd strand cDNA synthesis → DNA ligase, RNase H, DNA polymerase

↓

phenol: chloroform: isoamyl alcohol extraction

↓

Phaselock gel to remove contaminating ethanol and protein from ds cDNA

↓

Precipitate with ammonium acetate and ethanol and resuspend in 12µl water

↓

In vitro transcription (IVT) – Enzo kit - Single stranded non radioactive RNA

↓

Fragment cRNA

↓

Hybridisation target and target clean up

3.4.2 RNA extraction

For each culture condition, monolayer HepG2 cells, Day 8 and 15 HepG2 cells in 3-D culture, RNA was extracted from an equal number of cells and for each culture condition n=3.

1 unit =40μg/ml for RNA samples taking into account the dilution factor

Sample	Dilution	Ab_{260}	Ab_{280}	$Ab_{260:280}$	μg RNA	Total volume (μl)	μg / μl
Day 8	1:50	0.123	0.063	1.94	18	80	0.23
Day 8	1:50	0.112	0.054	2.07	16	80	0.20
Day 8	1:50	0.143	0.072	1.99	20	80	0.25
Day 15	1:50	0.135	0.068	1.99	37.8	160	0.24
Day 15	1:50	0.123	0.065	1.90	51.06	240	0.22
Day 15	1:50	0.105	0.054	1.94	29.4	160	0.18
Monolayer	1: 25	0.187	0.096	2.00	26.18	160	0.16
Monolayer	1:25	0.102	0.051	2.00	14.28	160	0.09
Monolayer	1:25	0.101	0.047	2.14	14	160	0.10

Table 7 The values given are after the RNeasy clean up stage after isolation with Promega Total RNagents system. Pure RNA has an Ab ratio at $_{260/280}$ of between 1.80-2.0.

3.4.3 Clean up of total RNA for Microarray

3.4.3.1 Materials

- Qiagen RNeasy Mini kit (Qiagen, UK Cat. No. 74104)
- Components of Buffer RLT, RW1 and RPE are proprietary

  RLT- contains a guanidine thiocynate

  RPE- no indication

  RWL-contains guanidium thiocynate and ethanol
- DNase I (Pharmacia)
- 10X DNase buffer

  1M Tris pH 7.5

  1M $MgCl_2$

  1M DTT
- Recombinant RNasin Ribonuclease Inhibitor (Promega)
- β-Mercaptoethanol 14.3M (Sigma Aldrich).

3.4.3.2 Method

RNA samples were subjected to DNase digestion to prevent any carry over of contaminating DNA. For each sample (from monolayer, Day 8 and Day 15 cultures in 3-D), 72.5µl of sample containing 1-2µg/µl of RNA, nuclease free water, 1X DNase buffer, 2 units of DNase, 100 units of recombinant RNasin RNase Inhibitor, was mixed gently with a pipette or inverted gently (vortexing was avoided to prevent degradation), and incubated at 37°C for 30 minutes. The samples were placed on ice, prior to loading onto spin columns supplied with the Qiagen RNeasy Mini kit.

While samples were incubating at 37°C, 10µl of β-mercaptoethanol was added per 1ml of Buffer RLT. This solution was stable for 1 month. 4 volumes of neat ethanol were added to buffer RPE. All centrifugation steps were carried out at room temperature.

To each RNA sample (in 1.5ml eppendorf tubes), 350µl of buffer RLT was added and mixed thoroughly. 250µl of ethanol was added to the lysate, mixed well by pipetting, without vortexing. The sample was transferred (total 700µl) to a minispin column placed in a collection tube and centrifuged at 10000g for 1 minute (Eppendorf 5402, Germany). The collection tube was discarded with the flow through. The column was transferred into a new 2ml collection tube. 700µl of buffer RWL was added and centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany). 500µl of buffer RPE was added to the columns, centrifuged for 2 minutes at 10000g (Eppendorf 5402, Germany) to dry the column. The column was removed carefully in order to avoid contact with the flow through resulting in carry over of ethanol and placed in an RNase free 1.5ml eppendorf with the lid taken off. The samples were centrifuged again at 10000g for 1 minute (Eppendorf 5402, Germany). The column was transferred into a 1.5ml tube supplied. 30-50µl of RNase free water (supplied) was placed directly onto the membrane.

The column and tube were centrifuged for 2 minutes at 10000g (Eppendorf 5402, Germany) to elute. This procedure was repeated as it was expected that the RNA yield would be greater than 30µg. The total RNA in solution was quantitated and analysed for purity measuring its optical density at 260nm and 280nm. RNA was

aliquotted and stored at –80°C.

3.4.4 Checking RNA integrity

As described in section 3.3.2.

3.4.4.1 RNA integrity

RNA samples run on an ethidium bromide formaldehyde agarose gel separating the RNA into its subunits determined the integrity of total RNA prepared from each culture condition. The 28S ribosomal subunit compared to the 18S ribosomal subunit should show an approximate 2: 1 ratio.

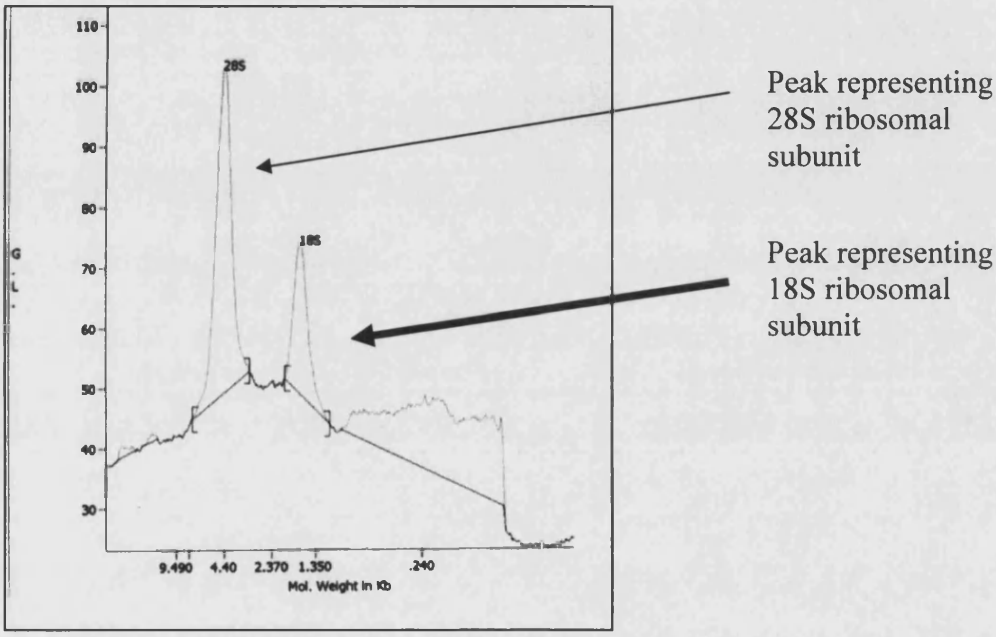
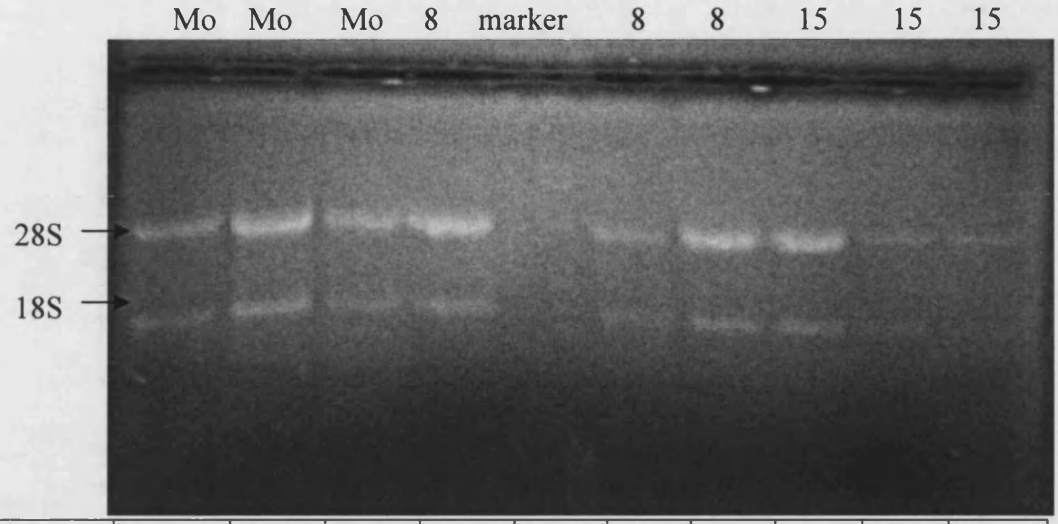


Fig. 10 Graph representing a typical RNA sample demonstrating the 28S and 18S ribosomal subunits. Values given in the table represent the area under curves of peaks.

Mo =Monolayer HepG2 cells
8 =Day 8, 3-D HepG2 culture
15 =Day 15, 3-D HepG2 culture



Amount in 28S band	33.8	51.8	41.5	31.8		32.1	35.9	41.2	28.4	25.2
Amount in 18S band	14.8	20.7	16.2	14.9		12.3	15.5	16.6	12.8	12.1
Ratio of 28S : 18S	2.28	2.50	2.57	2.56		2.6	2.31	2.49	2.21	2.08

Table 8 Agarose gel electrophoresis to determine integrity of total RNA prepared from each culture condition.

The methods to generate the target RNA for hybridising to complementary sequences on the microarray chips are described in full in the Appendix.

3.5 Results

3.5.1 Gene expression.

Genes considered relevant were those with a ≥ 2 fold change occurring in ≥ 44% of chips (representing 4 out of 9). This reflected a true change in expression and not occurring by chance or as a result of a single faulty chip. Using these criteria, the search of the database for liver enriched transcription factors, demonstrating a significant change in mRNA expression comparing the following culture conditions:-

A) Day 15 spheroidal 3-D HepG2 culture (diminished function) vs. Day 8 spheroidal 3-D culture (peak function).

B) Day 8 spheroidal 3-D HepG2 culture (peak function) vs. Monolayer HepG2 culture (less differentiated function),

produced the following result:-

Day 15 vs. Day 8		
	DECREASE ± S.D	% of chips showing this change
Transcription factor HNF-3	-2.17 ± 0.22	44

Table 9 Gene expression of liver enriched transcription factors.

The only change detected using the parameters defined was a decrease in HNF-3 expression, which was downregulated in Day 15, 3-D HepG2 cultures compared to Day 8, 3-D HepG2 cultures.

A change in HNF-3 mRNA levels was not detected in Day 8, 3-D cultures compared to monolayer HepG2 cultures.

The U95Av2 Affymetrix chip contained arrays for the following liver enriched transcription factor genes including of course, HNF-3, as shown above:-

- HNF-1
- HNF-4
- C/EBPα
- C/EBPβ

It should be noted that HNF-6 was not represented on the chip.

3.5.2 Protein expression

The Powerblot data from the protein array was investigated for changes in expression of protein levels of liver enriched transcription factors, with the parameters defined in chapter 2. Of the liver enriched transcription factors, only an antibody to HNF-4 was present in the Powerblot protein array, and although it was expressed, a significant change between the culture conditions described was not detected.

3.6 Discussion

The results obtained from the RNase protection assay were inconclusive, predominantly due to technical problems. There were problems with insufficient digestion of the positive controls with RNase, and the film had to be exposed to the gel for a longer period in order to detect the protected fragments corresponding to the transcription factors in RNA samples from the different culture conditions. Results could not be interpreted, as protected probes could not be distinguished from undigested probes. In addition, although it is a sensitive technique, in retrospect it may not have provided the sensitivity or specificity required for quantitating transcription factor activation.

In order to overcome these problems RNA probes for the transcription factors were modified by the addition of extra nucleotides, so that in the control samples, with the addition of RNase, of the two bands, which would be present for each probe, if there was incomplete digestion, only a top band should be present. The presence of a slightly lower band would be due to protection of the probe. The same problem occurred again and in addition HNF-4 did not transcribe efficiently. As the gel had to be exposed for longer, the control samples with the yeast RNA and RNase showed some protection. Normally this signal is taken away from the experimental samples and was subtracted as background from experimental samples, which was carried out for the RPAs with liver specific proteins and urea cycle enzymes (Khalil, 2000 personal communication). However, as can be seen in Fig.6 these control samples showed protection and the signals were stronger than the experimental samples or they could not be detected at all (Fig.9). This residual full-length probe in the RNase treated control lane is rarely due to using a suboptimal RNase concentration and this was increased in the samples. Using too much RNase can lead to overdigestion of the probe/target hybrid and loss of sensitivity.

Another cause for high background in the assay is using too much probe. For rare target such as these transcription factors, less probe is needed to be in molar excess. The explanations for these problems could be due to transcription factor RNA being present at very low levels, making it difficult to detect in a multiprobe

analysis. RPA was selected over northern blot analysis, as it was required to detect multiple targets in a sample and to improve signal and sensitivity over northern blotting. Nevertheless, this may be an option for the future as transcription factors in samples have been detected in this way (Elizondo and Medina-Diaz 2003;Tacchini *et al.* 2001).

With the advent of Microarray technology, the experimental procedure was modified (Lemaigre *et al.* 1996;Shoemaker and Linsley 2002;Unger *et al.* 2001). Microarray analysis was carried on high purity intact total RNA extracted from monolayer HepG2 cells, Day 8 and Day 15 HepG2 cells in 3-D culture, in order to increase the sensitivity of detection, and the database generated was interrogated for changes in expression of liver-enriched transcription factors in the culture samples. The only change detected was for HNF-3, which showed a decrease in Day 15, 3-D HepG2 cultures compared to Day 8, 3-D HepG2 cultures. This transcription factor binds and transactivates numerous liver specific genes such as albumin and AAT (Cerosaletti and Fournier 1996;Hafenrichter *et al.* 1994). This parallels the observed decrease in albumin and AAT protein secretion seen from Day 8 onwards in Fig. 2a and decreased mRNA levels of this transcription factor may be contributing to the observed downregulation of function with extended culture.

However, as described there are other liver-enriched transcription factors which work in a regulatory manner with HNF-3 to activate liver specific genes (Landry *et al.* 1997;Vallet *et al.* 1995). As examples, AAT gene contains binding sites for HNF-1, HNF-3, HNF-4 and HNF-6, that have been shown to interact with the liver enriched transcription factors HNF-1α, HNF-3β, HNF-4α and HNF-6β (Kanda *et al.* 2003;Samadani and Costa 1996). Furthermore, the transcription factors that bind to the AAT regulatory sequence also influence the transcriptional activity of each other. The HNF-3 factors have been implicated in the nucleosomal arrangement of the albumin gene, whose transcription is also transactivated by HNF-1 and HNF-4 (Griffo *et al.* 1993;Vallet *et al.* 1995). Therefore, not only do transcription factors have to be present, they have to be activated at the protein level.

Currently, widely used and available methods, which were not available during the course of my research for measuring active transcription, include electromobility shift assay (EMSA) (Brundage *et al.* 2003;Peng *et al.* 2003) and colorimetric ELISA based assays (Thelen *et al.* 2004). Traditionally, functional analysis of transcription factors have required the use of EMSA, which determines the binding interaction between DNA and DNA-binding proteins, such as the interaction of transcription factors with regulatory regions (Auyeung *et al.* 2003;Huang *et al.* 2002;Marten *et al.* 1996). The assay is based on the observation that protein-bound DNA will migrate slower though a non-denaturing polyacrylamide gel than free DNA. However, these assays can only investigate one transcription factor per reaction and are not amenable to high throughput applications. Colorimetric ELISA based assays can have low sensitivity (Allicotti *et al.* 2003).

The use of reporter genes has contributed greatly to the study of eukaryotic gene expression and regulation, and they are most frequently used as indicators of transcriptional activity in cells (Rosenthal 1987). The DNA regulatory region under investigation, can be subcloned upstream of a reporter, such as luciferase, which can then be transfected into a variety of cell types. The luciferase enzyme catalyses a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+} resulting in light emission. Light is produced from an enzymatic chemical reaction, which can be measured with a luminometer. The total amount of light measured during a given time interval is proportional to the amount of luciferase reporter activity in the sample. To determine whether genes are activated by specific transcription factors, and play a role in a particular signalling pathway, one can transfect a luciferase construct bearing the promoter elements that bind to these complexes into cells. If the transcription factors under investigation were activated in these signalling pathways, they would then bind to and induce transcription of the luciferase gene (Park and Waxman 2001;Wang *et al.* 1996).

The Microarray and Powerblot array, using the defined parameters did not provide sufficient information on the presence of or changes in levels of transcription factors when comparing the culture conditions, to support the initial hypotheses. One looked further at the Microarray and Powerblot array data, as it

was known that you could obtain more information on gene and protein expression between the HepG2 culture conditions, and perhaps give an indication of other pathways or mechanisms associated with the activation of liver enriched transcription factors, or contributing to the optimal and diminished function observed.

In part, the role of the stress response and its subsequent effects after activation, were considered in maintaining function in 3-D HepG2 culture. An initial search of the Microarray database for "stress response" genes showed a decrease in MAPK protein kinase 3 mRNA levels in Day 15, 3-D HepG2 cultures compared to Day 8, 3-D HepG2 culture. MAPK protein kinase 3 induces the phosphorylation and activation of the nuclear transcription factor CREB, which acts as a coactivator for HNF-4 (Dell and Hadzopoulou-Cladaras 1999;Maizels *et al.* 2001;Soutoglou *et al.* 2001). Although changes in HNF-4 mRNA levels were not detected between HepG2 culture conditions, as MAPK protein kinase 3 can activate HNF-4, it could therefore influence a possible decrease in the levels of HNF-4 mRNA in Day 15, 3-D culture compared to Day 8, 3-D culture. Both HNF-4 and HNF-3 are important for liver specific gene expression and decreased levels of these could account for the diminished function observed at Day 15.
The following chapter explores further, in more detail, stress response pathways and mechanisms and its role in maintaining differentiated function in 3-D HepG2 culture.

Chapter 4

The Stress Response and maintenance of function in HepG2 culture

Chapter 4- The Stress Response and maintenance of function in HepG2 culture

4.1 Hypothesis

In this chapter, the Microarray and Powerblot data was explored further to look at the expression of stress induced genes and proteins. Two general hypotheses were formulated: - 1) stress response activation after prolonged activation causes the downregulation of function observed in later times of 3-D culture and 2) stress response genes and proteins are involved to ensure the function maintained at Day 8, 3-D culture, either as an adaptive response to the successful environment created, or as a consequence of increased metabolic function. Therefore a complex relationship between stress and performance would be anticipated. The possible explanations for diminished function were investigated, in particular stresses that might be anticipated in culture. This chapter therefore starts off with a general introduction to stress response and stress proteins, and in particular hypoxic stress and oxidative damage.

4.2 General introduction to the Stress Response

The stress response - Changes in environment, injury, disease, even growth and differentiation place organisms under stress and organisms have evolved to cope with many different forms of stress by means of the "cellular stress response" (Mager and De Kruijff 1995;Pockley 2001).

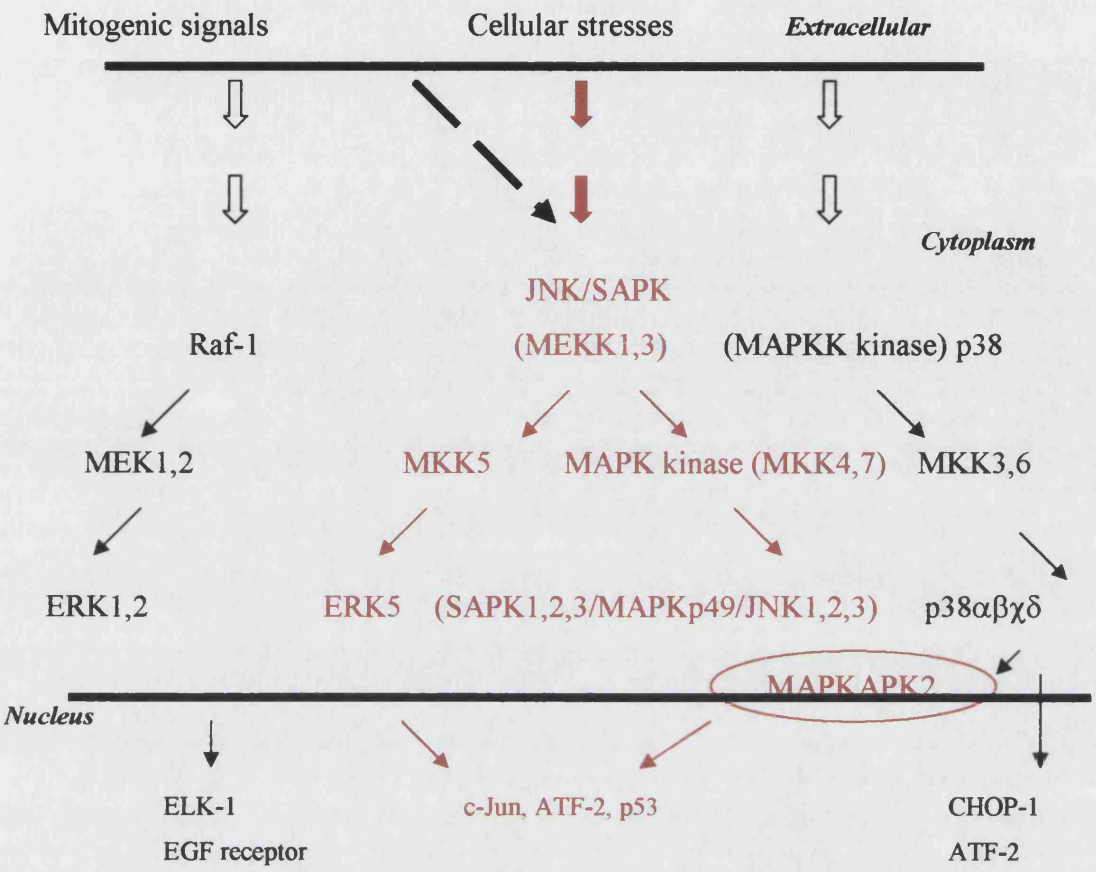
Stress response genes and proteins- In response to adverse stresses, including bacterial endotoxins, UV irradiation, heat and chemical shock, hyperosmosis and hypoxia, multiple kinases act as important stress-activated elements. These kinases are activated by detection of stress factors at the cell surface, involving phosphorylation cascades, containing pathways of kinases. These in turn are activated and undergo nuclear translocation, where they activate multiple transcription factors to regulate gene expression in response to the external stress (Powell *et al.* 2003).

Paradoxically, many stress proteins and genes are switched on or off which

perform "house-keeping functions"; examples are heat shock proteins and genes, which constitute the archetypal stress response, and molecular chaperones that help nascent polypeptides assume their proper configuration (Mager and De Kruijff 1995). Enzymes are expressed due to normal increased metabolic activity or other types of stress-responsive proteins, such as the haemoxygenase (HO) family and cyclooxygenase (Cox); these enzymes catalyse the formation of end products that protect against cellular stress (Otterbein *et al.* 2003;Steer and Corbett 2003).

MAPKinase Signalling Pathways- This large group of protein kinases contains over a dozen members that participate in many eukaryotic regulatory pathways. The extracellular signal-regulated protein kinase (ERK) is also referred to as the mitogen-activated protein kinase (MAPK) superfamily of enzymes. These enzymes comprise at least three parallel, yet interwoven, signal transduction cascades that are differentially regulated in response to mitogens, growth factors, cytokines and various forms of stress. Each cascade consists of a minimum of three enzymes activated in series: a MAP kinase/ERK kinase kinase or MEKK (a MEK activator), a MAP kinase/ERK kinase or MEK (a MAP kinase activator), and a MAP kinase/ERK homologue (Yang *et al.* 2003).

Four distinct MAPK pathways have been characterised 1) extracellular signal-related kinase 1 (ERK1), ERK2 also referred to as p42/44 MAPK), 2) the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), 3) p38 and 4) ERK5 kinase (Eckert *et al.* 2003).



Schematic representation of MAP kinase activation. MAP kinases are involved in phosphorylation cascades initiated by detection of stress factors at the cell surface. In red, the main stress activated pathway.

The ERK1, 2 pathway (Raf→MEK1, 2→ERK1, 2) is activated by mitogens via Ras and by phorbol esters via protein kinase C. Growth signals such as epidermal growth factor and platelet derived growth factor activate ERKs by tyrosine phosphorylation of their receptors. Depending on the cell type, ERK activation results in either proliferation or differentiation (Santen *et al.* 2002). ERK5 is activated by cellular stress as well as mitogens such as growth factors and serum (Gutkind 2000). The MAPK pathways JNK/SAPK (MEK kinase 1,3→MAPK kinase 4,7 [MKK4,7]→JNK1,2,3) and p38 (MAPK kinase kinase [MAPKKK]→MKK3,6→p38α,β,χ, δ) are activated by cellular stresses, e.g. UV

light, osmotic and oxidative stress, hypoxia, and inflammatory cytokines (Irving and Bamford 2002).

MAPKs are activated by phosphorylation of their threonine and tyrosine residues by dual specificity MAPK kinases, which in turn are activated by phosphorylation of two serine residues by upstream MAPKKKs (Torres *et al.* 2004). Activities of MAPK kinases are inhibited by dephosphorylation by phosphatases (Ratcliffe *et al.* 1998;Zhu *et al.* 2002). Modulation of gene expression involving transcription factors is a crucial mechanism in response to cellular stress. Transcription factors, such as NFκB, and AP-1, which is the transcription factor complex formed by c-jun and c-fos, are predominantly the targets of the SAPK/JNK pathway (Moriguchi *et al.* 1997;Shi and Kehrl 1997). Another important transcription factor, hypoxia inducible factor (HIF)-1α is stimulated by conditions of oxygen deprivation and activates the expression of multiple genes responsible for maintaining oxygen homeostasis (Gupta *et al.* 2000a).

4.3 Hypoxia as a possible cause for the downregulation of function observed in Day 15 of 3-D HepG2 cultures

It was hypothesised that downregulation of function observed in Day 15, 3-D HepG2 cells compared to Day 8, 3-D HepG2 cells could be attributed to hypoxia as oxygen solubilisation in culture medium is possibly insufficient and may be rate limiting when spheroids are larger as they are at later times of 3-D culture. As mentioned in chapter 3, an initial investigation of the microarray database for stress response genes, demonstrated a decrease in HNF-3 in Day 15, 3-D HepG2 cultures compared to Day 8, 3-D HepG2 cultures. This transcription factor binds and transactivates numerous liver specific genes such as albumin, AAT, and AFP, and hypoxic stress has also been shown to reduce AFP expression (Lee *et al.* 1999;Mazure *et al.* 2002). A pattern of decreased liver specific protein secretion is observed from Day 8, 3-D culture to Day 15, 3-D culture.

4.3.1 Expression of hypoxia related genes

The Microarray database was interrogated for increases and decreases for genes specifically associated with hypoxia, hypothesising that the spheroids in 3-D culture at Day 15 are hypoxic, as sufficient oxygen may not diffuse through the medium into the beads as the spheroids become larger at later time points.

Day 15 vs. Day 8	FOLD INCREASE ± S.D	% of chips showing this change
Vascular endothelial Growth Factor (VEGF)	+1.97 ± 0.19	100

Table 10 Expression of genes associated with hypoxia

There was an increase in VEGF in Day 15, 3-D culture compared to Day 8, 3-D culture. As mentioned previously, HIF-1α is activated in cells exposed to hypoxia, although HIF-1α itself was not detected by microarray analysis, with the parameters defined in section 3.5.1 However, VEGF does contain enhancer elements with functionally critical sites for HIF-1α. Ratcliffe *et al* 1998 reported a number of genes, which contain binding sites for the HIF-1α transcription factor, which are upregulated upon HIF-1α activation to modulate their environment in response to hypoxic stress.

The Microarray database was therefore interrogated for the expression of these genes comparing diminished function at Day 15, 3-D culture with Day 8, 3-D culture. The results in Table 11 show that there is an increased expression of these genes in Day 15, 3-D HepG2 culture compared to Day 8, 3-D HepG2 culture.

Table 11 Expression of genes with binding sites for HIF-1α, comparing Day 15, 3-D cultures with Day 8, 3-D cultures.

Gene	% of chips	Increase or Decrease with Fold Change	Pubmed Accession number	Standard Deviation ± S.D, n=9	Description
Enolase (Eno 1)	55	↑ 3.4	-	4.92	Glycolysis
Lactate Dehyhydrogenase A	55	↑ 2.14		2.92	Glycolysis
Adolase A, Fructose biphosphate	55	↑ 4.01	X05236	5.71	Glycolysis
Pyruvate kinase	44	↑ 1.56	M26252	1.85	Glycolysis
Glucose-6-phosphatase Catalytic (glycogen storage disease)	77	↑ 3.57	U01120	1.87	Glucose transporter
Adenylate kinase-3	55	↑ 7.78	Y09788	7.3	High energy phosphate metabolism
Short chain alcohol dehydrogenase family member	44	↑ 1.87	U31875	2.71	Glucose transporter Carbohydrate metabolism
Transferrin	55	↑ 5.57	S95936	7.7	Iron transport
Human insulin like growth factor binding protein	66	↑ 2.2	M74587	0.75	
Quiescin (growth factor)	66	↑ 5.28	L42379	3.64	Cell growth, maintenance, proliferation
Platelet-derived growth factor beta polypeptide (simian)	66	↑ 17.5	M12783	25.59	Angiogenesis and tumour progression
Prostate differentiation factor	55	↑ 1.78	AB000584	1.64	
Inhibin, beta B	55	↑ 5.34	M31682	8.33	
Insulin like growth factor binding protein 2 (36kDa)	66	↑ 1.37	X16302	2.46	Binds IGF-1,2. Found in periportal liver
VEGF gene	66	↑ 3.22		2.4	
VEGF mRNA	100	↑ 1.97	AF 024710	0.19	Angiogenesis
Transforming Growth factor, beta 1	66	↑ 2.67	M38449	1.03	Anti-apoptosis Cell-Cell signalling

To complement the Microarray analysis, Western blot analysis was performed for a panel of proteins upregulated during hypoxia.

4.3.2 Detection of stress related proteins
4.3.2.1 Methods

Total Protein Determination by the Lowry method (Schacterle and Pollack 1973).

Protein lysates were prepared from an equal number of cells from monolayer HepG2 cells, Day 8 and Day 15, HepG2 cells in 3-D culture.

Sample	Total Volume (ml)	Concentration (mg/ml)	Dilution to give final concentration of 1mg/ml (Protein + 2 x sample buffer)
Monolayer (A)	4.5	1.889	1ml protein + 0.889ml buffer
Monolayer (B)	4.5	3.2634	1ml + 2.2634ml buffer
Day 8 (C)	4.5	3.562	1ml + 2.562ml buffer
Day 8 (D)	4.0	3.507	1ml + 2.507ml buffer
Day 8 (D)	4.5	3.098	1ml + 2.098ml buffer
Day 15 (E)	4	2.0356	1ml + 1.0356ml buffer
Day 15 (F)	4	1.8124	1ml + 0.8124ml buffer
Day 15 (G)	4	1.916	1ml + 0.916ml buffer

Table 12 Concentrations of protein lysates from the different culture conditions.

The monolayer, Day 8 and Day 15 cultures were pooled and aliquotted into 100µl and 500µl samples.

Protein concentrations were assessed a second time in the diluted samples to confirm equal loading on gels. Once the samples were diluted it was not possible to determine the protein concentration by the Lowry method as the colour from

the sample buffer interfered with the readings from the plate reader.

Protein estimation was therefore carried out using a method by Warburg and Christian by measuring the absorbance at 260nm and 280nm and then multiplying by a published factor for the ratio to give a final protein concentration (Stoscheck 1990). This demonstrated further that the protein lysates had all been diluted to the same concentration.

HepG2 Culture	Ab_{260}	Ab_{280}	$Ab_{280/260}$	Factor	Ab_{280}x Factor
Monolayer HepG2 cells	0.066	0.075	1.136	0.886	1.006mg/ml
3-D Day 8	0.060	0.071	1.183	0.911	1.008mg/ml
3-D Day 15	0.057	0.068	1.193	0.918	1.009mg/ml

Table 13 Protein concentrations from the different culture conditions.

The presence of the housekeeping, constitutively expressed β-actin protein in equal amounts in the samples, serves as a further loading control for the subsequent proteins detected by western blot analysis and confirms the integrity of the protein lysates.

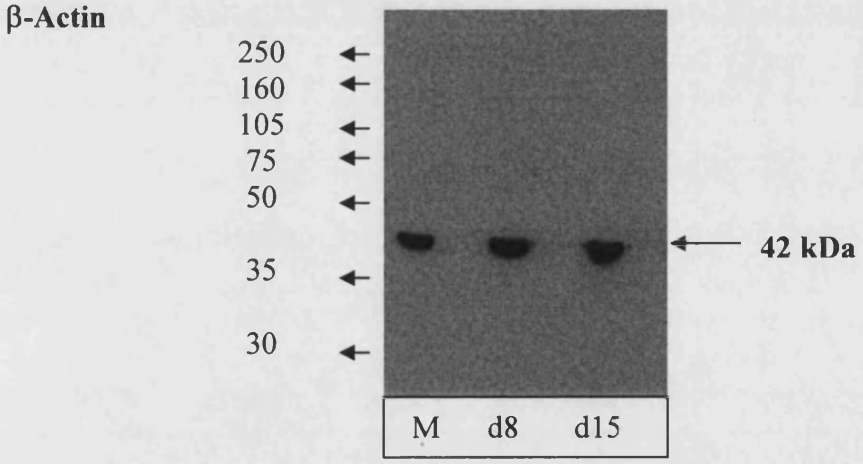


Fig. 11 Expression of β-actin in HepG2 cultures.

4.3.2.2 Analysis

Equal amounts of protein lysates from monolayer HepG2 cells (M) Day 8 (d8) and Day 15 (d15) HepG2 cells in 3-D culture, and control lysates supplied commercially for the relevant antibodies (+) were separated by SDS-PAGE, blotted and probed with specific primary antibodies and an appropriate enzyme linked secondary antibody prior to detection by chemiluminescence.

Comparisons were made of the abundance of these stress proteins in the different culture conditions comparing Day 8, 3-D cultures (peak performance) with Monolayer HepG2 cells (poor performance) and Day 15, 3-D cultures (diminished function) with Day 8, 3-D cultures (peak performance). The evidence to demonstrate that hypoxic stress was contributing to the diminished function observed was investigated at the protein level.

Western blots of hypoxia related proteins

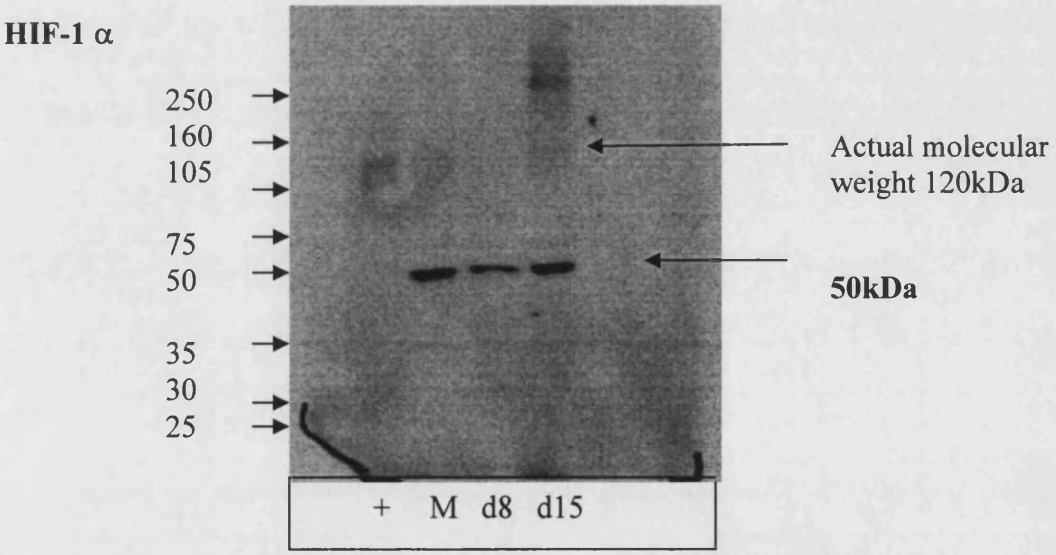


Fig. 12 Expression of HIF -1α in HepG2 cultures.

HIF-1 is a heterodimeric protein, and though the actual molecular weight of the protein is 120kDa, the band at 50kDa probably represents a proteolytic cleavage product or some post-translational modification of the protein. From both bands the protein is more abundant in the monolayer sample and Day 15 samples and an

expected band from the positive control sample was observed with lysate from HeLa cells (+).

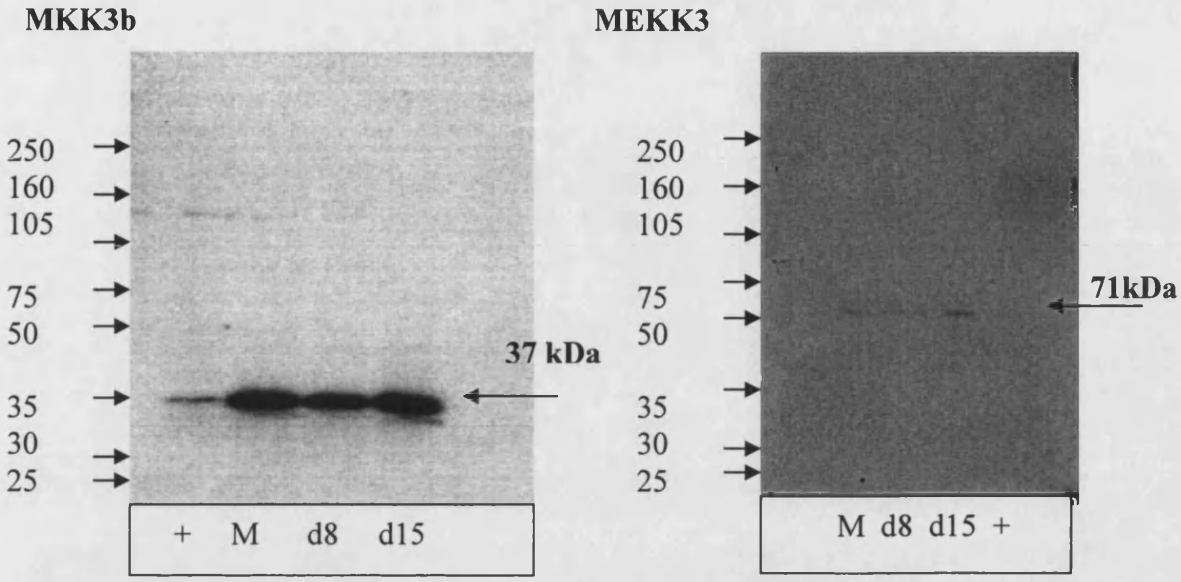


Fig.13 Expression of hypoxia related proteins in HepG2 cultures.

MKK3b protein was detected in all samples with the strongest signals observed with monolayer HepG2 cells and Day 15, 3-D samples. A faint signal was observed with the positive control lysate from HeLa cells (+).

MEKK3 (MAPK/ERK kinase kinase) protein was detected weakly in the samples but the strongest signal was observed in the Day 15 sample, a very weak signal was observed with the positive control lysate from SW-13 cells (+).

4.3.3 Densitometry

Densitometry allows for the generation of both semi-quantitative, and fully calibrated density or intensity data from experimental samples. Densitometry was carried out to confirm the differences in protein expression demonstrated, using the GS-670 Imaging Densitometer (Bio-Rad Laboratories). This scanner is supported by the Molecular Analyst PC image analysis software, allowing for image display and quantitation. The software was used to identify and select the relevant bands in each sample by marking around each band. This provided many parameters for each band, including mean absorbance (OD) values, and OD volume x mm^2

The results below show that the densitometry measurements confirm a) constitutively expressed β-actin protein is present in equal amounts in the samples and b) hypoxia related proteins are more highly expressed at times of diminished function.

Protein	Monolayer OD x mm^2	Day 8 OD x mm^2	Day 15 OD x mm^2
β-actin	16.78	17.754	17.370
HIF-1α	2.429	0.901	2.616
MKK3b	12.683	9.337	11.901
MEKK3	0.067	0.065	0.071

Table 14 Densitometry measurements for protein expression in monolayer, D8 and D15 lysates, probed with β-actin, HIF-1α, MKK3b, MEKK3.

The results from the western blots and the densitometry measurements suggest that hypoxia in later times of 3-D culture, at Day 15 and monolayer HepG2 cell culture was contributing to the downregulation of function observed, therefore it was attempted to correct hypoxia by increasing ambient oxygen concentration in order to enhance cell performance.

4.4 Manipulating the 3-D culture system, by modulating the environment to improve cell performance

4.4.1 The hypoxic response

The Western blot data and Microarray analysis identified genes and proteins implicated in a hypoxic stress response. This led to the hypothesis that downregulation of function observed in Day 15, 3-D HepG2 cells compared to Day 8, 3-D HepG2 cells could be attributed to lack of oxygen in the medium. To explore this, culture in three oxygen concentrations were investigated, 35%, 80% and 50%, compared with ambient oxygen concentration at ~21%.

4.4.2 Increasing oxygen concentration to 35%

The results demonstrate that maintaining 3-D cultures at 35% O_2 did not improve protein function and there was not a significant change in the proliferation of 3-D HepG2 spheroids at either time point under the different culture conditions. There was also no change in the morphology or viability of the spheroids at either time point.

4.4.2.1 Effect on cell proliferation

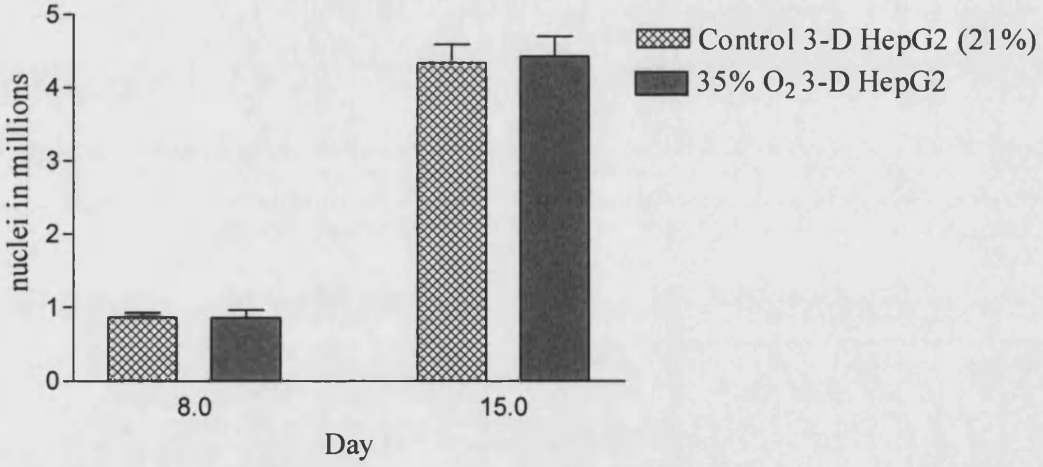


Fig. 14 Cell proliferation in 3-D HepG2 cultures in 35% O_2 vs. 21%. Mean values ± S.D, n=6 for each time point.

4.4.2.2 Protein synthesis

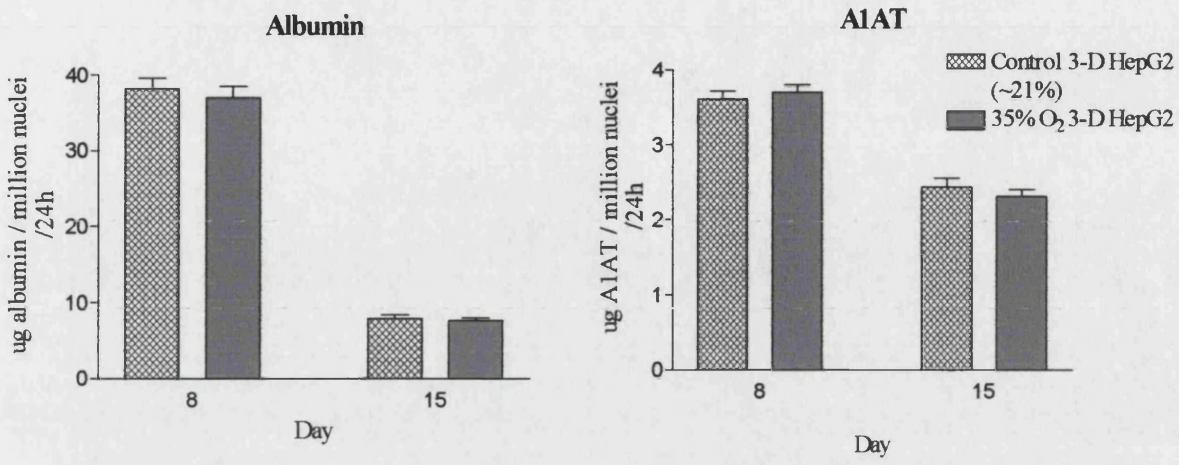


Fig. 15 Protein secretion from 3-D HepG2 cell cultures maintained at 35% O_2 vs. 21% O_2 concentration. Mean values ± S.D, n=6 for each time point.

4.4.2.3 Morphology and viability

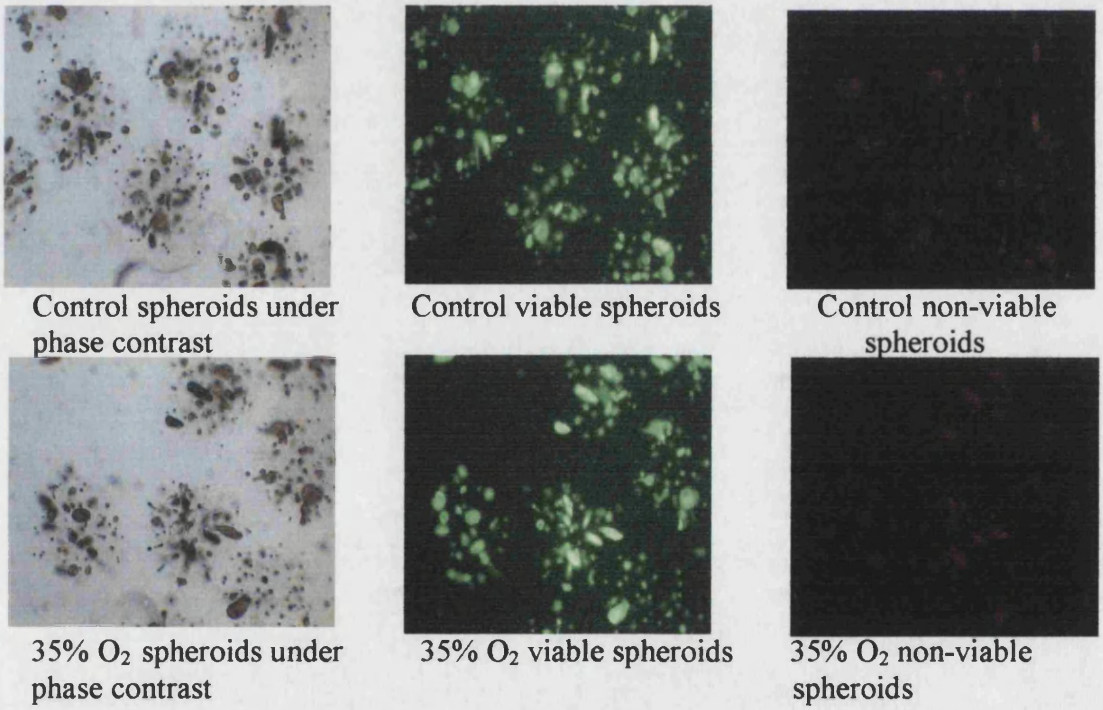


Fig. 16 Morphology of 3-D cultures at Day 8 in the 2 different oxygen concentrations taken at x4 magnification and cell viability assessed by PI and FDA staining.

As 35% was neither beneficial nor detrimental, the experiment was repeated in a much higher O_2 concentration at 80%.

4.4.3 Increasing the oxygen concentration to 80%

The results in Fig. 17 show that maintaining the 3-D cultures at 80% O_2 did not improve protein function and in addition had a detrimental effect on cell proliferation and protein function per cell.

The oxygen level generated more non-viable cells as a proportion of the total number of spheroids and the morphology of the spheroids under phase contrast microscopy again verified that the cells were not proliferating.

4.4.3.1 Effect on cell proliferation

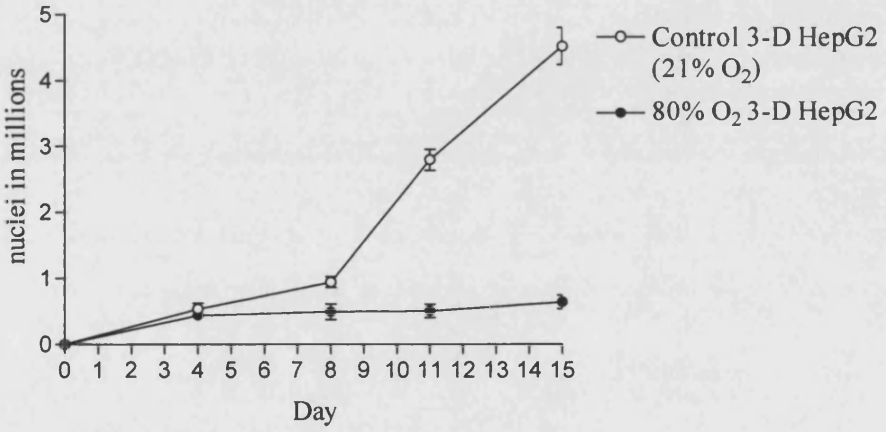


Fig. 17 Proliferation of HepG2 spheroids in 3-D culture over 15 Days at 80% O_2 vs. 21% (ambient) O_2 concentration. Mean values ± S.D, n=6 for each time point.

4.4.3.2 Protein synthesis

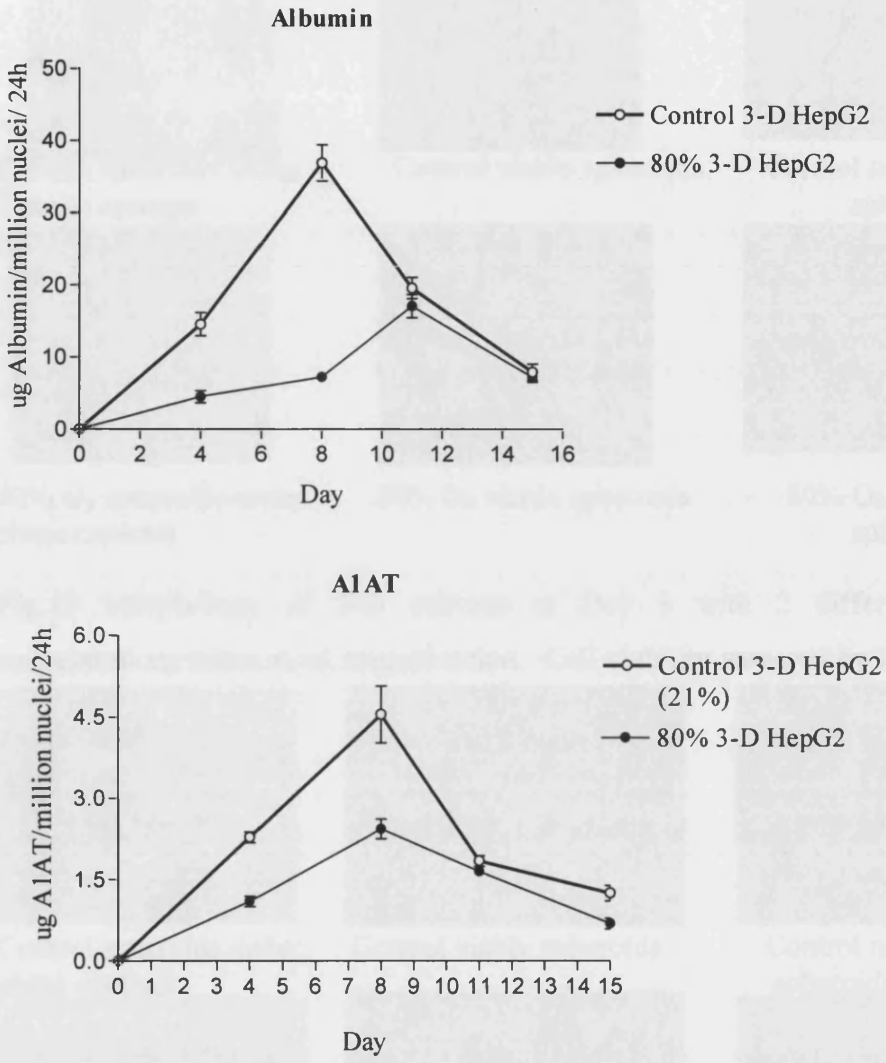


Fig. 18 Protein secretion of albumin and A1AT in 3-D HepG2 cultures maintained at 80% O_2 vs. 21% (ambient) O_2 concentration. Mean values ± S.D, n=6 for each time point.

4.4.3.3 Morphology and viability

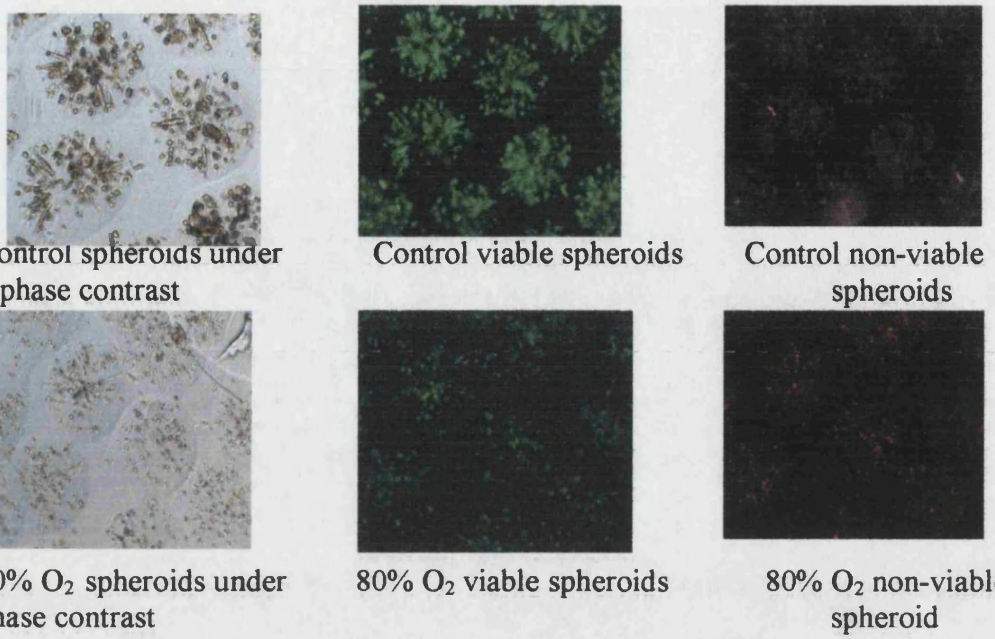


Fig.19 Morphology of 3-D cultures at Day 8 with 2 different oxygen concentrations taken at x4 magnification. Cell viability assessed by PI and FDA.

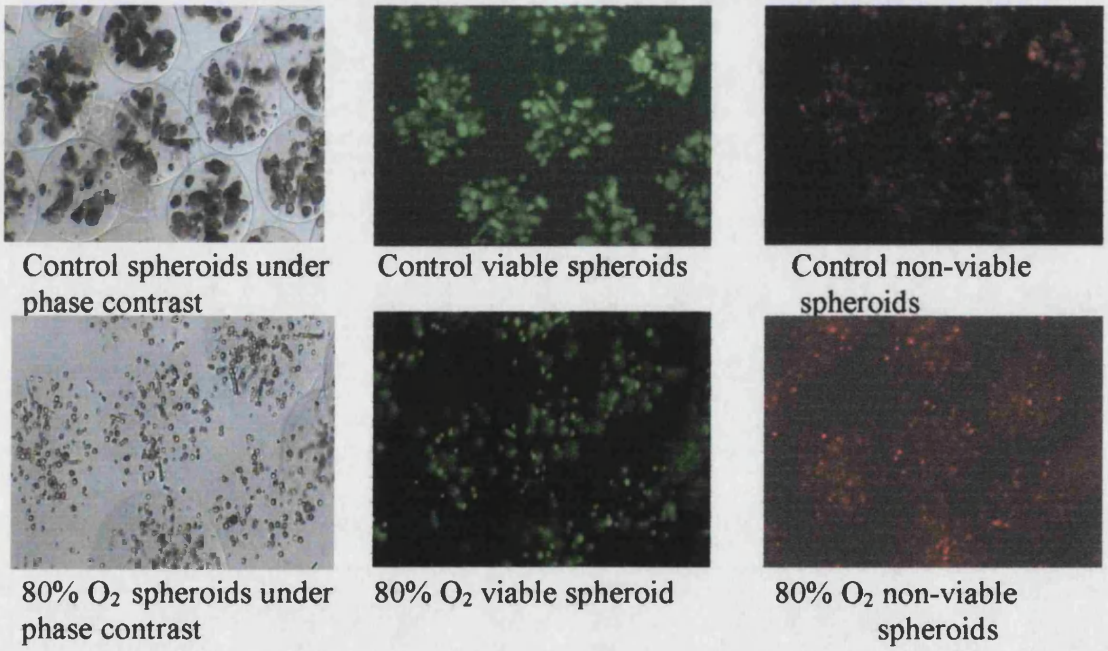


Fig. 20 Morphology of 3-D cultures at Day 15 in the 2 different oxygen concentrations taken at x4 magnification and cell viability assessed by PI and FDA staining.

It was decided to decrease the O_2 concentration lower than 80%, because as demonstrated, at that concentration, there was a detrimental effect on cell performance.

Therefore 3-D HepG2 cultures were exposed to 50% O_2 concentration, mid-way between ambient (21%) O_2 concentration and 80% O_2. This concentration is also higher than 35% O_2 concentration and at that concentration, although cell performance was not enhanced, it was not detrimental, so an improvement in cell performance was anticipated.

4.4.4 Changing the oxygen concentration to 50%

Maintaining the 3-D cultures at 50% O_2 did not improve protein function and also had a detrimental effect on cell proliferation and protein function per cell. In parallel the cells were maintained as monolayers and in increased oxygen concentration, their proliferation was also inhibited as shown in Fig.21.

4.4.4.1 Effect on cell proliferation in monolayer HepG2 culture

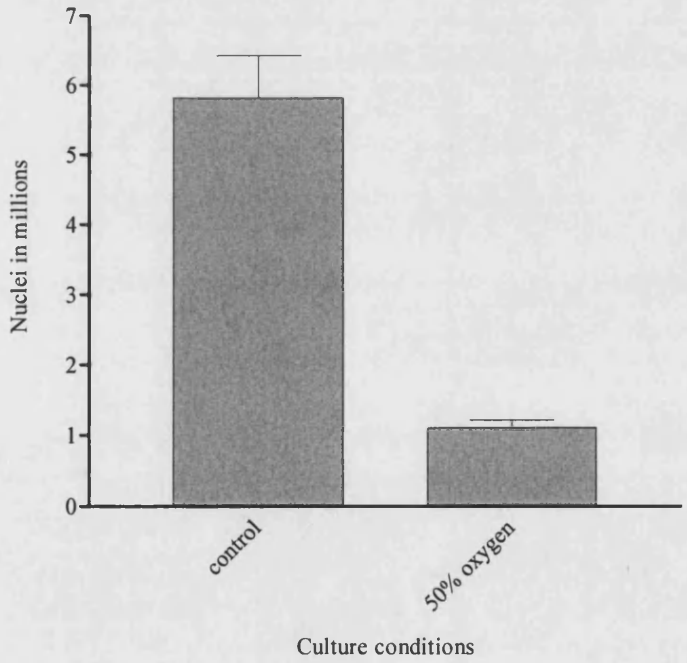


Fig. 21 Proliferation of HepG2 cells in monolayer culture at 2 different oxygen concentrations, 50% vs. 21%. Mean values ± S.D, n=6 for each time point.

4.4.4.2 Effect on cell performance in 3-D HepG2 culture

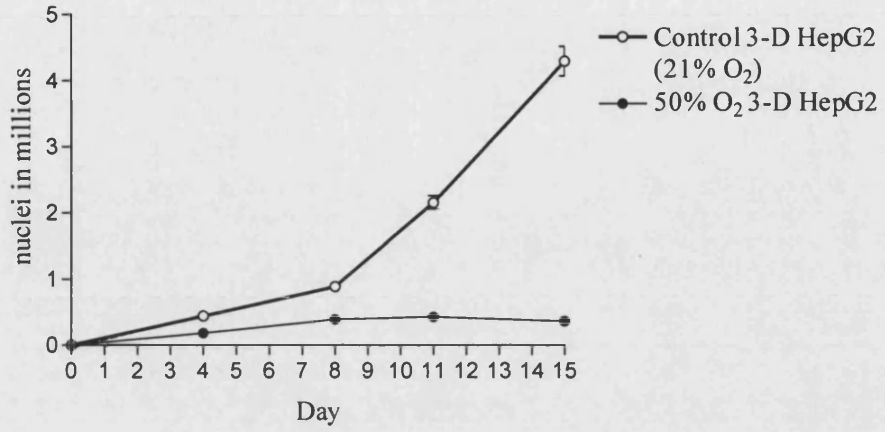


Fig. 22 Proliferation of HepG2 spheroids in 3-D culture over 15 days in two different oxygen concentrations, 50% vs. 21%. Mean values ± S.D, n=6 for each time point.

4.4.4.3 Protein synthesis

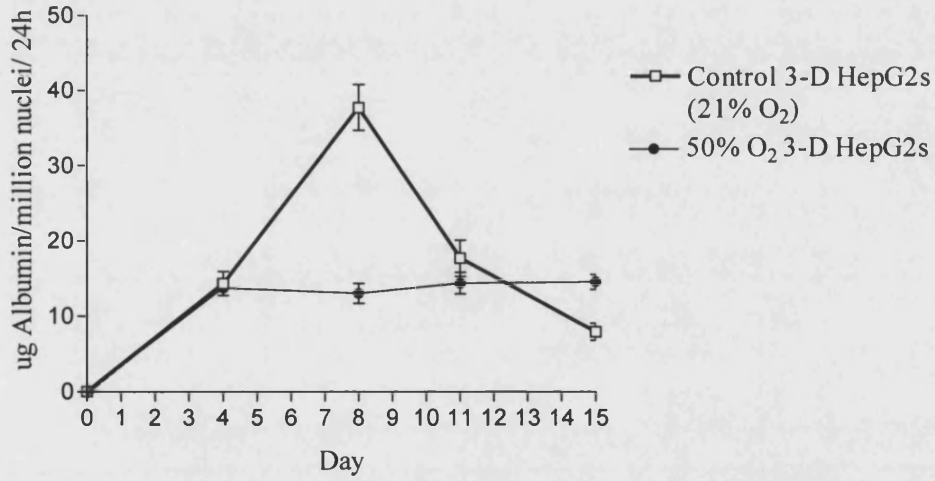
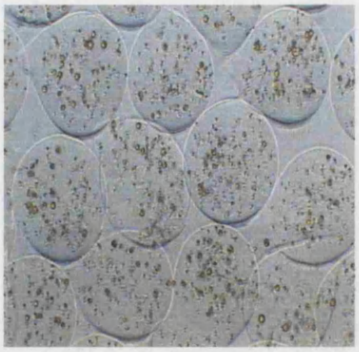
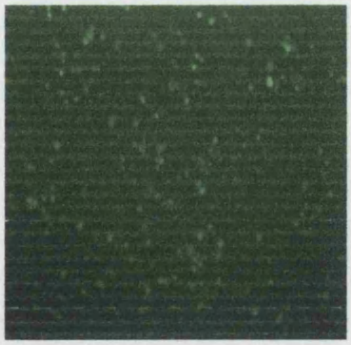


Fig. 23 Albumin secretion from HepG2 spheroids in 3-D culture over 15 days in two different oxygen concentrations, 50% vs. 21%. Mean values ± S.D, n=6 for each time point

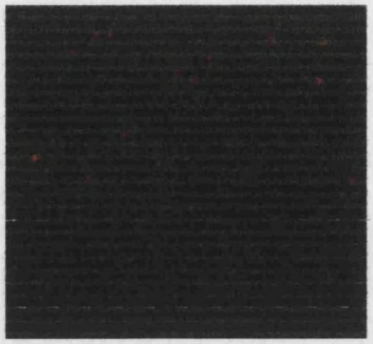
4.4.4.4 Morphology and viability



50% O_2 spheroids under phase contrast



50% O_2 viable spheroids



50% O_2 non- viable spheroids

Fig. 24 Morphology of 3-D cultures at Day 15 in the 2 different oxygen concentrations taken at x4 magnification and cell viability assessed by PI and FDA staining.

Overall, from the three different oxygen concentrations investigated, only the 35% oxygen concentration did not have an inhibitory function on proliferation or function but increasing the oxygen from 21% did not improve function per cell particularly at Day 15.

However, all known effects of signalling cascades can be activated by a variety of different stimuli, and 2 of these MAPKinases which were upregulated, indicative of a hypoxic stress response, can also be activated by an oxidative stress response and this was investigated at different stages in culture.

4.5 Oxidative stress is contributing to the downregulation of function observed at Day 15 of 3-D HepG2 culture

4.5.1 Background

In order to demonstrate whether oxidative stress could be contributing to the downregulation of function observed at Day 15, 3-D cultures, techniques can be employed to measure the products of modification of lipids and proteins undergoing oxidative stress. Reactive oxygen species (ROS) are produced by all aerobic cells and the higher the metabolic rate of an organism, the greater the production of ROS. The balance between ROS production and antioxidant defences determines the degree of oxidative stress (Finkel and Holbrook 2000;Finkelstein *et al.* 1983).

Consequences of this stress include modifications to cellular proteins (carbonyl formation), oxidative modifications of amino acid residues which include derivatization of those such as proline, arginine, and lysine to carbonyl derivatives, which can be detected by ELISA and Western blotting (Verbeke *et al.* 2001). Modifications to lipids undergoing oxidative stress can be assessed by measuring the breakdown products of lipid peroxidation including malondialdehyde (MDA) and 4-hydroxynonenal (HNE). MDA is a bi-product of polyunsaturated fatty acid peroxidation (Ling *et al.* 2003;Lorenzo *et al.* 1996).

4.5.2 Western blotting to show levels of phosphorylated MAPKinase in 3-D and monolayer HepG2 cultures activated in response to stress stimuli.

Western blot analysis of key MAP kinases expressed in HepG2 cultures showed increased levels of these proteins in monolayer HepG2 and/or Day 15, 3-D culture where differentiated function is low or diminished compared to peak function at Day 8, 3-D culture. In particular, MAPKp49/SAPK1/JNK1 is a downstream regulator of the JNK/SAPK stress activated pathway, whose activation is most sensitive to increased ROS (Turpaev 2002), and a downstream end terminal kinase, which when activated, results in the activation of

transcription factors, c-Jun, ATF-2 and p53 which in turn regulates gene expression to adapt to their survival in a new environment.

An equal amount of protein lysate from monolayer HepG2 culture, Day 8 3-D HepG2 culture and Day 15 3-D HepG2 culture was loaded onto a gel. By Western blot analysis, the levels of phosphorylated MAPKp49/SAPK1/JNK1 in these HepG2 cultures was measured and compared to the levels of the unphosphorylated form of this protein in these HepG2 cultures. It was demonstrated that this stress pathway is activated and at increased levels in monolayer and Day 15, 3-D culture.

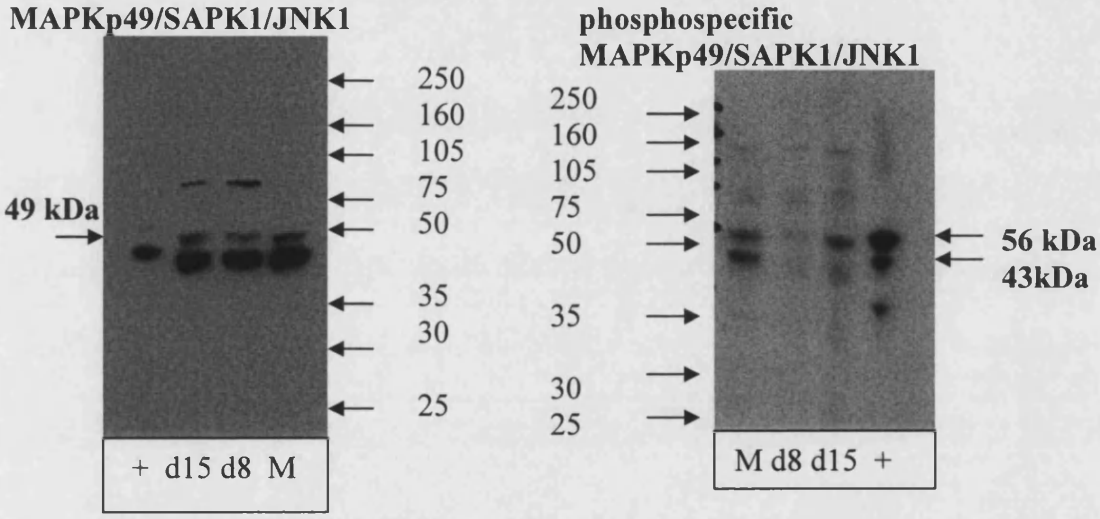


Fig. 25 MAPKp49/SAPK1/JNK1 and phosphorylated MAPKp49/SAPK1/JNK expression in monolayer HepG2 and 3-D HepG2 cultures (+ = cell lysate commercially supplied with antibody to serve as a positive control for the antibody).

Protein	Monolayer OD x mm^2	Day 8 OD x mm^2	Day 15 OD x mm^2
MAPKp49/SAPK1/JNK1	11.526	9.128	13.146
Phosphospecific MAPKp49/SAPK1/JNK1	4.366	0.466	3.505

Table 15 Densitometry measurements from protein expression of MAPKp49/SAPK1/JNK1 and phosphorylated MAPKp49/SAPK1/JNK expression in monolayer HepG2 and 3-D HepG2 cultures to confirm the observations made from Western blot detection.

4.5.3 Western blotting to measure protein oxidation in HepG2 culture

Proteins are one of the major targets of oxygen free radicals and other reactive species. The OxyBlot™ Kit was used to investigate protein oxidation through the detection of carbonyl groups (aldehydes and ketones) on proteins that occurs at and modifies the side chains of lysine, arginine, proline or threonine residues, and forms cysteine disulfide bonds as a result of several types of oxidative damage.

The carbonyl groups in the protein side chain are derivitised to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) which are then separated by polyacrylamide gel electrophoresis, followed by Western blotting. Oxidised proteins are revealed by an anti-dinitrophenyl antibody and subsequent procedures following a Western blotting procedure. This method was used as a semi-quantitative method for measuring oxidative stress in the samples.

4.5.3.1 Materials

- Protein Lysis Buffer- Final concentration, 50mM Tris-HCl pH 8.0,
  300mM NaCl, 0.5% Triton X-100-X, 0.1% SDS,
  1mM EDTA,
  2% β-mercaptoethanol,
  Protease inhibitor mix (10μg/ml leupeptin,
  10μg/ml pepstatin, 10μg/ml aprotinin, 1mM PMSF,
  10μg/ml antipain)
- 4mM EDTA in 0.15M NaCl pH 7.4
- Oxyblot™ Oxidised Protein Detection Kit, (Chemicon International, USA) containing- 10X 2, 4-Dinitrophenylhydrazine
  Neutralisation solution
  1° Antibody : Rabbit Anti-DNP Antibody
  2° Antibody : Goat Anti-Rabbit IgG (HRP-conjugated)
  10X Derivitisation-Control Solution
- NuPage Bis Tris 12% gels (Invitrogen, Scotland)
- 20X MOPS SDS Running Buffer
- NuPage Transfer Buffer

- 1% Bromophenol Blue in 2M Tris Base
- 10X PBS
- ECL+ Detection Kit (Amersham, UK)

4.5.3.2 Method

4.5.3.2.1 Positive control for oxidative stress assays

As a positive control for the assay, cells were made hypoxic by incubating them in deoxygenated medium, in which the medium was degassed by bubbling argon throughout the medium, and maintaining the cells overnight in an hypoxic environment at 37°C with the addition of argon gas. The cells were reperfused with oxygen for one hour prior to harvesting.

4.5.3.2.2 Monolayer Culture

Cells in a T175 cm^2 flask, ~25 x 10^6 cells were washed twice with ice cold PBS, and 3mls of lysis buffer was added. Cells were scraped off and transferred to a Nunc tube, vortexed and homogenised thoroughly with a Potter Elvjeham manual glass homegeniser. The samples were sonicated on ice for 10-20 seconds (Soniprep 150, Sanyo, Leicestershire, England) and centrifuged at 16000g at 4°C for 30 minutes (MSE Europa) to pellet the cells. The supernatant was removed and a protein concentration was determined. The sample was aliquotted and stored at -80°C. A protein concentration of at least 4μg/μl was required to obtain a 20μg protein sample in a 5μl sample. A protein concentration of more than 10μg/μl would render the protein sample insoluble with SDS.

4.5.3.2.3 3-D HepG2 culture

Beads, equivalent to ~ 25 x 10^6 cells for each time point, Day 8, Day 11 and Day 15 were transferred to a Nunc tube and allowed to settle. They were washed twice with ice cold PBS. Ice cold EDTA solution was added to the beads (1.9mls/0.25mls of beads) and mixed manually until the alginate dissolved and the spheroids were released. The solution was centrifuged at 200g for 10 minutes at 4°C (Heraeus, UK) to pellet the cells. The supernatant was removed and 2mls of lysis buffer was added. The samples were vortexed and then processed as for preparation of monolayer cultures.

4.5.3.2.4 Sample preparation for SDS-PAGE

The 20µg of protein (5µl) was denatured and solubilised by the addition of 5µl of 12% SDS, to obtain a final concentration of 6% SDS. After brief vortexing, 10µl of 1X DNPH solution (for derivatisation of carbonyl groups) was added to each tube, and 10µl of 1X Derivitisation-Control Solution to the negative control samples. After brief vortexing, the samples were incubated for 15 minutes at room temperature. To stop the derivitisation reaction, 7.5µl of neutralisation solution was added to each tube. A further neutralisation step was carried out by adding 4µl of 1% bromophenol blue in 2M Tris Base.

4.5.3.2.5 SDS-PAGE and Western blot Transfer

The samples were loaded onto a 12% Bis Tris gel and run for 1 hour at 200V. The gel was transferred to a PVDF membrane for 2 hours at 30V, and the membrane incubated for 1 hour at room temperature in 1% BSA in PBS with 0.1% Tween 20 with gentle agitation to block non-specific binding sites. The 1° antibody was diluted, 1:150 in dilution buffer (1% BSA with 0.05% Tween 20) just before use and the membrane incubated in this solution for 2 hours at room temperature with gentle agitation. The membrane was washed every 5-10 minutes in 1x PBS with 0.1% Tween 20 over 1 hour at room temperature with gentle shaking. It was incubated with 2° antibody 1:300 in dilution buffer for 1 hour at room temperature with gentle agitation, and again washed every 5-10 minutes in 1X PBS with 0.1% Tween 20 over 1 hour at room temperature with gentle shaking. After washing, the membranes were transferred to a dark room, excess wash buffer was drained and the signal developed by immersion in active ECL+ reagent and exposed to autoradiographic film. Initial exposures were of 10 seconds and extended as necessary. Exposed films were developed in a bench top developer (Compact X4, X-ograph Imaging Systems, Malmesbury, Wiltshire).

4.5.4 Results

The data below shows the increased levels of oxidised proteins with prolonged time in 3-D HepG2 culture. There were significant increases in levels of oxidised proteins ($p \leq 0.05$) in both Day 15, 3-D HepG2 culture compared to Day 8, 3-D culture and Day 11, 3-D culture compared to Day 8, 3-D culture.

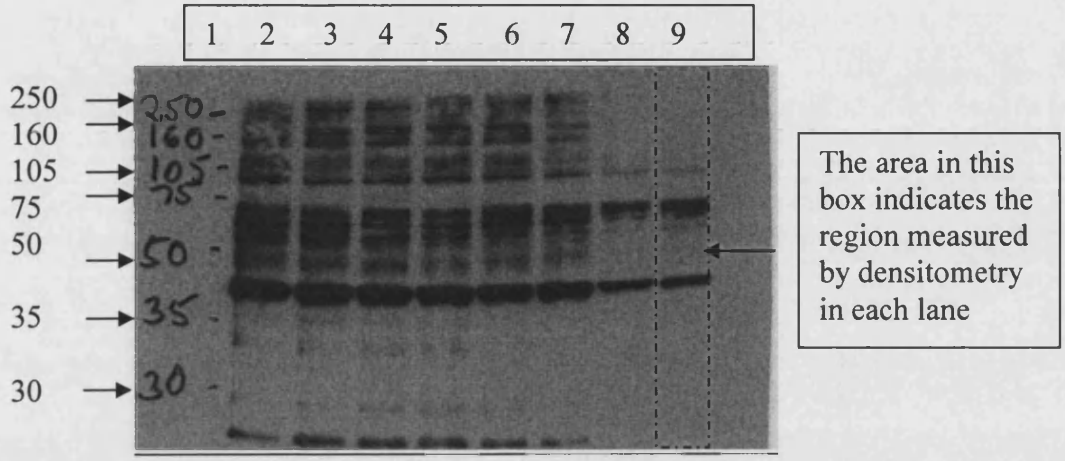


Fig. 26 Western blot for detection of oxidised proteins in 3-D HepG2 cultures.

Lane Number	Sample	Densitometry OD x mm^2
1	Protein marker (kDa)	n/a
2	Positive control for assay- Day 8 spheroids subjected to hypoxia/reperfusion	80.934
3		92.562
4	Day 15 (*)	74.861
5		65.976
6	Day 11 (*)	66.543
7		45.190
8	Day 8	13.1439
9		14.500
10	Negative control	

Table 16 Densitometry values of oxidised proteins in 3-D culture. Statistics (*) represents comparisons between Day 15 & 8, & then Day 11 & Day 8 $p \leq 0.05$ = *

To demonstrate further the enhanced performance of HepG2 cells in 3-D culture compared to monolayer HepG2 cell culture in protecting HepG2 cells from oxidative stress, oxidised proteins were measured in HepG2 cells in 3-D culture and monolayer HepG2 cell culture. Levels of oxidised proteins were compared in 3-D HepG2 cultures compared to HepG2 cells in monolayer culture. The protective function of HepG2 cells in 3-D culture was demonstrated further when HepG2 cells in 3-D and monolayer culture were both subjected to hypoxia then reperfusion, and then similar comparisons of levels of oxidised proteins were made.

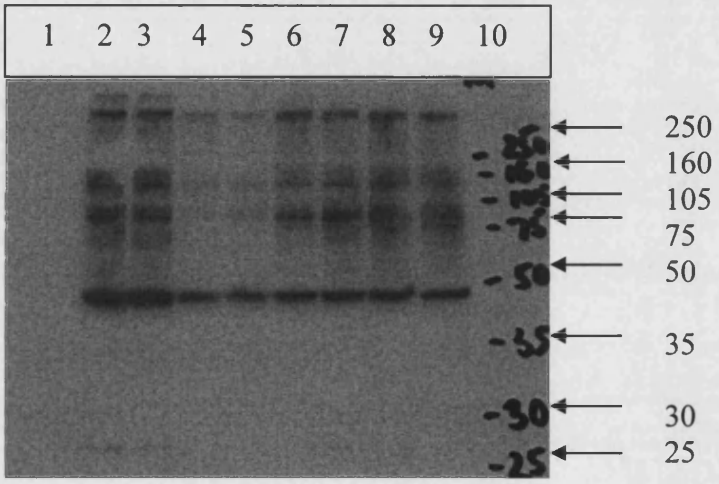


Fig. 27 Western blot for detection of oxidised proteins in monolayer cultures and Day 8, 3-D cultures under normoxia and subjected to hypoxia/reperfusion.

Lane Number	Sample	Densitometry OD x mm^2
1	Negative control	n/a
2	Monolayer HepG2 cells subjected to hypoxia/reperfusion (*)	43.472
3		46.137
4	Day 8, spheroids	9.270
5		7.858
6	Day 8 spheroids subjected to hypoxia/reperfusion (***)	24.163
7		30.241
8	Monolayer HepG2 cells	26.092
9		35.502
10	Protein marker (kDa)	n/a

Table 17 Densitometry values of oxidised proteins in Day 8, 3-D culture compared to monolayer HepG2 culture under control and hypoxic conditions. Statistical analysis (*) represents comparisons between hypoxic Day 8 vs. control Day 8, and hypoxic monolayer HepG2 cells vs. control HepG2 monolayer cells. $p \leq 0.05$ = *, $p \leq 0.0005$= ***

The results below in Fig.28 show the increasing levels of oxidised proteins with prolonged 3-D HepG2 culture as demonstrated before, but also human liver as an additional control for the experiment.

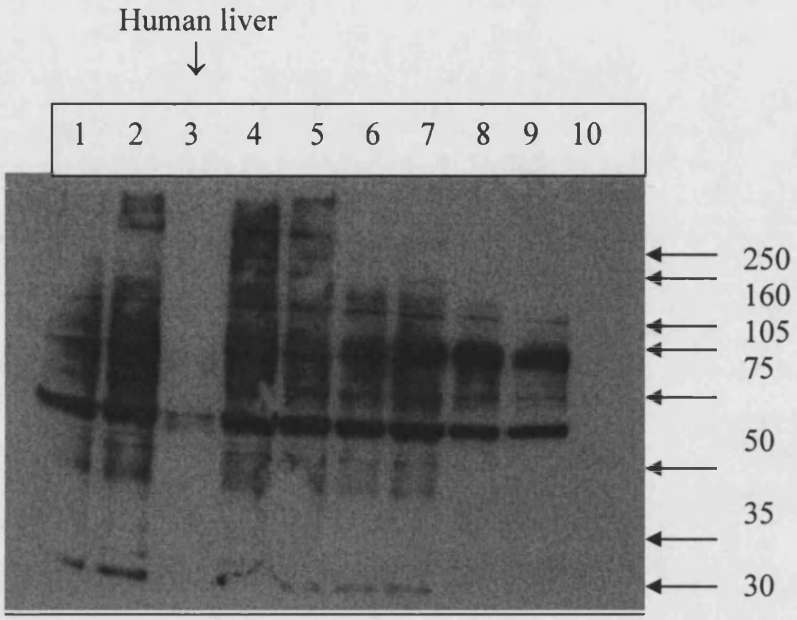


Fig. 28 Western blot for detection of oxidised proteins in 3-D HepG2 cultures, with human liver as a negative control to show lack of protein oxidation in normal human liver compared to Day 15.

Lane Number	Sample	Densitometry OD x mm^2
1	Day 8 spheroids subjected to hypoxia/reperfusion	65.401
2		114.751
3	Human Liver	4.791
4	Day 15 spheroids (*)	118.794
5		67.448
6	Day 11 spheroids	52.181
7		66.276
8	Day 8 spheroids	43.379
9		32.858
10	Protein marker (kDa)	n/a

Table 18 Densitometry values of oxidised proteins in 3-D cultures, with comparison to human liver as a negative control. Statistics (*) represents comparisons between Day 15 vs. Day 8, 3-D culture. $p \leq 0.05$ = *

4.5.5 The Thiobarbituric acid reactivity test to measure lipid peroxidation in HepG2 culture

One of the most commonly applied assays to measure the products of lipid peroxidation is the thiobarbituric acid (TBA) test. The sample under test is heated with TBA at a low pH, and the assay involves the reaction between malondialdehyde and TBA to yield a pink chromogen which is measured colorimetrically at 532nm using a spectrophotometer.

4.5.5.1 Materials

- Hank's x MDA (0.015g $CaCl_2$, 1.6g glucose, 0.4g KCl, 0.06g KH2PO4, 0.2g MgSO4. $7H_2O$, 8g NaCl, 0.048g Na_2HPO4, 0.35g NaHCO3) to a final volume of 1 litre pH 7.4
- 10% TritonX-100
- cold 4mM EDTA in 0.15M NaCl pH 7.4
- 50% TCA
- 1,1,3,3-tetramethoxypropane (malonaldehyde) Sigma
- HCl
- 5% TBA –Made in 1M NaOH and left to stir on a hot plate for 1-2 hours until it dissolves- turns a hazy yellow colour
- 10% Butylated hydroxytoluene (BHT)
- 1mM menadione sodium bisulphite
- 100mM hydrogen peroxide
- n-butanol
- Argon cylinder
- Oxygen cylinder

4.5.5.2 Method

4.5.5.2.1 Monolayer cultures

HepG2 cells in monolayer culture were grown to 70-80% confluency. Cells were washed twice with Hanks x MDA and to the flask 7.5mls of Hanks x MDA alone was added with or without the pro-oxidant, 100μm menadione sodium bisulphite to induce oxidative stress and serve as positive controls. As a further positive

control for the assay, a hypoxic positive control was used for the assay as described. The method adapted from Iguchi *et al* 1993 and Bassi *et al* 1998, allows us to measure the intra and extracellular Thiobarbituric Acid Reactive Substances (TBARS) released from the cells. After the incubation period, 150ul of 10% TritonX-100 was added to give a final concentration of 0.2% TritonX-100. The flasks were incubated for a further 5 minutes at 37°C to lyse the cells. The cells and medium were transferred to a Nunc tube and homogenised thoroughly and kept on ice.

4.5.5.2.2 Human liver

7.5mls of Hanks x MDA was added to small piece of human liver tissue (5-10mg), with 150ul of 10% TritonX-100 to give a final concentration of 0.2% TritonX-100. The tissue was homogenised thoroughly with a glass homogeniser and the samples were kept on ice.

4.5.5.2.3 3-D HepG2 cultures

2mls each of beads from Day 8, 11 and 15 of 3-D culture were transferred to a Nunc tube. The beads were allowed to settle and then washed twice with Hanks x MDA. 7.5mls of Hanks x MDA was added to each of the 2mls of beads with or without the pro-oxidant and transferred to T80cm^2 flasks and incubated for 2 hours at 37° C. 3-D HepG2 cultures were subjected to hypoxia, then reperfusion injury to serve as another positive control for the assay as described for monolayer culture. The beads and Hanks x MDA medium were transferred to Nunc tubes and once the beads had settled the conditioned medium with the released TBARS, was transferred to fresh Nunc tubes and kept on ice. To the beads, 15.2mls of cold 4mM EDTA in 0.15M NaCl pH 7.4 was added to chelate the calcium ions out of the alginate and release the spheroids. The tubes were mixed manually until the alginate had dissolved and centrifuged at 200g for 10 minutes at 4°C (Heraeus, UK) to obtain a pellet of spheroids. The supernatant was removed and to the remaining pellet, the conditioned medium was added with 150µl of 10% TritonX-100 to give a final concentration of 0.2% TritonX-100 in order to lyse the cells. The spheroids were homogenised thoroughly with a glass homogeniser and

the samples were kept on ice.

4.5.5.3 Assay for measuring lipid peroxidation

All samples were protected from light. To 1ml of cell suspension, 2mls of solution was added to give a final concentration of 0.375% TBA, 15% TCA, 0.25N HCl and 0.01% BHT in Hanks x MDA buffer. BHT acts as an antioxidant to prevent further lipid peroxidation in the samples.

The samples with the TBA solution were mixed thoroughly and boiled for 30 minutes, and cooled to room temperature with tap water. 3mls of n-butanol was added and each sample was vortexed thoroughly for 30 seconds to extract the TBARS formed during heating. The samples were centrifuged at 1250g for 10 minutes at room temperature (Heraeus, UK) to separate the organic layer (TBARS). This supernatant was carefully removed and placed in fresh tubes wrapped in aluminium foil. The fluorescence intensity of the TBARS released was read at 515nm excitation and 552nm emission with a flourimeter (Spectroflourophotomoeter, RF-5001 PC, Shimadzu, Japan).

4.5.5.4 Results

A standard curve was plotted using a serial dilution of known concentrations of 1,1,3,3-tetramethoxypropane (malonaldehyde) in Hanks x MDA using 1ml of this standard solution and 2mls of the TBA solution mix and processed in the same way as the HepG2 samples. Sample optimal densitometry (OD) readings were measured against the curve to obtain a known value in nm. A small amount from the remaining sample was removed and measured for total protein content by the Lowry method and TBARS were expressed as nmol/mg protein.

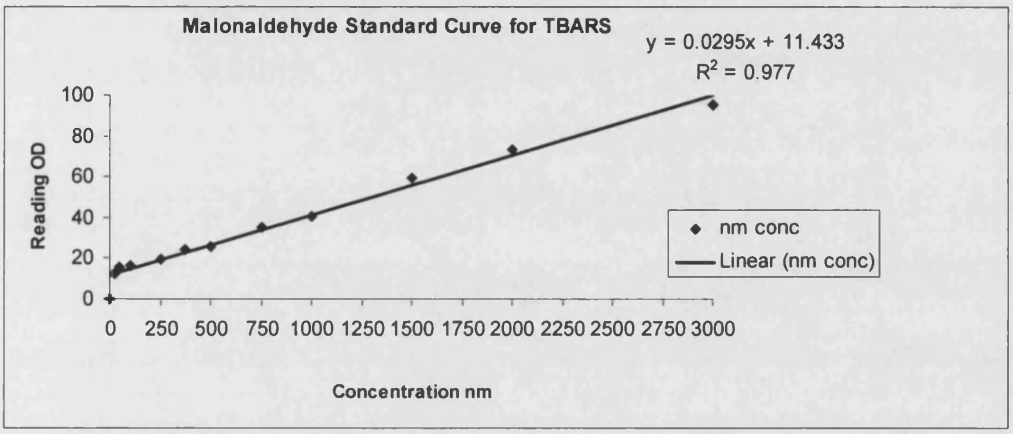


Fig. 29 Standard curve of absorbances corresponding to known concentrations of malondialdehyde

The data in Fig.30 shows the increasing level of lipid peroxidation with prolonged time in 3-D HepG2 culture. There were significant increases in levels of lipid peroxidation ($p \leq 0.05$) in Day 15, 3-D HepG2 culture compared to Day 8, 3-D culture.

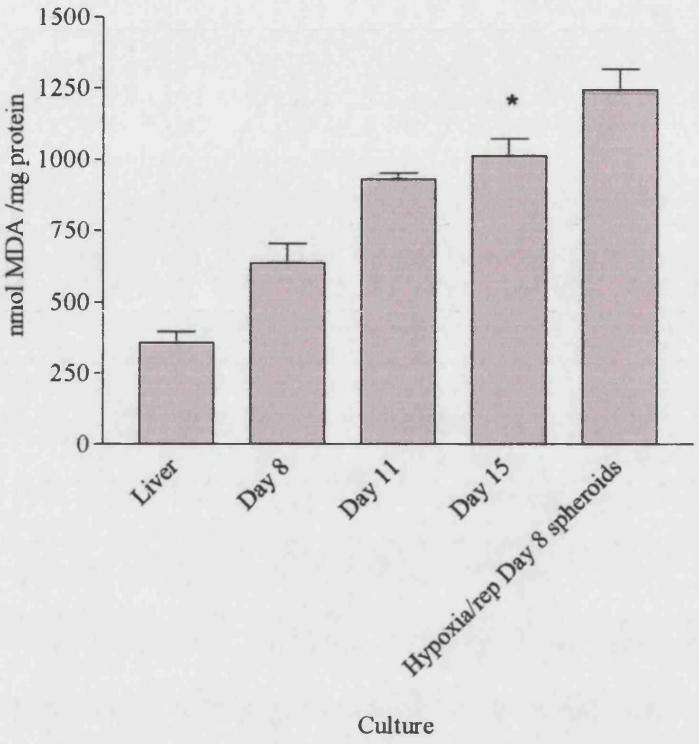


Fig. 30 Lipid peroxidation in 3-D HepG2 cultures over time, with liver as a negative control. Mean values ± S.D, n=3 for each time point. Statistics (*) represent comparisons between Day 15 and Day 8. $p \leq 0.05$ = *

To demonstrate further the enhanced performance of HepG2 cells in 3-D culture compared to monolayer HepG2 culture in protecting HepG2 cells from oxidative stress, lipid peroxidation was measured in HepG2 cells in 3-D culture and monolayer HepG2 culture. Levels of lipid peroxidation were compared in 3-D HepG2 cultures compared to HepG2 cells in monolayer culture. The protective function of HepG2 cells in 3-D culture was demonstrated further when HepG2 cells in 3-D and monolayer culture were both subjected to hypoxia then reperfusion, and then similar comparisons of levels of lipid peroxidation was made.

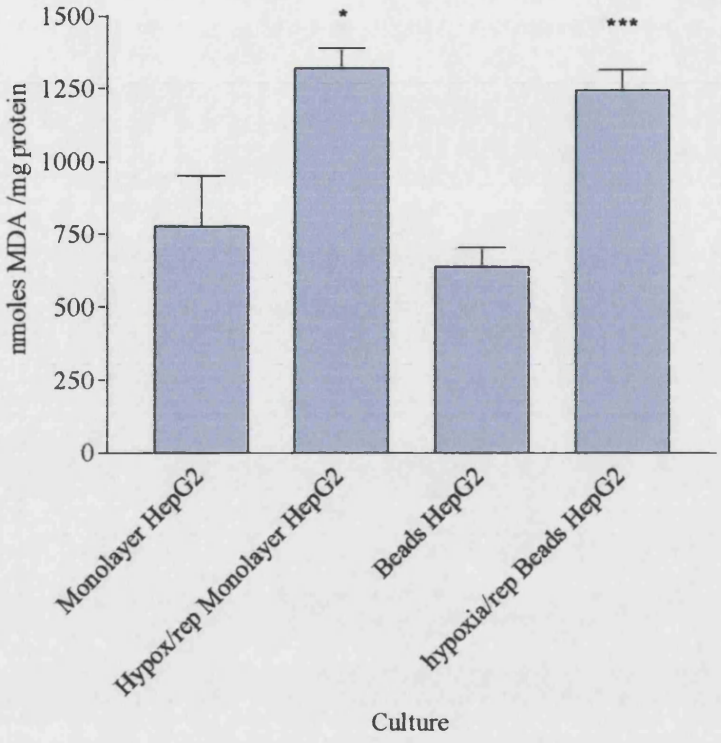


Fig. 31 Lipid peroxidation in monolayer HepG2 cells compared to 3-D HepG2 cells to show that HepG2 cells are protected against oxidative stress in 3-D culture compared to monolayer culture. Mean values ± S.D, n=3 for each time point. Statistics (*) represent comparisons between hypoxia/reperfusion cultures and control cultures. $p \leq 0.05$ = *, $p \leq 0.0005$= ***

4.6 Overcoming oxidative stress observed at later times of 3-D culture with supplementation of additional anti-oxidants

Oxidative stress has been observed at later times of 3-D culture from Day 11 onwards, when function starts to diminish as demonstrated by increased protein oxidation and lipid peroxidation from Day 11 onwards. In addition, it was shown that phospho-specific MAPKp49/SAPK1/JNK1 is activated and showed increased expression in monolayer and Day 15, 3-D culture compared to Day 8, 3-D culture. Therefore, in order to overcome the oxidative stress observed, with the aim of increasing function per cell at times of diminished function or increasing cell

numbers at times of peak function, the medium was supplemented with additional anti-oxidants just prior to culture time points when function starts to diminish. The culture medium was already supplemented with anti-oxidants e.g. sodium selenite so anti-oxidants were not added at the beginning to prevent disturbance of the oxidant/anti-oxidant balance.

4.6.1 Materials

Glutathione (GSH)	(Sigma)
Vitamin E	(Sigma)
DMSO	(BDH)
N-acetylcysteine (NAC)	(Sigma)

4.6.2 Methods

3-D HepG2 cultures were generated as previously described and maintained up to 15 days. At Day 8, of 3-D culture, beads were harvested as previously described. 24 hours prior to the Day 11 harvesting time point, the control plate was set up with 5mls of HG medium, as before and 3 additional 6 well plates, the medium was supplemented with 50μM Vitamin E (dissolved in DMSO), 30mM NAC, and 20mg/litre GSH respectively (Goasduff and Cederbaum 1999;Waris *et al.* 2001). For beads harvested at Day 15, a control plate and 3 additional 6 well plates were also set up, and the medium was supplemented with the corresponding anti-oxidants at Day 10 and 12 in 8mls of medium as before, and Day 14, 24 hours prior to harvesting in 5mls of HG medium. Conditioned medium was collected for measurement of liver specific proteins by ELISA and cell number was elucidated by crystal violet staining.

4.6.3 Results

The results below show that with the supplementation of additional anti-oxidants, an increase in cell number or protein secretion per cell was not demonstrated, particularly from Day 11 onwards, when function starts to diminish.

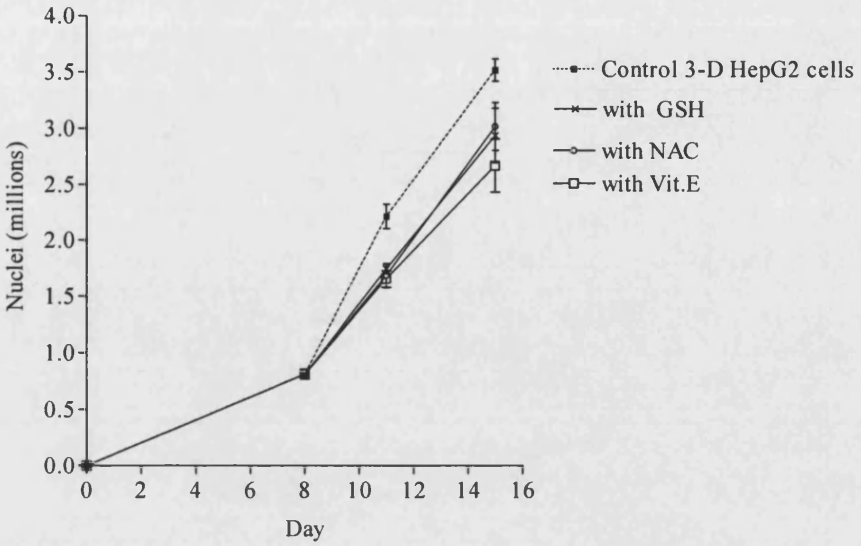


Fig. 32 Cell proliferation in 3-D culture with and without the supplementation of additional anti-oxidants. Mean values ± S.D, n=6 for each time point.

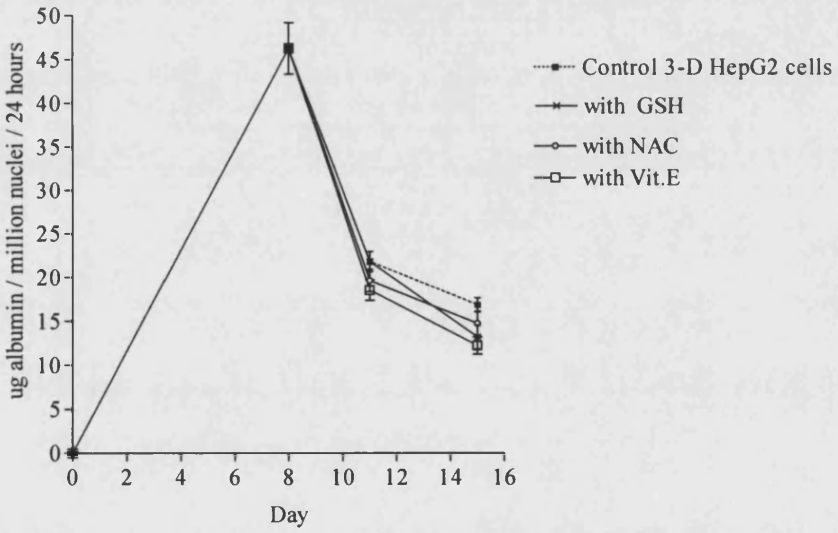


Fig. 33 Albumin secretion in 3-D culture with and without the supplementation of additional anti-oxidants. Mean values ± S.D, n=6 for each time point.

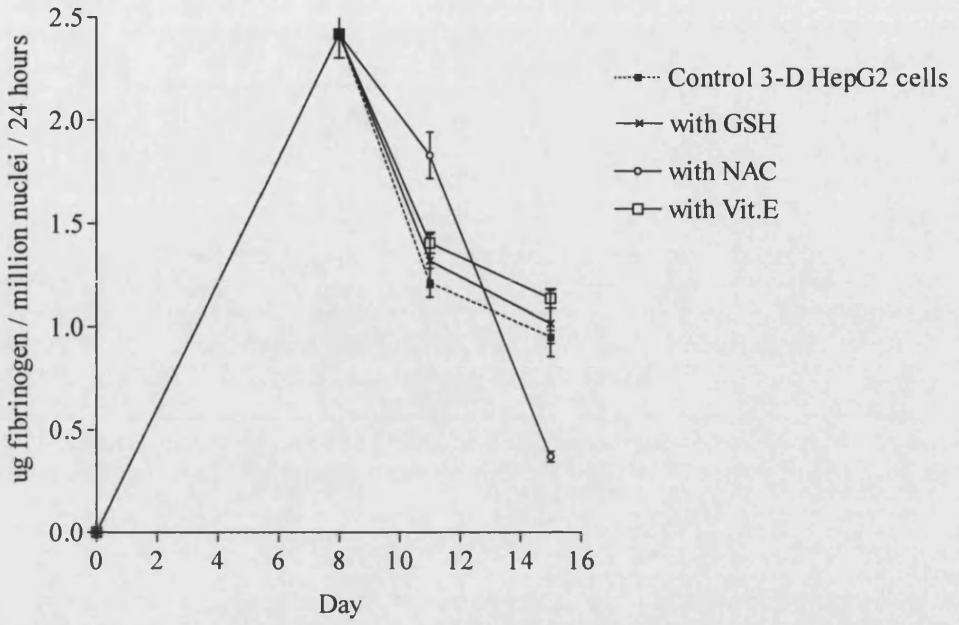


Fig. 34 Fibrinogen secretion in 3-D culture with and without the supplementation of additional anti-oxidants. Mean values ± S.D, n=6 for each time point.

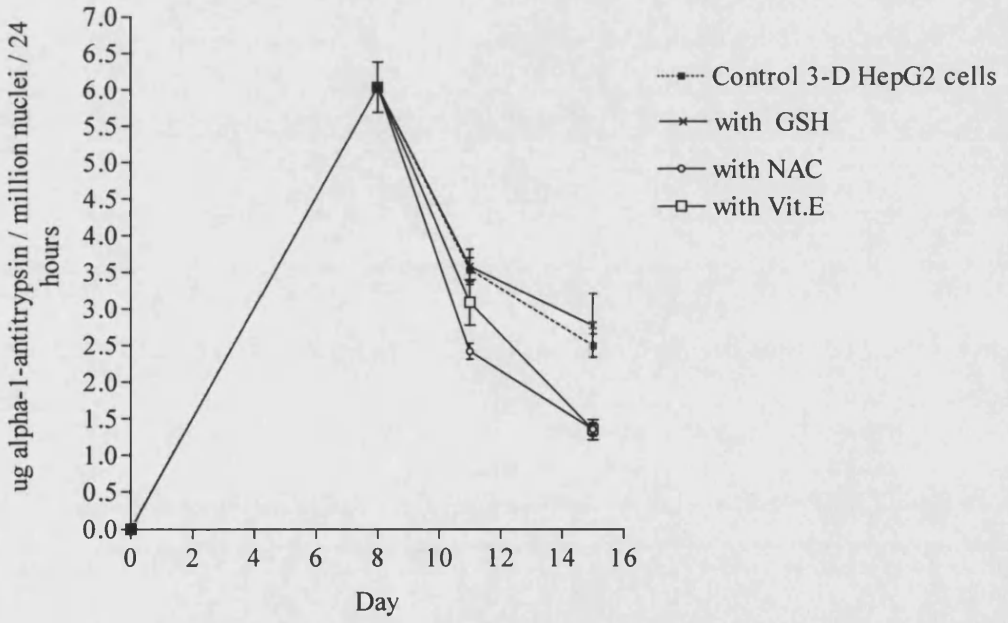


Fig. 35 Alpha-1-antitrypsin secretion in 3-D culture with and without the supplementation of additional anti-oxidants. Mean values ± S.D, n=6 for each time point.

Although there was evidence of hypoxic and oxidative stress, attempts to alleviate this stress were unsuccessful. The Microarray database was searched further and western blots were carried out to look for changes in genes and proteins, which triggered a general stress response.

4.7 Evidence for a general stress response in HepG2 culture

4.7.1 From Microarray analysis

	Fold Change ± S.D	% of chips showing this change
Day 8 vs. Monolayer		
INCREASE		
Heat shock protein 70 (HSP 70)	+ 3.46 ± 2.50	100
Catalase	+ 2.44 ± 0.30	100
MHC Class I	+ 3.20 ± 0.60	100
DECREASE		
HSP 47	-3.15 ± 0.94	55
RNA Polymerase II	-2.38 ± 0.24	55
Day 15 vs. Day 8		
DECREASE		
Glutathione-S-transferase	-3.83 ± 1.98	44
MAPK protein kinase 3	-4.45 ± 1.39	44

Table 19 Expression of genes associated with cellular stress by Microarray analysis

4.7.2 By Western blotting

Western blots were carried out with total cellular protein from monolayer HepG2 cells, Day 8 and Day 15, 3-D culture for the presence of key proteins expressed as a result of these stress inducing stimuli to complement the Microarray analysis.

MAPKp49/SAPK1/JNK1

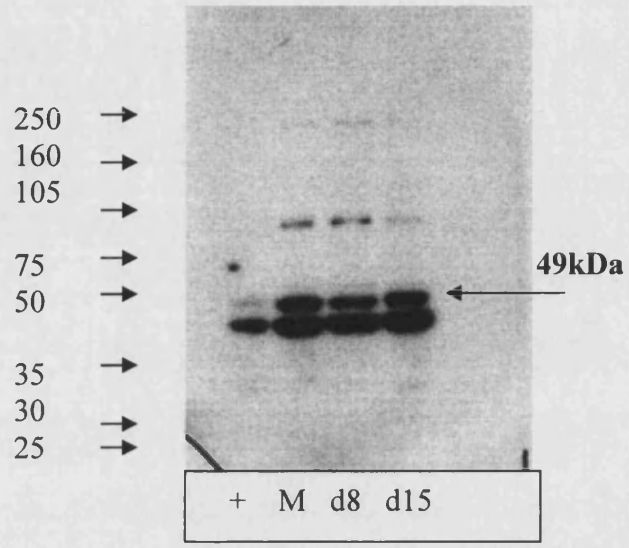


Fig. 36 MAPKp49 protein expression in HepG2 cultures

MAPKp49 protein is detected as 2 strong bands in all the samples including the control. The upper band is the band of interest as the lower band is a post-translational modification of the protein. Stronger signals were detected in monolayer and Day 15 samples than the Day 8 sample.

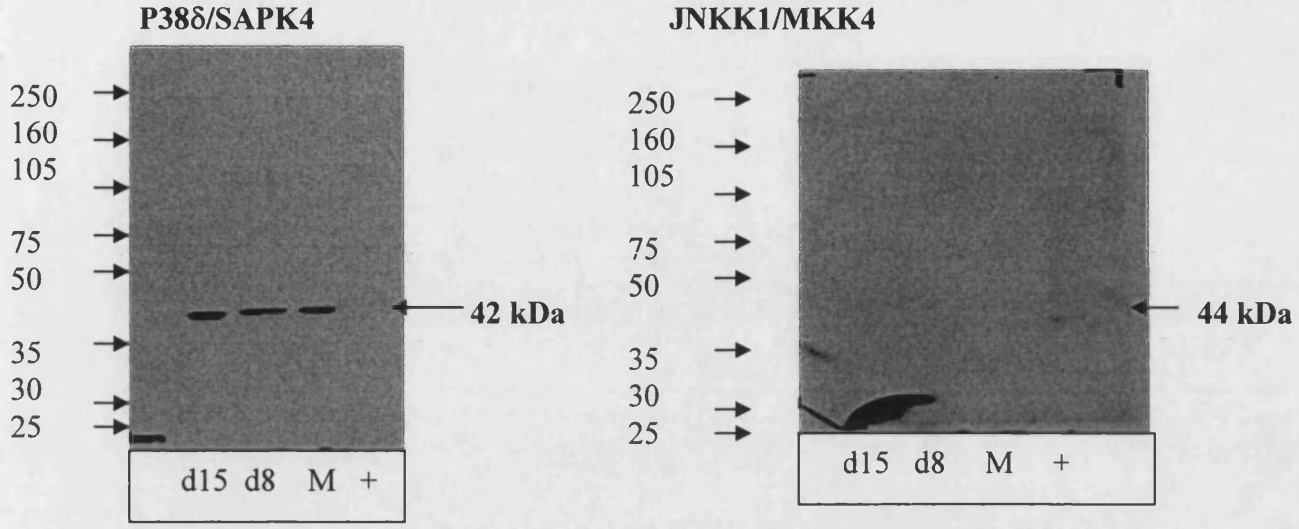


Fig. 37 P38δ and JNNK1 protein expression in HepG2 cultures

JNKK1 or MKK4 protein was not detected in any of the samples but a faint signal was observed with the positive control lysate from rat cerebellum (+).

P38δ/SAPK4 protein was present in equal amounts in all the samples and showed no change in signals, a faint signal was observed with the positive control lysate from A431 cells (+).

ST1/Hop-p60

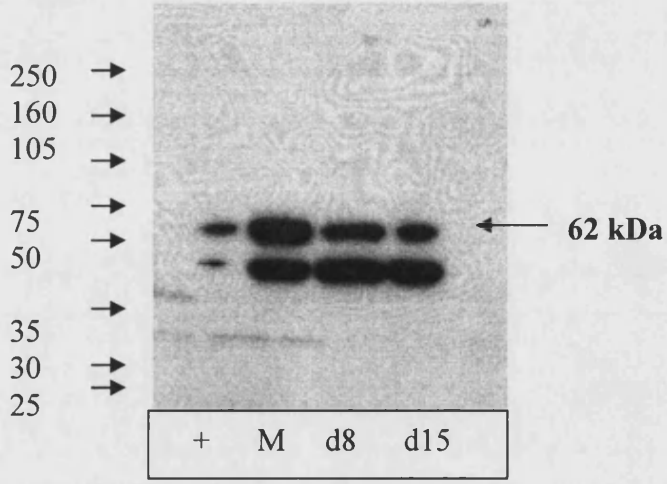


Fig. 38 ST1 protein expression in HepG2 cultures

ST1 (Hop p60) shows a similar pattern of expression to MAPKp49 and again, an expression of a slightly smaller band is due to post-translational modification of

that protein. The strongest signal here was shown in the monolayer samples compared to Day 8 and Day 15. A clear signal was shown with the positive control lysate from A431 cells (+).

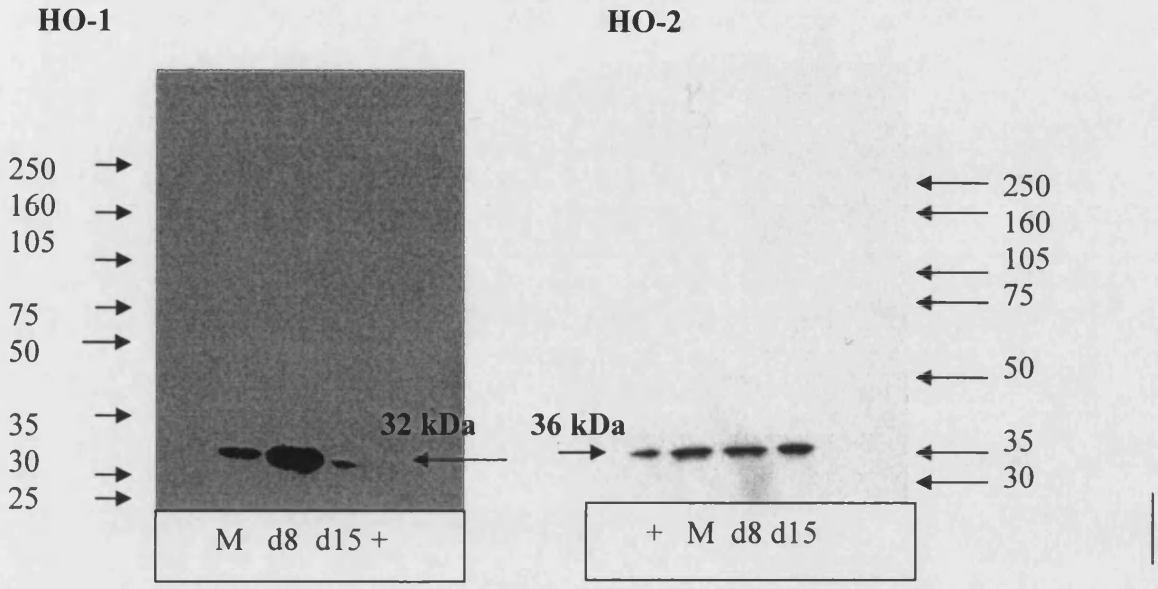


Fig. 39 HO-1 and HO-2 protein expression in HepG2 cultures

Haemoxygenase (HO)-1 protein was abundant in the Day 8 sample and gave the strongest signal compared to the monolayer sample and Day 15 sample. A very weak signal was observed with the positive control lysate from SW-13 cells (+).

HO-2 showed no change in signal strength between all the samples and a clear signal was seen with the positive control lysate from SW-13 cells. The expected expression of HO-2 confirmed equal loading of samples as HO-2 is constitutively expressed in mammalian cells, whereas the expression of HO-1 is induced upon external stresses (+).

Cyclo-oxygenase (COX)-2 protein was not detected in any of the samples but a strong signal was observed with the positive control lysate from macrophages (figure not shown).

The densitometry measurements quantify the observations made from Western blot detection that these "stress" proteins showed increased levels of expression at times of diminished function in monolayer and Day 15, 3-D HepG2 culture.

Densitometry

Protein	Monolayer OD x mm^2	Day 8 OD x mm^2	Day 15 OD x mm^2
MAPKp49	9.749	8.364	11.288
P38δ / SAPK4	2.595	2.754	2.653
ST1-Hop60	13.992	7.697	4.585
HO-1	5.501	13.367	5.873
HO-2	2.979	2.891	3.154

Table 20 Densitometry measurements for general stress protein expression

4.7.3 Powerblot-Protein Array

As a complement to the Microarray, a protein array was carried out. Approximately 8mg of protein, from all culture conditions was analysed by Western blot, using the Becton Dickinson (USA) Powerblot, which includes antibodies to more than 700 antigens. The comparisons made were.

1) 3-D Cultures at optimal function at Day 8 vs. monolayer (M) cultures
2) 3-D cultures at Day 15 (Diminished Function) vs. Day 8 (Optimal Function)

The Powerblot results were comparable with the results obtained from the western blots carried out in the laboratory for the following proteins;

Day 8 vs. Monolayer

Protein	Cat. No	Fold change in expression
Heme oxygenase (HO) 1	H59320	INCREASE 4.84
MAPKp49	M54920	DECREASE 2.54

Day 15 vs. Day 8

Protein	Cat No.	Fold change in Expression
STI / Hop-p60	S65720	INCREASE 1.84
Heme oxygenase (HO) 1	H59320	DECREASE 2.51

Tables 21 Densitometry measurements of stress response proteins from the Powerblot

The Powerblot results were interrogated further and the most significant results are shown, grouped into various biological functions. Confidence levels range from 5-1 so changes in level 5 and 4 are shown as the most significant, as a result of reproducibility of results and fold increases.

Tables 22, 23 and 24 below demonstrate a complex result; some stress proteins were turned on at time of poor performance, indicating the role of stress response mechanisms during diminished function. Other stress proteins were turned on at the time of enhanced function, or perhaps more likely that this increased expression was an adaptive response to enhanced performance.

4.7.3.1 Day 8 vs Monolayer-Adaptive Stress

Table 22 Most significant results when comparing **Day 8 vs. monolayer** showing proteins expressed as a result of the enhanced metabolic performance and *in vivo* like morphology observed at Day 8, 3-D cultures

Adaptive stress proteins	Catalog. No	Confidence Level	Fold Change	Function
p115	P67420	5	INCREASE 2.09	Vesicular transport from the ER to Golgi
Plectin –144	P92020	5	INCREASE 3.04	Filament binding protein-crosslinking in cytoskeleton
E-cadherin	C377020	5	INCREASE 1.98	Calcium dependent adhesion molecule, Increase reduces invasive carcinoma, important for epithelial junction formation
Fibronectin	F14420	5	INCREASE 4.03	ECM protein –via integrin binds to collagen and attachment to cell, cellular signalling
HSP60	H99020	4	INCREASE 2.01	Heat shock protein, constitutively expressed in normal and apoptotic cells
Annexin IV	A29920	4	INCREASE 2.44	Family of calcium and phopsholipid binding proteins
Adaptin alpha	A43920	4	INCREASE 2.21	Recruits membrane proteins-vesicular transport
REF-1	R64820	4	INCREASE 2.51	Redox factor. Increase by AP-1 DNA binding-DNA repair
HRF	H42020	5	DECREASE 6.37	Histamine releasing factor
Nip-1	N79420	4	DECREASE 1.95	Pro-apoptotic proteins-target proteins to mitochondria

4.7.3.2 Day 8 vs Monolayer-Cellular stress

Table 23 Most significant results when comparing **Day 8 vs. monolayer** showing proteins expressed as a result of cellular stress

Cellular Stress Response Protein	Catalog. No	Level	Fold Change	Function
Cathepsin D-29	C47620	5	INCREASE 9.2	Tissue remodelling in response to oestrogen
CDC42	C70820	5	DECREASE 4.3	Rho protein- activates MEKK1
HSF4 – 38	H65520	5	DECREASE 4.25	Heat shock factor- mediates transcription of HSPs, increases when there is a decrease of HSP70, 90

4.7.3.3 Day 15 Vs Day 8-Cellular Stress

Table 24 Most significant results when comparing **Day 15 vs. Day 8** showing proteins expressed as a result of cellular stress

Cellular stress Response Protein	Catalog. No	Level	Fold Change	Function
Gelsolin	G37820	5	INCREASE 5.54	Severs actin filaments in a calcium dependent manner
Annexin IV	A14020	5	INCREASE 4.52	Calcium and phospholipid binding proteins. Unphosphorylated forms causes aggregation of chromatin granules
STI-1	S65720	4	INCREASE 1.84	Human Stress Inducible factor-induced by heat shock
panERK	E17120	4	INCREASE 2.21	Extracellular signal related kinase
Mek1	M17020	4	INCREASE 2.80	MAP / ERK kinase
HsPBP1	H98620	4	INCREASE 6.6	Heat shock 70 binding protein. Negative regulator of HSP70
Bid cl.7	B97920	5	DECREASE	Member of BCl-2 family. Pro-apoptotic
Rap2	R23020	5	DECREASE	Member of Ras family –activated by growth factors, mitogenic stimuli

This complex relationship between stress and performance was demonstrated further at the functional level, by carrying out enzyme activity assays, of key genes at the mRNA level activated by a general stress response.

4.8 Assays to demonstrate functional activity of genes at the mRNA level activated by a general stress response, contributing to the maintenance of optimal function in 3-D HepG2 culture

Amongst the changes observed in levels of gene expression of genes associated with a general cellular stress response was an increase in catalase mRNA in Day 8 spheroidal 3-D HepG2 cultures compared to monolayer HepG2 culture and a decrease in glutathione-S-transferase mRNA levels in Day 15 spheroidal 3-D HepG2 cultures compared to Day 8 spheroidal 3-D cultures.

In order to investigate whether these changes were also occurring at the functional level, assays were carried out in the HepG2 cultures to assess enzymatic activities of their gene products.

4.8.1 Catalase Activity Assay

Enzyme reaction

4.8.1.1 Materials

- HBSS on ice
- 1X PBS on ice
- 4mM EDTA in 0.15M NaCl pH 7.4 on ice
- *Protein Homogenisation buffer*

  0.32M sucrose

  1mM EDTA

  10mM Tris pH 7.4
- 0.5M KH_2PO_4
- Hydrogen peroxide solution (30%)
- Catalase (from bovine liver, Sigma cat. C-40)

Apparatus

- UV 300-Dual Beam Spectrophotometer (Unicam, Cambridge, UK)
- Waterbath.

4.8.1.2 Method

4.8.1.2.1 For 3-D cultures

Beads were cultured in 6 well plates as previously described. On the day of harvesting for Day 8 and Day 15, 3-D cultures, the beads were transferred from each cell strainer containing 0.25ml of beads into respective sterile microfuge tubes. The beads were washed once with ice cold HBSS and washed once with ice cold PBS. The beads were allowed to settle and 1ml cold 4mM EDTA in 0.15M NaCl pH 7.4 was added. The tubes were mixed manually until the alginate dissolved and released the HepG2 spheroids. The samples were centrifuged at 85g for 10 minutes at 4°C (Eppendorf 5402, Germany). The supernatant was removed and 1ml of homogenising buffer (0.32M sucrose, 1mM EDTA, 10mM Tris pH 7.4) was added. The samples were homogenised thoroughly with a Potter Elvjeham manual glass homegeniser to homogenate the samples. At this stage, 120µl of the homogenate was removed and kept on ice. The total protein content of the homogenate was measured by the Lowry method. The remaining sample was centrifuged at 350g for 10 minutes at 4°C (Eppendorf 5402, Germany). The supernatant was removed and placed in a fresh tube and kept on ice. The total protein content of the supernatant was determined by the Lowry method and assayed for enzymatic activity.

4.8.1.2.2 For monolayer culture

HepG2 cells were seeded in 6 well plates at the same time of preparation of 3-D culture and at the same initial seeding density. For protein preparation, cells were washed with cold HBSS and once with cold PBS. The cells were trypsinised on ice (1ml per well) and after gentle agitation with a cell scraper, cells were transferred to a microfuge tube and centrifuged at 120g for 4 minutes at 4°C (Eppendorf 5402, Germany). The supernatant was removed and 1ml homogenising buffer was added. The sample was homogenised thoroughly and processed as described for 3-D culture.

4.8.1.2.3 For liver tissue

Normal human liver tissue was homogenised in 1ml protein homogenising buffer and the sample was processed as described above.

4.8.1.3 Measuring catalase enzyme activity

Catalase activity was determined at 25°C, by measuring the rate of decomposition of hydrogen peroxide by ultraviolet spectroscopy at 240nm (UV 300-Dual Beam Spectrophotometer (Unicam, Cambridge, UK).

The reaction was performed using a solution of 50mM KH_2PO_4, 30μg of protein homogenate and 20mM hydrogen peroxide added at the end in a final volume of 1ml. Before measuring each sample, n=6 for each culture condition, the spectophotometer was zeroed with the above solution excluding the protein. The specific activity of the catalase was measured against this reference. The rate of decomposition of hydrogen peroxide by catalase was measured over 10 minutes. A graph was plotted showing absorbance at 240nm over 10 minutes. The software calculated a rate from the slope of the graph. From the slope of the graph, optimal rate was observed, usually in the initial 3 minutes from the reaction initiating. Specific activity was calculated as (units/mg of protein/min) = ΔA_{240}nm (1 min) x 1000/43.6 x 0.3 mg protein. As positive controls, 10 and 30 units of catalase were used and human liver tissue.

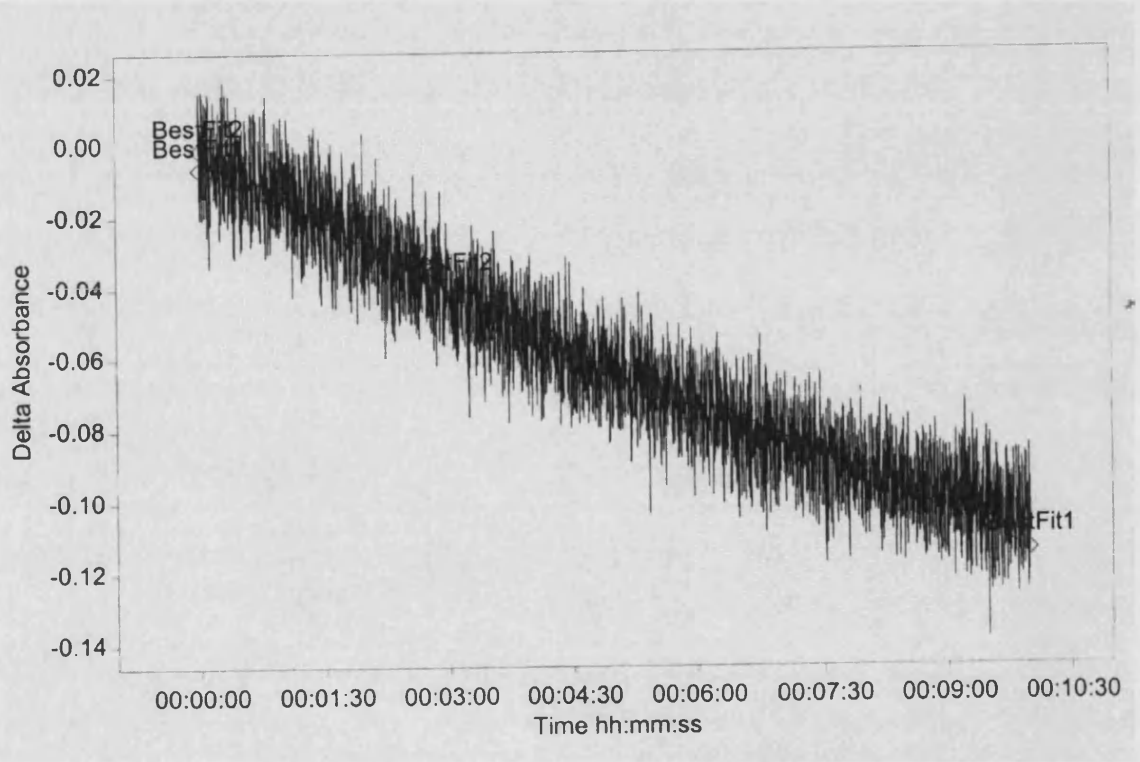


Fig. 40 A typical slope observed over time of decomposition of hydrogen peroxide by catalase over time.

4.8.2 Glutathione-S-transferase enzyme activity
Enzyme reaction

4.8.2.1 Materials

- HBSS on ice
- 1X PBS on ice
- 4mM EDTA in 0.15M NaCl pH 7.4 on ice
- 1-chloro-2,4-dinitrobenzene (CDNB), Sigma Cat No. C6396)
- Glutathione reduced form (GSH), Sigma Cat No. G4251.

4.8.2.2 Methods

Sample preparation

Protein homogenates from monolayer HepG2 cells, 3-D culture and human liver tissue were prepared as previously described for the catalase enzyme assay. 30μg of cytosolic protein was used in each assay.

4.8.2.3 Measuring glutathione-S-transferase (GST) enzyme activity

GST activity was determined at 25°C by measuring the rate of conjugation of GSH to CDNB with ultraviolet spectroscopy at 240nm (UV 300-Dual Beam Spectrophotometer (Unicam, Cambridge, UK). Final substrate concentrations were 1mM CDNB and 0.1M potassium phosphate buffer pH 6.5 in a final volume of 1ml. The reaction was started with the addition of 25μl of 40mM GSH. Before measuring each sample, the spectrophotometer was zeroed with the above solution excluding the protein. The specific activity of the protein sample was measured against this reference. As a positive control, human liver tissue was used. The change in extinction at 340nm with incubation time was measured. Results were expressed as units of specific activity defined as the amount of the enzyme that produces the conjugated product per minute per milligram of protein using the extinction coefficient 9.6 /mM/cm at 340 nm. Therefore GST activity was calculated as $\Delta A3_{40}$nm (1 min) x 9.6 x 0.03 mg protein.

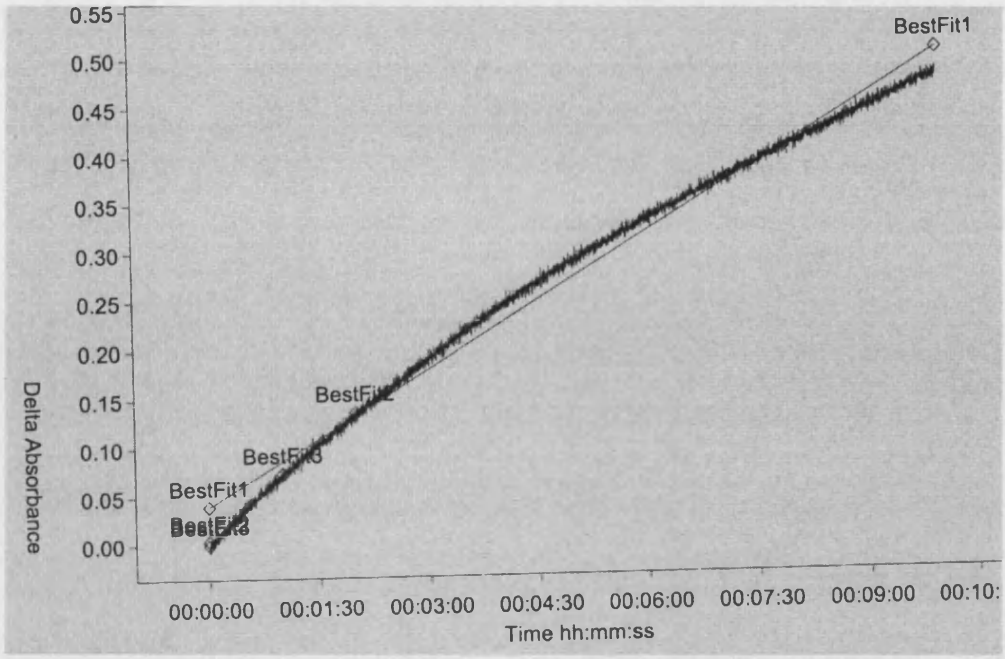


Fig. 41 A typical slope observed from the conjugation of GSH to CDNB to measure glutathione-S-transferase activity over time.

4.8.3 Results

4.8.3.1 Catalase Assay

The results show that catalase activity is optimal in Day 8, 3-D cultures, and is increased at Day 8, 3-D culture compared to monolayer HepG2 culture. The fold increase is comparable to the fold increase observed at the mRNA levels for this gene. Catalase activity is also upregulated in Day 15, 3-D culture compared to Day 8, 3-D culture.

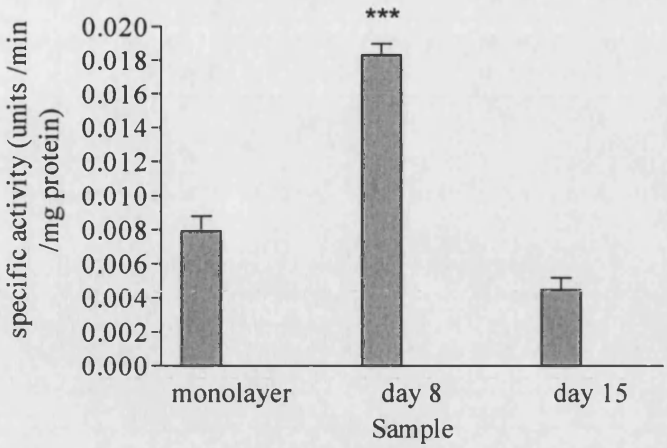


Fig. 42 Specific activity of catalase in 3-D HepG2 cultures and monolayer HepG2 cultures. Statistics (*) represents comparisons between Day 8 to Day 15 and then Day 8 to monolayer. Mean values ± S.D , n=6 for each time point.

p≤0.0005= ***

4.8 3.2 GST activity

The results show that GST activity is optimal in Day 8, 3-D cultures, and is increased at Day 8, 3-D culture compared to monolayer HepG2 culture. GST activity is also greater in Day 15, 3-D culture compared to Day 8, 3-D culture.

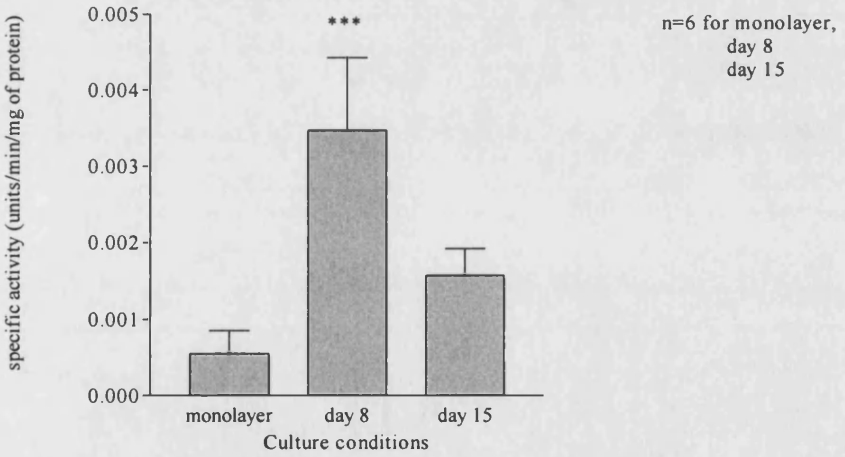


Fig. 43 Specific activity of glutathione-S-transferase in 3-D and monolayer HepG2 cultures. Statistics (*) represents comparisons between Day 8 to monolayer and then Day 8 to Day 15, 3-D culture.

Mean values ± S.D, n=6 for each time point. p≤0.0005=***

4.9 Discussion

From the results obtained from Microarray and Western blotting it was concluded:-

(1) stress associated genes and proteins were present at times of peak function, 3-D culture (at Day 8) compared to monolayer culture.

(2) The stress responses at later time points of culture, Day 15 spheroids in 3-D culture (diminished function) compared to Day 8 spheroids in 3-D culture (peak function) causing downregulation of function, could be caused by cellular stresses such as oxidative stress and/or hypoxia.

(3) in 3-D cultures at optimal function (Day 8), there are some cellular stress responses which may be turned on as an adaptive response as a consequence of increased metabolic activity (Nilsson *et al.* 2002). These stress responses early on may reflect increased function of healthy cells.

The results were complex, with some stress-induced genes proteins being more strongly expressed at the time of peak performance, at Day 8, 3-D culture, while others were strongly expressed in Day 15, 3-D cultures as performance diminished. An example is the catalase gene which constitutes an anti-oxidant defence in cells (Bai *et al.* 1999;Bai and Cederbaum 2000). There was an increase in expression of this gene at the mRNA level and functionally at the protein level, in Day 8, 3-D cultures compared to monolayer HepG2 culture and functionally at the protein level in Day 8, 3-D culture compared to Day 15, 3-D culture, reflecting both the optimal function at Day 8 and the adaptive response of the cells, requiring more catalase due to increased metabolic activity at Day 8, 3-D culture.

Stress Response- Day 15 HepG2 3-D cultures (diminished function) vs. Day 8 HepG2 cultures (optimal function)- The changes in expression of genes and proteins between Day 15 and Day 8 are associated with liver enriched transcription factors and various forms of cellular stress including both hypoxia and oxidative stress.

Western blots detected various proteins associated with cellular stress in 3-D

cultures at Day 8, Day 15 and monolayer HepG2 culture. MAPKp49/SAPK1/JNK1 is activated by an upstream MAPKK such as MEKK1 (Boldt *et al.* 2003;Cuenda and Dorow 1998) and in its corresponding western blot analysis, the protein was abundant in the culture conditions. The upper band illustrates the activated form of the protein was most abundant in monolayer HepG2 culture and in Day 15, 3-D culture. MKK4 directly activates the c-jun NH2 terminal kinases (JNK) in response to cellular stresses and proinflammatory cytokines. These MAP kinases are involved in phosphorylation cascades initiated by detection of stress which include mitogenic stimuli and cellular stresses (schematic diagram p.126). However, Rap2, a member of the Ras superfamily of GTPases which could activate MKK4 via mitogenic stimuli (Boldt *et al.* 2003;Yue and Mulder 2000) rather than cellular stresses, showed decreased levels of expression in Day 15 3-D culture compared to Day 8 3-D culture (Table 24) suggesting that the JNK/SAPK pathway, which constitutes MKK4, is activated by cellular stresses rather than mitogenic stimuli (Ellinger-Ziegelbauer *et al.* 1997;Haussinger *et al.* 1997). MKK4 also activates p38δ *in vitro* (Cuenda 2000). Interestingly, MKK4 protein was not detected in the culture conditions, and although p38δ was evident, there was no difference in expression between the culture conditions. p38δ is also activated by MKK3 and MKK6 (Fleming *et al.* 2000;Keesler *et al.* 1998), but there may not have been any difference in levels of expression of p38δ in the culture conditions because MKK4 was not present in the culture conditions.

ST1/hop p60 is a stress-inducible phosphoprotein and also complexes with HSP 70 and HSP 90 (Lassle *et al.* 1997). The strongest signal was observed in monolayer HepG2 cultures, although it was also evident in 3-D cultures.

The Powerblot data highlighted proteins comparing Day 15 vs. Day 8 cultures, increases of more stress inducible protein kinases, panERK and MEK1, which increase to phosphorylate microtubule associated proteins. Specific cellular stresses were then investigated in more detail, with respect to evidence obtained from microarray analysis, powerblot and western data analysis.

Hypoxic stress response

It was hypothesised that hypoxia could be contributing to the downregulation of function in spheroids at Day 15 of culture. There was an increase in VEGF in Day 15, 3-D culture compared to Day 8, 3-D culture. VEGF is implicated in angiogenesis and vascular tone, and more importantly is activated or upregulated as a consequence of hypoxic stress (Carmeliet *et al.* 1998;Stein *et al.* 1995).

Oxygen is assumed to be one of the key factors in the maintenance of the metabolic functions of cultured hepatocytes, as the liver is known to consume a large quantity of oxygen *in vivo* (Else 1991;Kaur *et al.* 2000).

Hypoxia is known to upregulate expression of several genes including erythropoietin (EPO) (Fisher 2003), tyrosine hydroxylase (Hohler *et al.* 1999), vascular endothelial growth factor (VEGF) (Carmeliet *et al.* 1998), platelet derived growth factor (Gorlach *et al.* 2001;Kourembanas *et al.* 1990), and lactate dehyrogenase A (Ebert and Bunn 1998). These and other biologically important genes have in common either promoter or enhancer elements that respond to hypoxia. Among these, the EPO gene has the most robust response to hypoxia (Greco *et al.* 2002).

VEGF contains a functionally critical site for HIF-1α, a DNA binding complex which transcribes VEGF in response to hypoxia, thereby enabling growth of new blood vessels needed for tissues to recover from oxygen deprivation (Ratcliffe *et al.* 1998;Zhu *et al.* 2002). VEGF contains enhancer elements with functionally critical sites for HIF-1α.

HIF-1α is activated in cells exposed to hypoxia although HIF-1α itself was not detected by Microarray analysis. Ratcliffe *et al* 1998 reported a number of genes, which contain binding sites for HIF-1α. The Microarray database was interrogated for the expression of these genes comparing diminished function at Day 15, 3-D culture with Day 8, 3-D culture. The results presented in Table 11 showed increases in all the genes reported with binding sites for HIF-1α. Glycolytic genes such as lactate dehydrogenase A and enolase were increased.

HIF-1 is a heterodimer composed of basic helix-loop-helix proteins. HIF-1 is composed of two subunits, α and β genes (Tsuchiya *et al.* 2002). The steady state

levels of both HIF-1α and β mRNA are not significantly affected by oxygen tension. At the protein level the β subunit is constitutively expressed and is not significantly affected by oxygen (Ratcliffe *et al.* 1998). However, in cells exposed to 21% oxygen, the HIF-1α subunit is unstable as it is controlled by an oxygen dependent degradation domain within HIF-1α (Ratcliffe *et al.* 1998). The degradation is catabolised through the ubiquitin-proteosome pathway (Huang *et al.* 1998). The steady state levels of HIF-1 α mRNA are not significantly affected by oxygen tension and in the majority of studies, mRNA was not found to be inducible by hypoxic stimulation which would explain why increases or decreases in the expression of HIF-1 α mRNA levels were not detected by Microarray analysis with the defined parameters, and that activation of HIF-1 involves post-translational or translational mechanisms (Bunn *et al.* 1998;Ratcliffe *et al.* 1998). Other growth factors demonstrating increased expression in Day 15, 3-D culture compared to Day 8, include insulin-like growth factor binding protein-1 (IGFBP-1). IGFBP-1 is primarily expressed in the adult liver and IGRBP-1 gene expression has been reported to be activated by hypoxia through a HIF-1-mediated pathway in HepG2 cells (Sugawara *et al.* 2000).

Therefore, Western blot analysis was carried out to detect HIF-1α protein in the HepG2 culture samples and it was shown that this protein was more abundant in monolayer HepG2 culture and Day 15, 3-D culture (diminished function) as shown in Fig.12 suggesting that hypoxic stress may be contributing to the downregulation of function observed.

Western blot analysis was also carried out for 2 MAP Kinase proteins induced in response to hypoxic stress; MEKK3 and MKK3b. Upstream regulatory components of these kinase cascades, are the *M*APK/*E*RK *k*inase *k*inases (MEKKK), MEKK3 activates SAPK activity and ERK pathways whereas the other isoform MEKK1 can only activate the SAPK pathway (Xu and Cobb 1997). MKK3b is an isoform of MKK3 (Han *et al.* 1997), and the protein is activated in all culture conditions but most abundant in monolayer HepG2 cells and Day 15 HepG2, 3-D culture.

HIF-1 activity can be induced by hypoxia in a variety of nonerythropoietin

producing cells but its activation may require phosphorylation as it is affected by alkaline phosphatases (Minet *et al.* 2001). Minet *at al* 2001 showed that c-jun protein accumulates in hypoxic HepG2 cells. AP-1 activation involves the phosphorylation of the amino-terminal domain of c-jun by the JNK SAP kinase. In the presence of a dominant negative mutant of MEKK1, an upstream kinase of the SAPK pathway, AP-1 activity is inhibited and northern blotting shows that hypoxia can induce the transcription of c-jun.

Interpretation of the stress response led to the hypothesis that downregulation of function observed in Day 15, 3-D HepG2 cells compared to Day 8, 3-D HepG2 cells could be attributed to lack of oxygen: oxygen solubilisation in culture medium is not very good and maybe rate limiting when spheroids are larger. Therefore, the culture environment was modulated with increasing oxygen concentration to improve cell performance.

Modulating the culture environment with increasing oxygen concentration to improve cell performance

There are many reports regarding the fate of cells when they are placed in hypoxic conditions, but there is little previous published work to indicate any measurements taken to modulate the environment for hypoxic cells (Yanagi and Ohshima 2001). Yanagi *et al.*, 2001 investigated the effect of oxygen concentration (10-40%) on metabolic functions of rat hepatocytes maintained up to 4 days. The albumin secretion rate of hepatocytes in the 40% oxygen concentration was 2.2 fold higher than in the 20% oxygen on Day 1. Oxygen is assumed to be one of the key factors in the maintenance of the metabolic functions of cultured hepatocytes because the liver is known to consume a large quantity if oxygen *in vivo*. The oxygen requirements of hepatocytes are normally very high (Jasmund *et al.* 2002). The maximum oxygen saturation rate of normal culture medium is 21%, but at the later time points there may not have been sufficient oxygenation for cells at the centre of the bead.

In order to overcome this problem, oxygen tension in which these cultures were maintained, was increased from ~21% ambient oxygen concentration to 35%, 80%, and finally 50%. Function was measured in terms of protein secretion, cell

proliferation, morphology and viability. 35% oxygen tension was chosen, as this was almost twice the ambient oxygen tension. However, as observed, increasing the oxygen tension to this amount had no improved effect on the parameters measured.

As no difference was observed at 35%, the oxygen concentration was increased to 80%. However, this was detrimental to the cells in terms of viability, proliferation and therefore, protein secretion per cell. The oxygen concentration was subsequently lowered to 50% as Nishikawa *et al* 1996 had reported that hepatocytes cultured as monolayers under an atmosphere of 10% to 50% oxygen maintained the same levels of cell viability and metabolic functions.

There was a detrimental effect on cell proliferation both on monolayer HepG2 cells and HepG2 cells in 3-D culture; this impacted on cell viability and therefore function per cell.

Although HepG2 cells in 3-D culture demonstrated an apparent hypoxic response, the observed negative detrimental effects of increasing oxygen concentrations suggested the spheroid had become predominantly glycolytic with extended culture, and therefore increasing oxygen concentration would not have any positive effect.

However, it has also been reported that HIF-1α has been upregulated in tumour cells under conditions of normoxia by peptides such as insulin and IL-1 and tumour necosis factor (TNF)α, angiotensin II and thrombin (Stiehl *et al.* 2002).

The Microarray database could be interrogated further, comparing Day 8 to Monolayer to demonstrate the changes in expression of these genes and to see whether a decrease in these genes are observed, as the data suggests that at Day 8 of 3-D culture (optimal function) HepG2 cells demonstrate normal metabolic activity and whether these genes are switched off or expressed at lower levels.

The negative effects of increasing levels of oxygen concentrations in HepG2 culture on cell performance may have been as a result of the oxidisation of essential metabolic intermediates such as thiols, the O_2 can "drain away" the reducing equivalents required for biosynthetic reactions within the cell. These oxidations often simultaneously reduce O_2 to oxygen free radicals and other toxic

oxygen derived species (Halliwell and Gutteridge 2002).

Although, the Western blot, Powerblot data and Microarray analysis identified genes and proteins implicated in a hypoxic stress response, manipulation of the environment to overcome this stress was unsuccessful. However, the signalling cascade, constituting many of the MAPKinases investigated, activated by hypoxic stresses, can also be activated by a variety of different stimuli, and 2 of these MAPKinases which were upregulated, MEKK3 and MKK3b, indicative of a hypoxic stress response (Kunz *et al.* 2001) can also be activated by an oxidative stress response (Bruckner and Estus 2002). In addition to this evidence suggesting that hypoxic stress was responsible for the diminished function observed, further investigation of the Microarray, Powerblot and Western blot data demonstrated additional evidence, which will be discussed further, that an oxidative stress was contributing to the diminished function observed.

Oxidative stress response

Changes in the levels of catalase in 3-D HepG2 culture compared to monolayer HepG2 culture, reflect not only changes in metabolic activity (Carlo, Jr. and Loeser 2003;Nebbia *et al.* 2004), but the decreased levels of catalase both at the mRNA level and at a functional level demonstrate the reduced anti-oxidant defence abilities of these cultures at Day 15. Hydrogen peroxide, one of the major reactive oxygen species is produced at a relatively high rate as a product of aerobic metabolism. Dismutation of superoxide generates hydrogen peroxide (Takada *et al.* 2002). The main cellular defence systems against hydrogen peroxide are the glutathione redox cycle and catalase (Bai and Cederbaum 2000;Bai and Cederbaum 2001). Activation of the JNK/SAPK stress activated pathway, whose activation is most sensitive to increased ROS° (Turpaev 2002) was shown via increased expression of phospho-specific MAPKp49/SAPK1/JNK1 in Day 15, 3-D culture and monolayer HepG2 culture.

In addition, the oxyblot results demonstrates the presence of increasing levels of oxidised proteins in 3-D HepG2 cultures over the culture period, and a significant increase at Day 15 compared to Day 8. Levels of oxidised proteins in Day 15 of 3-D HepG2 culture were parallel to levels observed in spheroids which served as

a positive control for the assay. The positive control for the assay was created by subjecting spheroids to hypoxia, then hyperoxia. However, the results also demonstrate the beneficial effects of 3-D culture than monolayer culture. In addition, albumin can also act as an anti-oxidant defence as it binds haem and copper ions and the anti-oxidant bilirubin is transported in an albumin bound form. As demonstrated, albumin, one measure of liver specific function is decreased considerably at Day 15, 3-D culture (Brass *et al.* 1998).

Modulating the culture environment with additional anti-oxidants to improve cell performance

The supplementation of additional anti-oxidants, to overcome the oxidative stress observed, may have decreased the overall levels of lipid peroxidation and protein oxidation (Locatelli *et al.* 2003) which would have to be elucidated further, but in terms of the ultimate end-point, it did not improve function per cell with respect to the measurement of secretion of liver specific proteins or increased cell numbers. The oxidative stress may be a result of the natural "ageing" process in cells (Golden *et al.* 2002;Hamet and Tremblay 2003).

A general stress response and its role in maintaining differentiated function

A general stress response which is activated and the effects triggering this response, and its contribution to diminished or enhanced function was then investigated. Many of the MAPKinases which showed a difference in expression between HepG2 cultures, MAPKp49, p38δ, MKK4 and ST1-p60 have been discussed previously, and are upregulated at times of diminished function. However, the Microarray and Western blot data highlighted proteins and genes which were involved in a complex relationship between the activation of the stress response, and its subsequent effects on cell performance. Where optimal function was observed between Days 8-10 of 3-D culture in comparison to monolayer culture, there was an increase in expression of genes and proteins, which contributed not only to the enhanced cell performance observed, but to the protective function observed in normal differentiated cells *in vivo* as a result of the successful environment created in 3-D culture, either morphologically or due to

increased metabolic activity or even as a combination of both.

The Adaptive Response– Day 8, 3-D culture vs. monolayer HepG2 culture- In Day 8 HepG2 cells in 3-D culture vs. monolayer HepG2 cells, an increase in HSP70 was observed. In monolayer HepG2 culture, during initial culture, HSP70 binds to unfolded proteins to prevent their accumulation as the accumulation of unfolded preoteins could potentially be toxic. HSP70 also functions as a molecular chaperone to enhance survival of the cell by preventing the denaturation of other cellular proteins and is essential for the initial folding of newly synthesised proteins (Papp *et al.* 2003;Patel *et al.* 1995). The elevated expression of HSP70, demonstrates that at Day 8, 3-D HepG2 culture it is serving a normal protective function and as a result of an adaptation of the increased function of healthy cells at Day 8, due to the successful environment created in 3-D culture (Bassi *et al.* 2000;Hosoi *et al.* 2002;Patel *et al.* 1995). Comparing Day 8 HepG2 cells in 3-D culture vs. monolayer HepG2 cells also showed an increase in MHC Class I antigen and catalase. MHC Class 1 Ag, as a cell surface marker, signals cytotoxic and helper T cells and is essential in eliminating infected cells (Basta and Bennink 2003). Therefore, demonstrating further that HepG2 cells in 3-D culture mimic the *in vivo* architecture of hepatocytes and the expression of this gene may simply reflect the "normal" morphology demonstrated by HepG2 cells in 3-D culture.

Hydrogen peroxide, one of the major reactive oxygen species is produced at a relatively high rate as a product of aerobic metabolism. The primary cellular enzymatic defence systems against hydrogen peroxide are the glutathione redox cycle and catalase. An increase in catalase, both at the mRNA level and functionally may reflect a greater need due to overall increased metabolic activity in 3-D culture at Day 8, and thus a requirement to produce more catalase (Bai *et al.* 1999;Bai and Cederbaum 2000;Patel *et al.* 1995).

Two isoforms of haemoxygenase proteins are present. Haemoxygenases break heme into carbon monoxide and biliverdin; HO-1 is the inducible form whereas HO-2 is constitutively expressed, and catalyses the formation of end products that protect against cellular stress, oxidative stress and its consequences (Elbirt and

Bonkovsky 1999;Ghattas *et al.* 2002). The HO product biliverdin is converted into bilirubin, a potent anti-oxidant and free iron is sequested into ferritin (Bunn *et al.* 1998;Westermarck *et al.* 2001).

HO-1, the inducible form which showed the strongest signal in the Day 8 3-D cultures, is induced by agents causing oxidative stress but can also function as a cytoprotective molecule against oxidative stress (Poss and Tonegawa 1997). Poss and Tonegawa (1997) demonstrated this function by generating HO-1 (-/-) mice by targeting deletion of the mouse HO-1 gene. The majority of these mice did not survive to adulthood and cells obtained from these mice were more vulnerable to oxidative stress induced by endotoxin. The Western blot shows no difference in signal of HO-2 between the different culture conditions and also served as a control to show there was equal loading of samples.

Gene Microarray studies also suggested that there were different stress mechanisms involved in the upregulation and the subsequent downregulation of function, some of which were protective and adaptive due to the increased metabolic function observed in Day 8, 3-D culture, and various stresses such as hypoxia and/or oxidative stress could be contributing to the downregulation of function. The Powerblot results confirmed early observations made by Western blot analysis of specific stress response proteins. For example, HO-1 expression was increased in the Day 8, 3-D culture vs. monolayer as shown in the Western blot and the fold change in expression was comparable to the Powerblot data. In the Day 15 vs. Day 8 cultures there was a similar decrease in HO-1, also shown in the Western blot carried out in the laboratory and from the Powerblot. MAPKp49 also showed comparable results from the Western blots carried out in the laboratory and the Powerblot data, as did ST1 and JNKK1, giving us the confidence to accept the Powerblot data.

In addition to HO-1 being detected in the Powerblot data comparing Day 8 with monolayer cultures, other adaptive stress proteins were present, E-cadherin, fibronectin and annexin IV, plectin -144 which reflect the normal *in vivo* like morphology of these cells in 3-D culture (Clark *et al.* 2003;Margulis *et al.*

2003;Schlaepfer and Haigler 1990). In response to stress, HSF was released from HSP 70 and forms a trimer that binds with high affinity to heat shock gene promoters. Heat shock factors (HSF) transcriptionally activate the heat shock proteins (Nakai *et al.* 1997), and as it was shown that there was an increase of HSP70 mRNA; as expected one observed a decrease in HSF4 (Table 23). Cycloxygenase (COX) converts arachidonic acid to prostaglandin H that is further metabolised to prostanoids. COX-2, the inducible form is induced by proinflammatory stimuli (Vane *et al.* 1994), as this protein was not detected in any of the culture samples, this suggested that inflammatory stress was not contributing to changing levels of function. In the Day 15 HepG2, 3-D cultures (diminished function) vs. Day 8, 3-D HepG2 cultures (optimal function) a decrease in HSP47 was observed. HSP47 is another member of the heat shock protein family, however, it is a collagen specific molecular chaperone and conditions that alter collagen levels also affect HSP47 levels, the only exception being prolonged heat shock stress, when HSP47 levels rise independently of collagen synthesis (Kawada *et al.* 1996;Thomson and Ananthanarayanan 2000). At Day 8, collagen, a component of the ECM is synthesised, and the group has shown previously that extracellular proteins are expressed and upregulated in 3-D HepG2 culture compared to monolayer HepG2 culture (Selden *et al.* 2000), reflecting the decreased levels of expression of this heat shock protein. This suggests that cells in 3-D culture compared to monolayer HepG2 cells, would be better protected from heat shock stress, should this stress be present, and the *in vivo* morphology demonstrated by the presence and upregulation of extracellular proteins, is as a result of the successful environment created in 3-D culture.

HepG2 cells are a good model for stress response studies, cDNA arrays have been used to monitor the expression of over 5,000 genes in response to toxic stress in the HepG2 liver cell line after treatment with cytotoxic doses of acetaminophen, caffeine or thioacetamide (Gore *et al.* 2000), and Timbrells group showed that liver spheroids could provide a model for studying stress protein expression in particular, heat shock protein expression (Dilworth *et al.* 2000).

The studies demonstrated that there is a complex relationship between activation of the stress response, and its effects to maintain differentiated function, and cell performance. However, in particular, attempts to alleviate hypoxic and oxidative stress were unsuccessful by manipulating the 3-D culture system to improve cell performance by increasing oxygen concentration and increasing the concentration of anti-oxidants.

In the next chapter, the cell cycle is investigated in the 3-D culture system, and its manipulation using a biological differentiating agent, ECM to increase cell numbers at times of peak function and/or improve cell performance at later times of 3-D culture.

Chapter 5

Investigating the role of the cell cycle in 3-D HepG2 culture

Chapter 5- Investigating the role of the cell cycle in 3-D HepG2 culture

5.1 Introduction

As mentioned previously, function could be improved in the 3-D cultures in either of two ways. Firstly, increasing the specific activity per cell i.e. at Days 11-15 when cell numbers are greater per bead, in order to use a smaller number of beads to replace function in the BAL, or secondly one could increase cell number at a time point when the cells are working optimally.

In this chapter, a previous observation made in the laboratory was investigated further. Previous data indicated that the addition of human placental ECM to 3-D HepG2 culture resulted in an increase in cell number and function per cell, particularly at later time points of 3-D culture i.e. at Day 11 when function per cell in conventional 3-D HepG2 culture starts to diminish. It is well known that ECM can help maintain differentiated function (cell organisation, migration and intracellular signalling pathways) through surface integrins which bind specific integrin ligands such as the arginine-glycine-aspartic acid (RGD) tripeptide found in ECM proteins (Hansen *et al.* 1994).

The aims of experiments presented in this chapter were to increase performance by culture with extracellular matrices of various origins and to assess proliferation immunohistochemically. Both DNA synthesis, analysed by 5-bromo-deoxyuridine (BrdU) incorporation and the number of cells in the growth fraction analysed by Ki-67 immunostaining are presented.
In addition, proliferation was investigated using Powerblot analysis of proteins, to demonstrate cell cycle related proteins. The Powerblot data demonstrated changes in expression of proteins associated with cell cycle regulation. HepG2 cells in 3-D culture initially demonstrate a slow rate of proliferation (lag) until Day 8 and then cell number doubles every 3-4 days (linear to 15 days) and this is reflected in the Powerblot data. The Powerblot data shows common groups of proteins expressed in HepG2 cultures, and many proteins that were expressed, were associated with the cell cycle.

5.2 AIM- Using Ki-67 and/or BrdU staining to show evidence of increased cell number and cell proliferation, with addition of ECM

5.2.1 Background

It has been previously shown that the addition of human placental ECM enhanced cell performance by increasing protein synthesis and cell number, when function starts to diminish. The increase in cell number was demonstrated by total nuclei staining with crystal violet, which quantifies viable cells and non-viable cells, in all parts of the cell cycle. However, cell proliferation can also be measured by assessing DNA synthesis *in situ* using BrdU incorporation and measuring the growth fraction with Ki-67 labelling.

During cell proliferation, DNA replication occurs before the cell is divided into two daughter cells. This close association between DNA synthesis and cell doubling makes the measurement of DNA synthesis very attractive for assessing cell proliferation, although in primary hepatocyte culture DNA synthesis can occur without subsequent mitosis (Cortes *et al.* 2003;Wright *et al.* 1999).

BrdU is a thymidine analogue which can be used for the study of DNA synthesis (cells in S phase) where it is incorporated into newly synthesised DNA strands of actively proliferating cells (Wright *et al.* 1999). Cells will incorporate BrdU and can be subsequently detected by immunohistochemistry (light microscopy) for the BrdU antigen in 3-D spheroids. The advantage of using this method, over methods which employ the use of [^{3}H]-TdR, is that it avoids radioactive isotope handling and storage problems. Immunohistochemical staining for BrdU also allows quantitative detection of S-phase cells, and counterstaining of the tissue simultaneously to reveal tissue morphology and position and pattern of BrdU incorporation. However one disadvantage of this technique is that BrdU must be administered before detection, so retrospective investigations of archival material cannot be performed (Gerlach *et al.* 1997).

The Ki-67 antigen reacts exclusively with nuclei of proliferating cells in human tissues. Detailed cell cycle analysis has demonstrated that this protein is expressed during all active parts of the cell division cycle (G1, S, G2 and M), but it is absent in resting cells (G0) (Gerdes *et al.* 1984). Therefore by using the

monoclonal antibody Ki-67, there is an additional advantage of being able to detect all proliferating cells in the cell cycle and not only those in the S phase. After initial analysis of DNA synthesis and the growth fraction in the established 3-D system, the effect of ECM on these parameters will be investigated.

5.3 Methods for measuring cell proliferation in 3-D HepG2 culture

5.3.1 Powerblot analysis (Chapter 2, Section 2.6)

5.3.2 Total cell count as described in section 2.1.4

5.3.3 BrdU incorporation

5.3.3.1 Materials

- 1% low gelling temperature agarose in 0.15M NaCl pH 7.4
- xylene (3 baths)
- Acid Alcohol
- 100% ethanol
- 70% ethanol
- Gills Haemotoxylin, (Sigma)
- Eosin
- 1X PBS
- 0.1% trypsin in 0.1% $CaCl_2$ in 0.05M Tris/HCl and 0.15M NaCl pH 7.8
- 0.3% hydrogen peroxide in 1X PBS
- DAKO Pen for immunohistochemistry (Dako Cat No. S2002)
- Rabbit Serum (Sigma Aldrich, Cat No. M0744)
- Mouse anti rat BrdU (Dako Cat No. M0744)
- Biotinylated rabbit anti mouse (Dako Cat No. E0464)
- Strept ABC complex/HRP (Dako Cat No. K0492)
- Sigma Fast 3,3'-Diaminobenzidine Tablet Sets (DAB Peroxidase Substrate) Cat no. D4293
- DPX for mounting slides.

5.3.3.2 Methods

5.3.3.2.1 Sample preparation

3-D HepG2 cultures were set up as previously described in section 2.1.3.5. Beads were seeded into 6 well plates containing 0.25mls of beads per well in 8mls of α-MEM complete medium with HG. One plate was set up for each time point, Day 8 and Day 15 of 3-D culture and for each concentration of BrdU and the two different types of medium (see below for further details). A negative control plate was set up in the absence of BrdU.

The medium was replenished every 48 hours as described earlier. Initially, three different concentrations of BrdU was added to the medium, 40μM (Sugiyama *et al.* 2002), 100μM and 200μM and fresh BrdU in these concentrations was added to these 6 well plates in 5mls of medium every 12 hours, for 60 hours prior to the harvesting time point. HepG2 cells in 3-D culture double their cell number every 3-4 days, so by ensuring that the BrdU was added 2 ½ days before harvesting, it should be incorporated during DNA synthesis. The cultures were maintained in normal α-MEM complete medium with HG until 60 hours before the harvesting time point, when BrdU was added to α-MEM complete medium with HG without ribonucleosides and deoxyribonucleosides, to assess whether ribonucleosides and deoxyribonucleosides in the medium would interfere with the sensitivity of the assay. At the highest concentration of BrdU, a parallel plate was set up in which BrdU was added at the same time intervals in normal α-MEM complete medium with HG.

Beads placed in strainers in each well of a 6 well plate were washed twice with 1X PBS and afterwards placed in a fresh 6 well plate and immersed in 10% buffered formalin for 1-2 minutes to fix the cells immediately. The beads in the cell strainers of 6 wells of each plate were pooled together and the pooled cell strainer now containing 1.5mls of beads was transferred to a fresh well. 5mls of 1% low gelling temperature agarose in 0.15M NaCl pH 7.4 was added to this well. The agarose was left for 40 minutes to set. The cell strainer was lifted out of the well with a spatula and the block placed upside down with the surface holding the beads facing upwards. This was placed in a fresh well and another 5mls of agarose was added to sandwich the beads in a solid block. After 40

minutes leaving it to set at room temperature, 5mls of formalin was added as a fixative. After overnight processing, the blocks were embedded in paraffin and 6μm sections were cut and placed on 3-aminopropyltriethoxysilane (APES) coated slides for immunohistochemical staining for the BrdU antigen in spheroids from Day 8 and Day 15.

5.3.3.2.2 BrdU staining in rat liver sections and 3-D HepG2 spheroids by mouse anti BrdU-Streptavidin /Biotin Complex (ABC) immunoperoxidase detection

As a positive control for the staining, one rat received, a BrdU injection at a concentration of 50mg/kg in 1ml every 6 hours from 15 -72 hours after partial hepatectomy and sacrificed 96 hours later to show active regeneration of the liver by proliferating hepatocytes. This work was carried out under the approval of Home Office Project License No. PPL 70/5115, "Use of growth factors to enhance liver function" which was approved on 24 May 2000.

5.3.3.3 Method

5.3.3.3.1 Dewaxing of paraffin embedded, formalin fixed tissues

- Xylene (3 baths)
- Acid Alcohol
- 100% ethanol
- 70% ethanol.

APES slides with embedded spheroids and liver tissue were dewaxed in 3 baths for xylene for 3 minutes each, and rehydrated in 1 minute of 100% ethanol, 1 minute 70% ethanol and 1 minute in running water. The slides were kept in distilled water.

5.3.3.3.2 Antigen demasking and immunostaining

After dewaxing, the sections were covered with warm 0.1% trypsin in 0.05M Tris/HCl, 0.15M NaCl pH 7.8 for proteolytic digestion of tissue and to demask antigens and allow epitope retrieval, for 30 minutes in a 37°C humid incubator. The reaction was stopped by rinsing sections with distilled water. The sections were placed in a trough and immersed in a 0.3% hydrogen peroxide in PBS for 20 minutes on a rotating platform to block endogenous peroxidase. The sections were washed three times with distilled water. Normal rabbit serum was diluted 1:20 in PBS to block non-specific binding sites.

With a paper tissue the sections were carefully dried and a wax pen used to mark around the sections to maximise antibody contact with the sample. It was important that the sections were not allowed to dry out thereafter.

The sections were incubated for 10 minutes at room temperature in a closed humid incubator with normal rabbit serum diluted 1:20 with PBS (approximately 200-250µl for each section). During this incubation period, the 1° antibody solution was prepared, mouse anti rat BrdU: at a dilution of 1:100 in PBS.

The serum was gently tapped off, without rinsing and immediately incubated with the 1° antibody for 2 hours at room temperature in a closed humid container. For the negative controls only PBS was added to the sections.

After incubation, the sections were washed well in 3 x 5 minute changes of 1X PBS in a trough on the shaking platform. The 2° antibody was prepared, biotinylated rabbit anti-mouse immunoglobulins at the optimal dilution 1:200 in 1X PBS. The sections were incubated with the 2° antibody for 90 minutes in a humid closed incubator at room temperature. The ABC was made up freshly less than 30 minutes before use, 10µl A + 10µl B (peroxidase) for every 1ml PBS.

The sections were washed well with 3 x 5 minute changes of PBS in a trough on the shaking platform and incubated for 1 hour with ABC at room temperature in a humid closed incubator, the sections were washed with 3 x 5 minute changes of PBS in a trough on the shaking platform. Before the third wash, the substrate was made up, consisting of two tablets, one consisting of the DAB peroxidase substrate and a tablet made up of urea hydrogen peroxide in Tris buffer in 5ml of

dH_20 deionised water. The sections were developed in this substrate with 250µl for each section for 20 minutes or for as long as there was a real colour change. The reaction was stopped by rinsing with distilled water. The sections were counterstained for 30 seconds with haemotoxylin, and then rinsed in running water for 1 minute. The sections were dehydrated for 1 minute with 70% ethanol, 1 minute with 100% ethanol and 3 baths of xylene for 3 minutes. The sections were mounted with dpx and left for at least 2 hours, before viewing under the microscope.

5.3.4 Ki-67 labelling
5.3.4.1 Materials

- As for section ***5.3.3.1*** and;
- 1X TBS
- 0.5% hydrogen peroxide in methanol
- 0.01M citrate buffer
- NCL-L-Ki-67-MM1 (Novacastra Labs, UK).

5.3.4.2 Method

5.3.4.2.1 Dewaxing of paraffin embedded, formalin fixed tissues
As described for BrdU labelling

5.3.4.2.2 Antigen demasking and immunostaining
After dewaxing, the sections were placed in a trough and immersed in a 0.5% solution of hydrogen peroxide in methanol for 10 minutes in a rotating platform to block endogenous peroxidase. The slides were loaded onto a metal rack and placed in 1500ml of 0.01M citrate buffer pH 6.0 which was heated to boiling point in a pressure cooker for 1 minute to demask the antigens and for epitope retrieval. The sections were removed from the heat as quickly as possible and placed in a cold water bath to stop the reaction. The sections were washed once in 1X TBS for 5 minutes at room temperature. Normal rabbit serum was diluted 1:20 in 1X TBS to block non-specific binding sites.

The sections were incubated for 10 minutes at room temperature in a closed humid incubator with normal rabbit serum diluted 1:20 with PBS (approximately

200-250µl for each section). During this incubation period, the 1° antibody solution was prepared, (NCL-L-Ki-67-MM1) at the optimal dilution, 1:100 in 1X TBS.

The serum was gently tapped off, without rinsing and immediately incubated with the 1° antibody for 1 hour at room temperature in a closed humid container. For the negative controls only TBS was added to the sections.

The sections were washed well in 2 x 5 minute washes of TBS. The sections were then incubated with the 2° antibody, biotinylated rabbit anti mouse immunoglobulins at the optimal dilution 1:100 in 1X TBS for 1 hour at room temperature. The ABC reagent was made as described for BrdU labelling and the following procedures were carried out as for BrdU labelling.

5.4 Methods for manipulating HepG2 culture, to enhance cell performance

To demonstrate the ability of ECM to enhance cell performance in 3-D HepG2 culture, ECM was added initially to monolayer HepG2 culture and cell performance was assessed with respect to liver specific protein secretion and cell proliferation.

5.4.1 Effect on proliferation and function from monolayer HepG2 cultured on EHS matrigel ECM

Ideally, ECM from a human source should be used in the cell culture system to prevent bioincompatibility from cells as the biological component of a BAL. However, human ECM is very difficult to obtain, so many optimisation experiments were carried out on EHS gel prepared from sarcoma produced in mice. It contains laminin as a major component, collagen type IV, heparan sulphate proteoglycan and entactin.

5.4.1.1 Materials

- ECM Gel (From EHS mouse sarcoma) Sigma, UK
- Dispase (protease), Roche, UK.

5.4.1.2 Methods

HepG2 cells were seeded as monolayers in 24 well plates either on plastic or on a layer of ECM gel and grown to confluency. 24 hour conditioned medium was collected from both plates and 1.5 units/ml of dispase was added to the cells remaining in the well to disassociate the cells from the gel. To the cells cultured on plastic, the cells were gently scraped out of the wells. The cells were centrifuged at 120g for 4 minutes at room temperature (Eppendorf 5402, Germany) and the pellet washed twice with 1X PBS. The pellet was resuspended in 200µl of crystal violet. A cell count was obtained and ELISAs were performed on the conditioned medium for levels of secreted proteins.

5.4.2 Effect on proliferation and function in 3-D HepG2 cells cultured with human placental ECM

An increase in cell proliferation of cells in monolayer on ECM was shown, so it was hypothesised that there would be an increase in cell proliferation with the addition of human ECM in 3-D culture, and that due to the cell-cell matrix interactions already present and the ECM proteins and integrins expressed, addition of exogenous ECM should enhance cell function further, particularly at Day 11 of 3-D culture.

5.4.2.1 Materials

- Human placental ECM (Becton Dickinson)
- 200µm BrdU.

Human placental ECM is comprised of laminin, collagen IV and heparan sulphate proteoglycan.

5.4.2.2 Methods

Two sets of 3-D HepG2 cultures were prepared as previously described. However, to one set 88ug/ml of ECM was added to the alginate/cell/medium suspension so that the exogenous ECM was present in alginate beads in which cells aggregated and formed spheroids. The beads were maintained in 6 well

plates and medium was changed as previously described. As function per cell in the 3-D culture starts to diminish from Day 11, this time point was specifically looked at. In order to obtain a growth fraction from Ki-67 labelling and to measure cells in the S phase of the cell cycle, 200um BrdU was added every 12 hours, 60 hours prior to harvesting. BrdU has a short half life and was therefore added every 12 hours. 2 wells with beads in each 6 well plate were fixed in agarose and processed for histology and immunohistochemistry for both antigens.

5.4.3 Addition of ECM to alginate/cell suspension mix and culture medium to increase cell proliferation and function per cell in 3-D HepG2 culture

In the event that adding ECM to the beads alone may not have provided a sufficient stimulus, ECM was also added to the culture medium, as well as in the bead preparation. ECM was added at 2 different concentrations albeit at slightly lower concentrations than when ECM was added only to the beads.

5.4.3.1 Materials

- ECM Gel (From EHS mouse sarcoma)
  (Sigma, UK).

5.4.3.2 Methods

Three sets of 3-D HepG2 cultures were prepared as previously described. In addition to the conventional 3-D culture preparation, 30ug/ml and 60ug/ml of ECM was added to the culture mix and the culture medium. The medium was changed every 2 days with fresh ECM and the cells were harvested at two time points; Day 10 when peak function is still maintained and Day 11 when function per cell starts to diminish.

Expression of a number of cell cycle related proteins was demonstrated by the Powerblot analysis. These are summarised in Tables 25 and 26.

5.5 Results

5.5.1 Evidence from Powerblot analysis to show regulation of the cell cycle in control of cell proliferation in HepG2 culture

Table 25 Most significant results when comparing **Day 8 vs. monolayer,** showing proteins associated with the cell cycle.

Cell Cycle Proteins	Product No.	Level	Change	Function
KAP-27	K32120	4	INCREASE 3.55	Cdk associated phosphatase-cell cycle regulation
NBS1	N10720	4	INCREASE 2.67	Nijmejin breakage syndrome-complex with Rad 50 and MRE11, important for both DNA damage and repair, and telomere length maintenance
Bub3 cl.31	B11520	5	DECREASE 3.46	Sensing kinetochore attachment to microtubules during prometaphase to metaphase transition
GCAP-1	G54220	4	DECREASE 6.74	Guanylate cyclase binding protein, cell cycle progression
NEK2-46	N52120	4	DECREASE 2.71	Nima related kinase-conditions –Controls entry of cells into S phase and mitosis
IAK1-46	I71320	4	DECREASE 2.68	Mammalian chromosome segregation
Cyclin B	C23420	4	DECREASE 2.26	Regulate the protein kinases of the cell cycle, by binding to cell cycle kinases

After comparing the Day 8 3-D HepG2 cell culture versus monolayer HepG2 cell culture, it was found as expected that the majority of the cell cycle proteins showed a decrease in expression and this was demonstated further in Day 15, 3-D cultures compared to Day 8, 3-D cultures. This was expected as the doubling time of HepG2 cells in monolayer decreased from 24 hours to 3-4 days when proliferating in 3-D culture. Control of cell proliferation is important in all multicellular organisms, as a number of pathological processes, such as cancer are characterised by failure of the normal regulation of cell turnover (Macallan *et al.*

1998).

***5.5.2* Table 26 Day 15 vs Day 8, 3-D cultures**

Cell cycle proteins	Product No.	Level	Fold change	Function
PLK-1	P47920	5	INCREASE 3.12	Poliolike kinase-DNA synthesis, increases in primary tumours, important for chromosome segregation
Rb2	R27020	4	INCREASE 2.29	Regulates transcription to control cell growth
BM28-117	B58720	5	DECREASE 2.25	Phosphorylated -important for entry into S phase and cell division
Cdk1/ Cdc2	C12720	5	DECREASE 4.43	Cyclin dependent protein kinase-Catalytic subunit of the MPF (M-phase promoting factor) and cyclin B acts as a regulatory component, binding of these two subunits - drives cells into mitosis.
GCAP-1	G54220	5	DECREASE 7.23	Guanylate cyclase binding-imp.for cell cycle progression
Eg 5	E83920	5	DECREASE 5.48	Mitotic motor protein-regulates spindle formation by phosphorylation
hsMAD2	H57520	5	DECREASE 3.92	Mitotic Arrest Deficient protein - important for spindle assembly.
MCM6	M13120	5	DECREASE 5.52	Minichromosome Maintenance Protein-Initiates DNA replication
Cyclin A	C88020	4	DECREASE 4.99	Required during S phase through G_2 -binds Cdc2 (Cdk10).
DNA Polymerase epsiln cat.	D86520	4	DECREASE 2.24	DNA replication and repair. With PCNA promotes primer recognition.
HRAD9	R90820	4	DECREASE 2.31	Checkpoint control protein, regulates cell cycle progression through DNA damage.
MSH6	G70220	4	DECREASE 1.68	DNA Mismatch Repair
Tau- 48.9	T57120	4	DECREASE 3.03	Microtubule associated protein
IAK-1	I71320	4	DECREASE 3.5	Mammalian chromosome segregation.

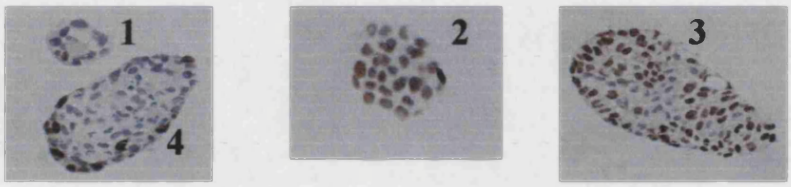
After comparing Day 15, 3-D cultures with Day 8, 3-D cultures, it was

demonstrated further that the majority of the cell cycle proteins showed a decrease in expression. The results presented in Table 26 demonstrate further the beneficial effect of 3-D culture with respect to cell performance, as not only is the cell proliferation rate more controlled, but HepG2 cells in 3-D culture retain the ability to proliferate longer, confirming Khalil's results (2000) had shown up to 22 days in culture, compared to up to 7-8 days in monolayer culture depending on the initial seeding density of HepG2 cells.

5.5.3 BrdU staining in rat liver and 3-D HepG2 spheroids

Initial experiments performed established typical staining patterns in HepG2 spheroids as shown in Fig.44.

Spheroids



1- **Top spheroid**: Majority unstained with occasional positively stained nuclei

2- Majority positively stained (~≥ 90%) with occasional unstained nuclei

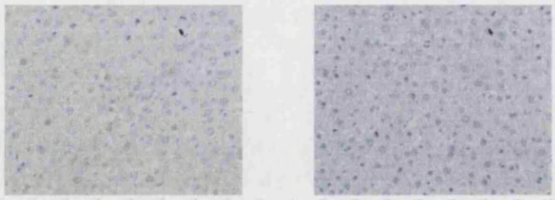
3- Staining around periphery and throughout the spheroid

4- **Bottom spheroid**: Staining around periphery

Fig. 44 Different patterns of staining displayed in spheroids in 3-D culture

Rat liver was used as a positive control for the immunohistochemistry and staining, demonstrated in Fig.45.

Rat liver

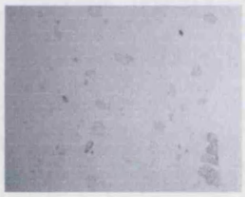


rat liver x40 mag rat liver x40 negative control

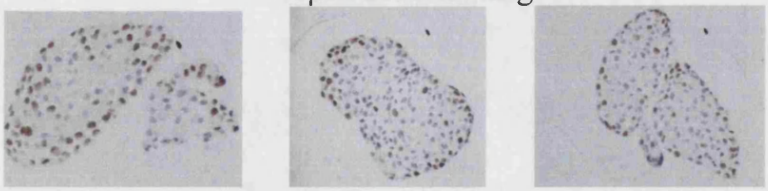
Fig. 45 BrdU staining in rat liver sections to serve as a positive control for staining

Several concentrations of BrdU were used to establish the optimal conditions of assessing DNA synthesis. The results are illustrated in Fig, 46, 47, 48, 49.

Spheroids



40μM BrdU x4 mag



40μM BrdU x40

Fig. 46 BrdU staining in 3-D spheroids with 40μM BrdU.

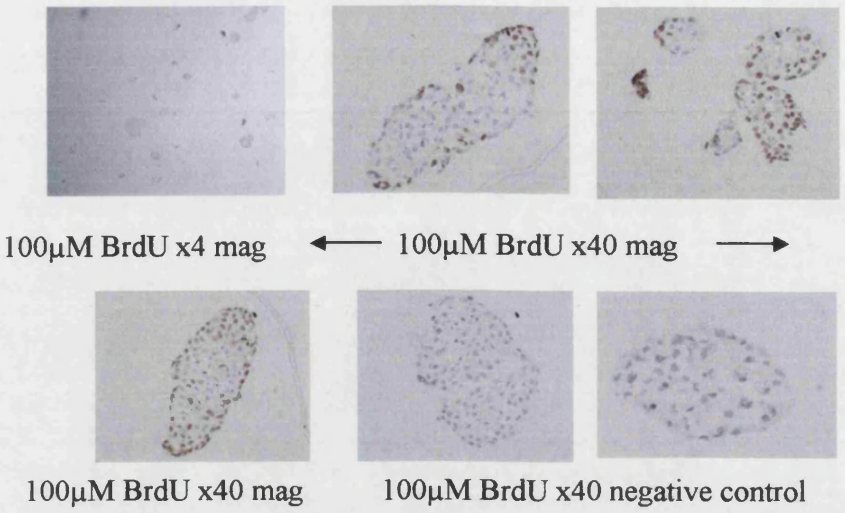


Fig. 47 BrdU staining in 3-D spheroids with 100μM BrdU.

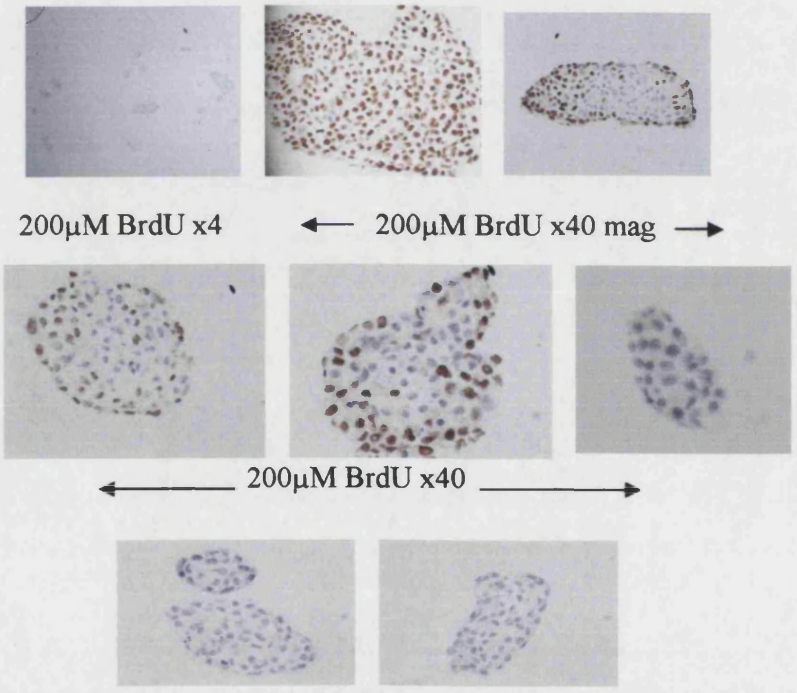
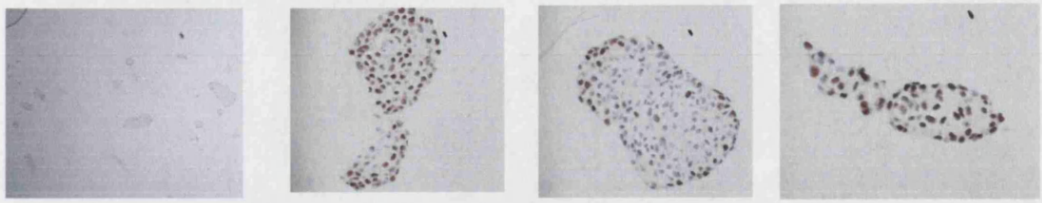
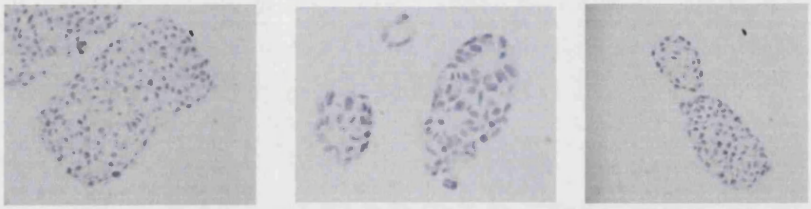


Fig. 48 BrdU staining in 3-D spheroids with 200μM BrdU.



200μM BrdU in normal medium x4 mag ← 200μM BrdU in normal medium x40 mag →



200μM BrdU in normal medium x40
negative control

Fig. 49 BrdU staining in 3-D spheroids with 200μM BrdU in α MEM medium.

5.5.4 Analysis of results from immunohistochemical staining

The pattern and intensity of staining appeared to be similar with the 3 different concentrations of BrdU and in the 200μM concentration of BrdU in which 3-D cultures were maintained in normal α-MEM complete medium with HG and 3-D cultures maintained α-MEM complete medium with HG without ribonucleosides and deoxyribonucleosides. Four different patterns of staining was observed, as shown with BrdU incorporation in spheroids, and this was expressed as a total percentage of all the cell positively stained in spheroids, and separately expressed, observing the two main patterns of BrdU staining observed; around the periphery and throughout the spheroid. The same was evident with Ki-67 labelling. It was also observed that similar levels of positive incorporation were observed in small and large spheroids as shown in Fig. 50 and Fig. 54, suggesting that incorporation of BrdU or labelling of Ki-67 is not determined by spheroid size.

The results showed that 3-D cultures maintained in α-MEM complete medium with HG without ribonucleosides and deoxyribonucleosides did not interfere with the uptake of BrdU and affect the sensitivity of the assay. There was also no significant difference when comparing the uptake of 40μm BrdU with 200μm BrdU (Fig. 51). It was therefore decided to use 200μm BrdU in subsequent studies.

5.5.5 Quantitation of BrdU staining in spheroids

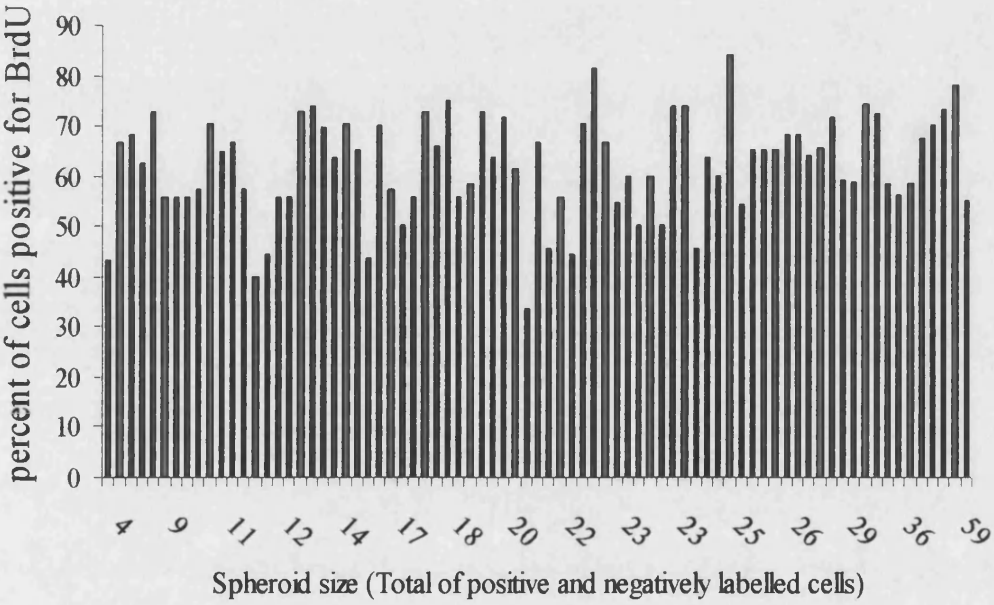


Fig. 50 Comparison of spheroid size with percent of BrdU incorporation in spheroids with positive staining around the periphery and throughout the spheroid.

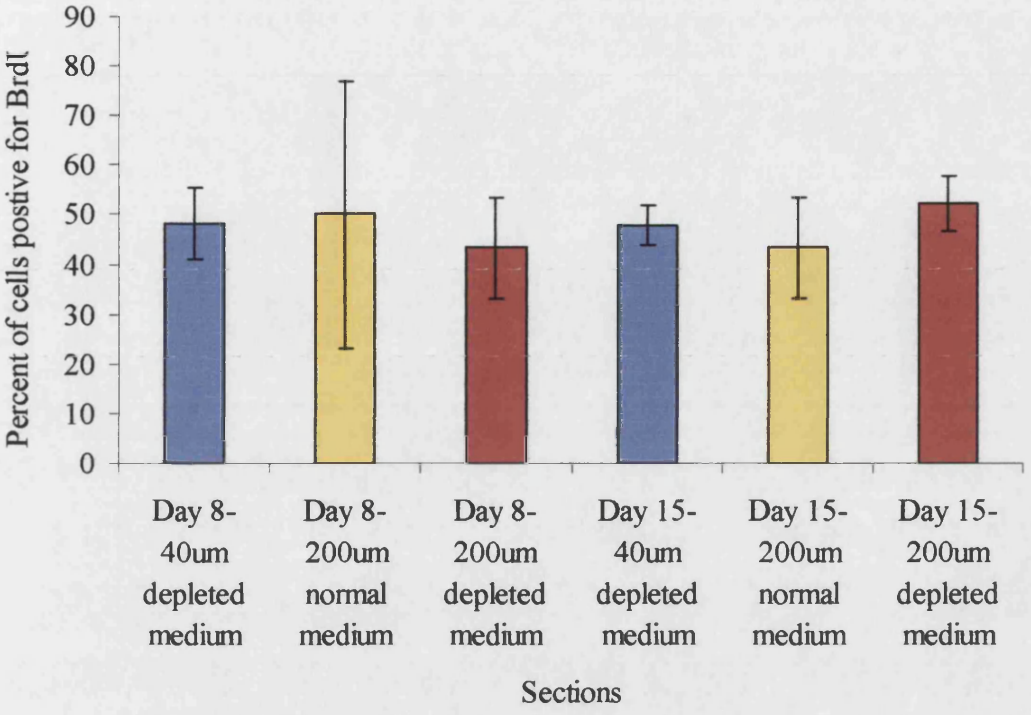
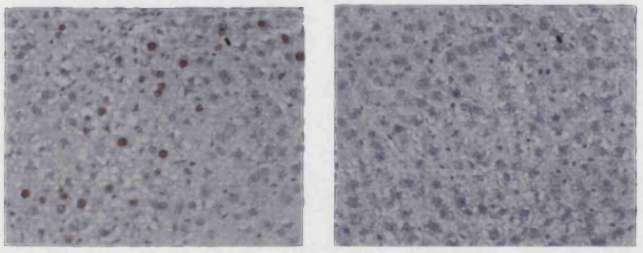


Fig. 51 Comparison of BrdU doses in normal and depleted HG medium.

5.5.6 Ki-67 labelling in rat liver sections and 3-D spheroids

Rat liver was used as a positive control for the immunohistochemistry and staining demonstrated in Fig.52.

Rat liver



rat liver x40 mag

rat liver x40 negative control

Fig. 52 Ki-67 labelling in rat liver sections to serve as a positive control for staining.

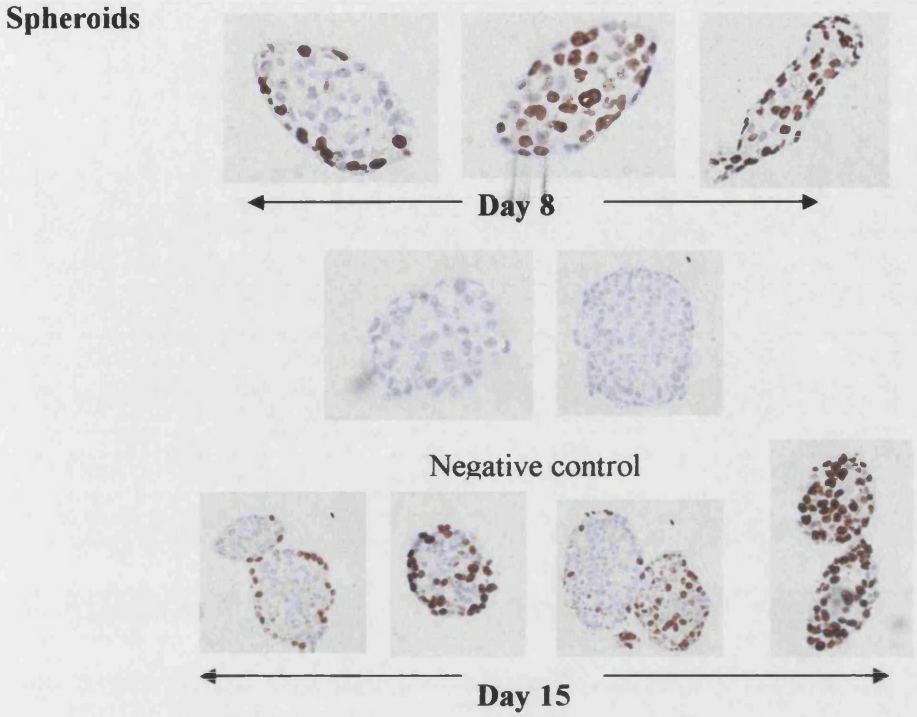


Fig. 53 Ki-67 labelling in day 8 and day 15 spheroids

5.5.7 Quantitation of BrdU and Ki-67 staining in spheroids

The result in Fig.53 showed similar percentages of positive staining of BrdU incorporation compared with Ki-67 staining. However, the main patterns of staining observed were similar to the patterns of staining from no. 3 & 4, section 5.5.3 demonstrating actively proliferating cells throughout the spheroids and not only the periphery. These patterns of staining were quantitated in BrdU incorporation and Ki-67 labelling in Day 8 and Day 15 spheroids.

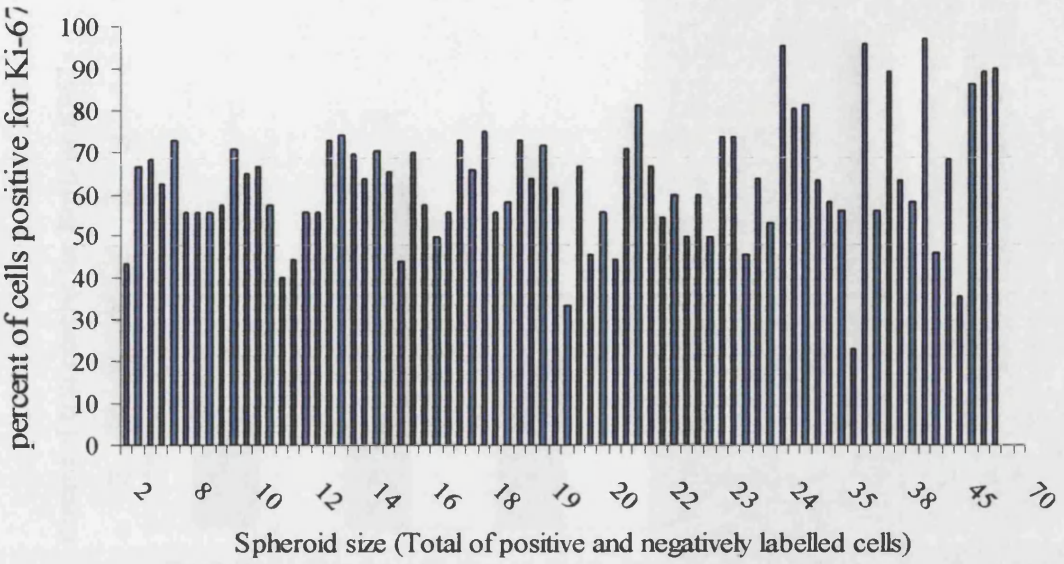


Fig. 54 Comparison of spheroid size with percentage of Ki-67 labelling in spheroids with positive staining around the periphery and throughout the spheroid.

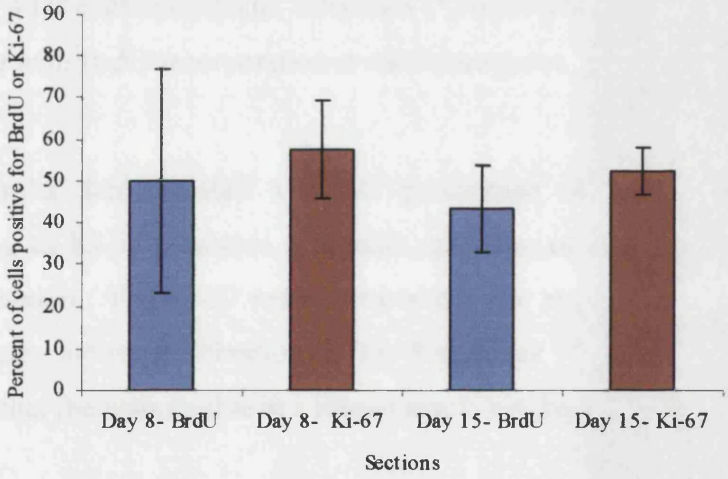


Fig. 55 Comparison of BrdU incorporation and Ki-67 labelling in Day 8 and Day 15 spheroids from positively stained cells, in total of all spheroids demonstrating all patterns of staining observed. Mean values ± S.D. n=4 for each time point.

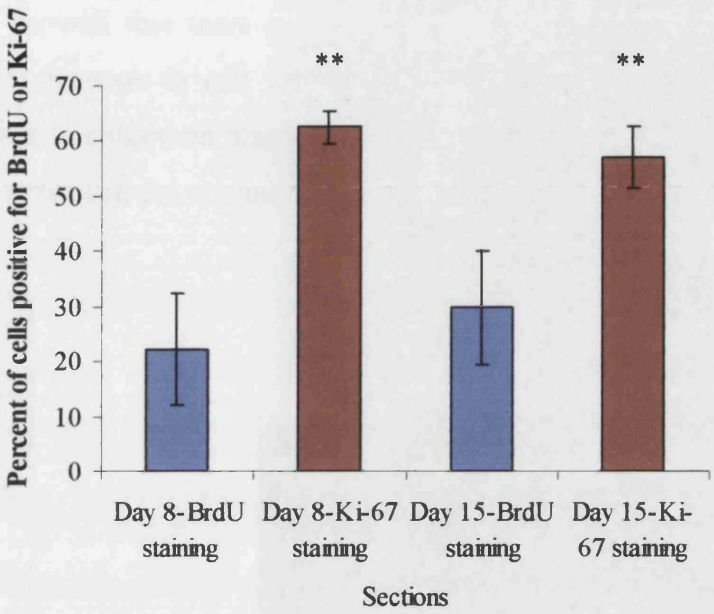


Fig. 56 BrdU and Ki-67 staining in Day 8 and Day 15 spheroids showing incorporation of positively stained cells throughout the spheroids. Mean values ± S.D. n=4 for each time point. Statistics (*) represent comparisons between Ki-67 staining with BrdU incorporation at each time point. $p \leq 0.005$ = **

The results demonstrated a higher percentage of incorporation from Ki-67 labelling as Ki-67 measures a broader spectrum of cell cycle stages than BrdU incorporation. The Ki-67 results also show that approximately half the cells are undergoing active proliferation in Day 8 and Day 15 spheroids and demonstrated further that the cells double at a rate of every 3-4 days.

5.5.8 Cell performance in monolayer HepG2 culture with additional EHS gel ECM

HepG2 cells as a monolayer were seeded on ECM and cell performance was assessed with respect to cell number and liver specific protein secretion. The results showed that there was an approximately 16% (statistically significant, $p \leq 0.005$) increase in cell proliferation when cells were cultured on ECM gel compared to culture on plastic. However, in terms of synthetic function, protein secretion per cell did not increase.

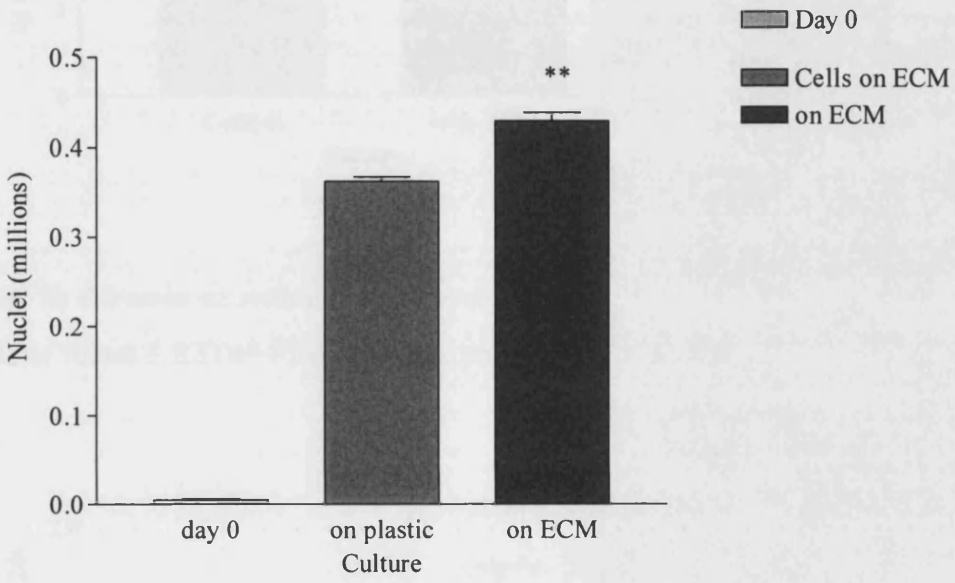


Fig. 57 Cell proliferation of monolayer HepG2 cells seeded on or without ECM. Mean values ± S.D. n=4 for each time point. Statistics (*) represent comparisons between cultures with ECM and control cultures. $p \leq 0.005$ = **

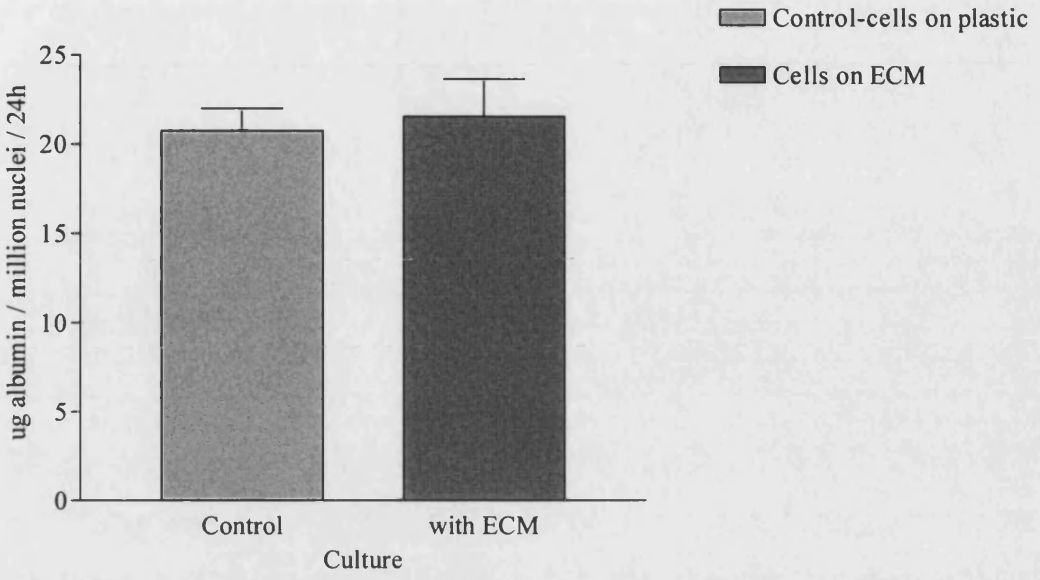


Fig. 58 Albumin secretion in monolayer HepG2 cells seeded on ECM and plastic. Mean values ± S.D n=4 for each time point.

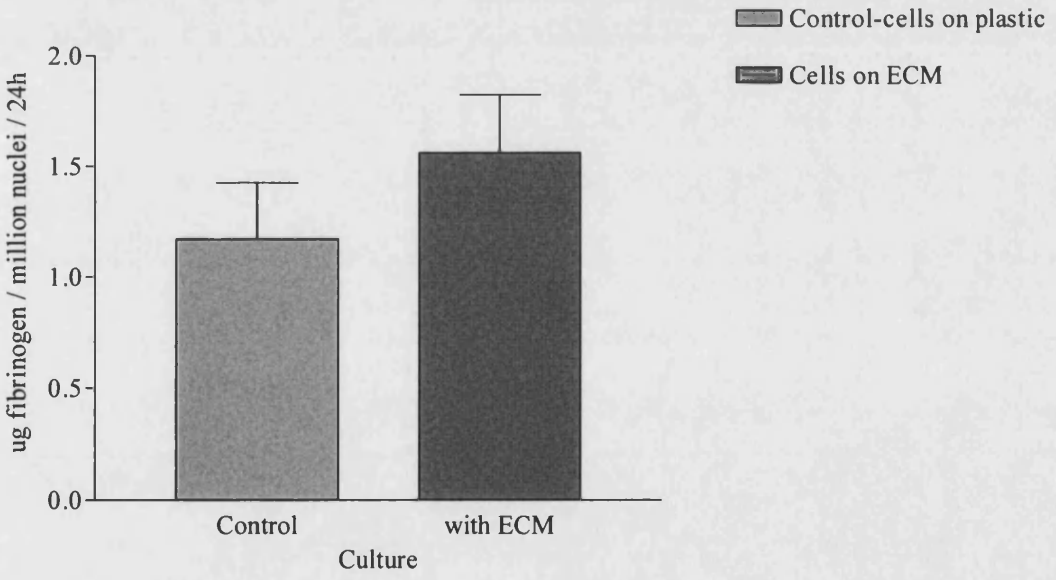


Fig. 59 Fibrinogen secretion in monolayer HepG2 cells seeded on ECM and plastic. Mean values ± S.D n=4 for each time point.

As increase in cell number of HepG2 cells in monolayer was demonstrated with EHS gel ECM. This source of ECM was used, as the difficulties of obtaining human ECM have been previously described, and therefore was used to optimise some experiments, in this case with HepG2 cells in monolayer. Many of the components that comprise EHS gel ECM are common to human placental ECM, so it was feasible to assume that the addition of ECM would have a beneficial effect in 3-D culture. However, as biocompatibility is a concern, for eventual use in a BAL and to demonstrate improved cell performance in 3-D culture, the following experiment was carried out with human placental ECM.
Human placental ECM contains laminin as a major component, collagen type IV, heparan sulphate proteoglycan and entactin.

5.5.9 Cell performance in 3-D HepG2 at Day11 with human placental ECM

An increase in cell number of 3-D HepG2 cells at Day 11 of 3-D culture, when human placental ECM was added, was observed. The data shows that there was a significant, ($p \leq 0.005$), 17.68% increase in cell number by total cell nuclei count with crystal violet. However, protein secretion per cell did not increase.

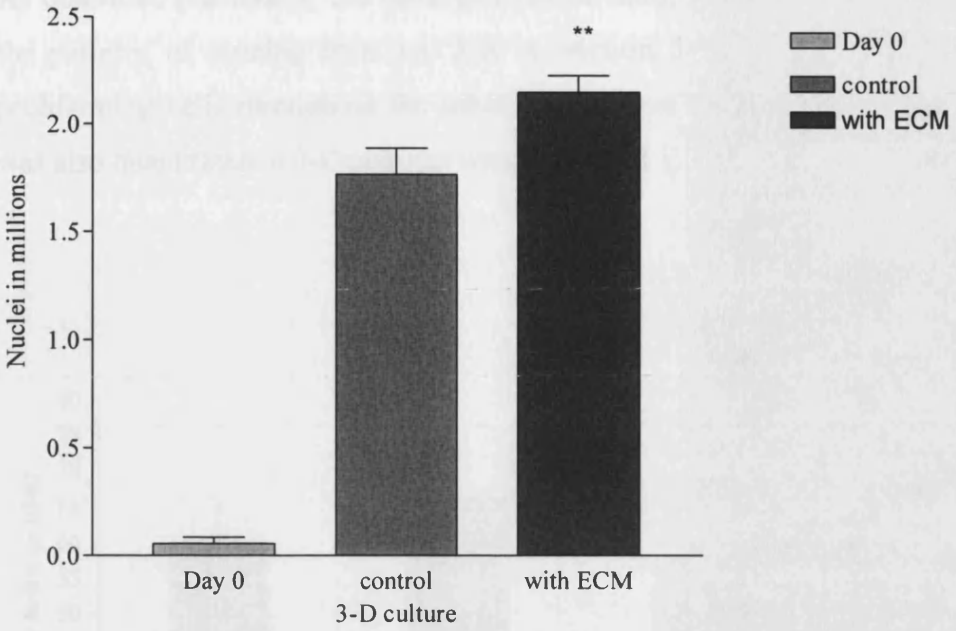


Fig. 60 Cell proliferation of 3-D HepG2 cells cultured with or without ECM. Mean values ± S.D. n=4 for each time point. Statistics (*) represent comparisons between cultures with ECM and control cultures. $p \leq 0.005$ = **

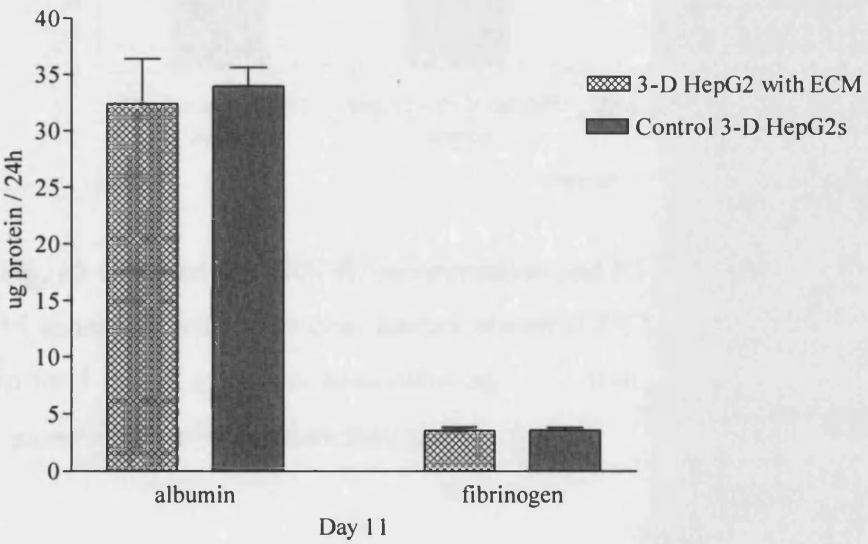


Fig. 61 Protein secretion in 3-D HepG2 cells cultured with or without ECM. Mean values ± S.D. n=4 for each time point

As described previously, the main patterns of staining observed were similar to the patterns of staining from no. 3 & 4, section 5.7.3 demonstrating actively proliferating cells throughout the spheroids and not only the periphery and this was also quantitated in 3-D cultures with additional ECM.

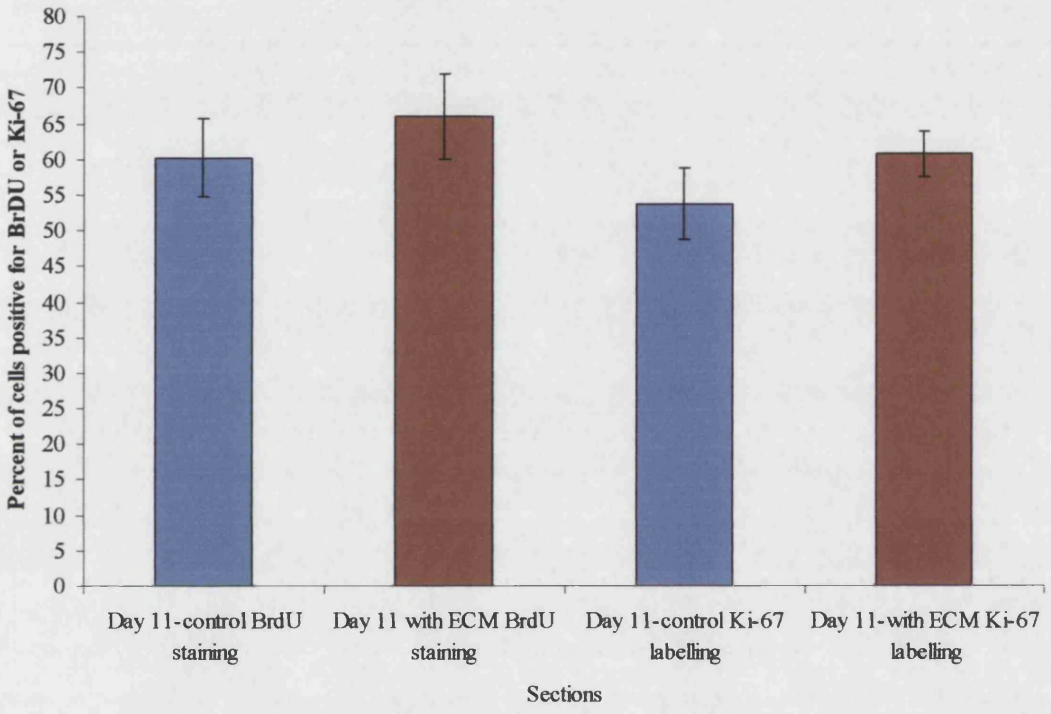


Fig. 62 Comparison of BrdU incorporation and Ki-67 labelling in Day 8 and Day 15 spheroids with additional human placental ECM from positively stained cells in total of all spheroids demonstrating all patterns of staining observed. Mean values ± S.D. n=4 for each time point.

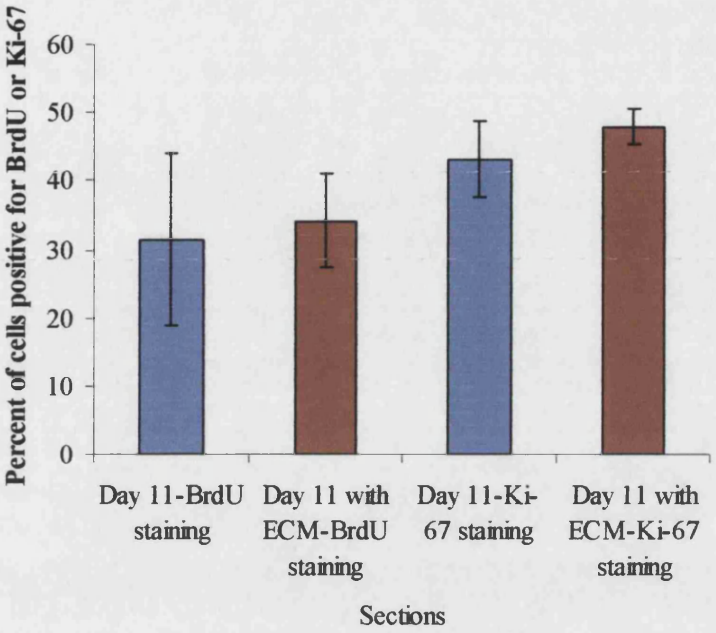


Fig. 63 BrdU incorporation and Ki-67 staining in Day 11 spheroids with or without the addition of ECM, showing incorporation of positively stained cells throughout the spheroids. Mean values ± S.D. n=4 for each time point.

The data shows that there was a significant, ($p \leq 0.005$), 17.68% increase in cell number by total cell nuclei count with crystal violet. This was similar to the 12.1% increase observed in Ki-67 expression with the addition of ECM. The pattern and intensity of staining was similar to that obtained from conventional 3-D culture as previously shown in this chapter.

As mentioned, ideally, ECM from a human source should be used in the cell culture system to prevent bioincompatibility from cells as the biological component of a BAL. However, human ECM is very difficult to obtain, so the following optimisation experiment was carried out with EHS gel ECM, which as for human placental ECM was added to the alginate/cell suspension mix at Day 0 of 3-D HepG2 culture, and additionally to the culture medium.

5.5.10 Cell performance in 3-D HepG2 culture with additional EHS gel ECM to alginate/cell suspension mix and culture medium.

The data shows that there was a significant, ($p \leq 0.005$), ~20% increase in cell number by total cell nuclei count with crystal violet. However, protein secretion per cell did not increase.

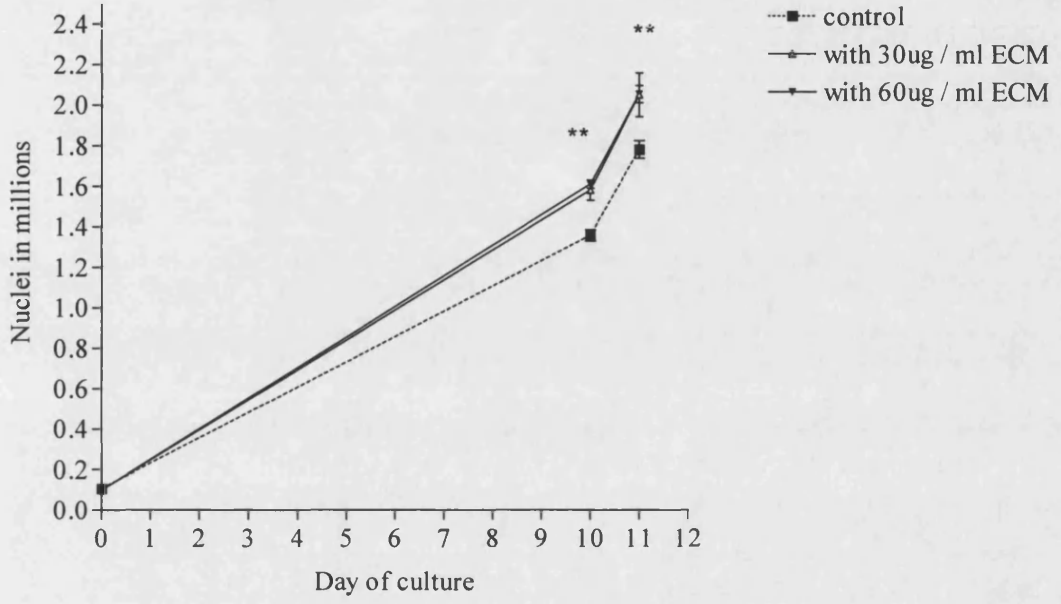


Fig. 64 Cell proliferation in 3-D cultures with EHS gel ECM in alginate suspension and culture medium. Mean values ± S.D. n=4 for each time point. Statistics (*) represent comparisons between cultures with ECM and control cultures. $p \leq 0.005$ = **

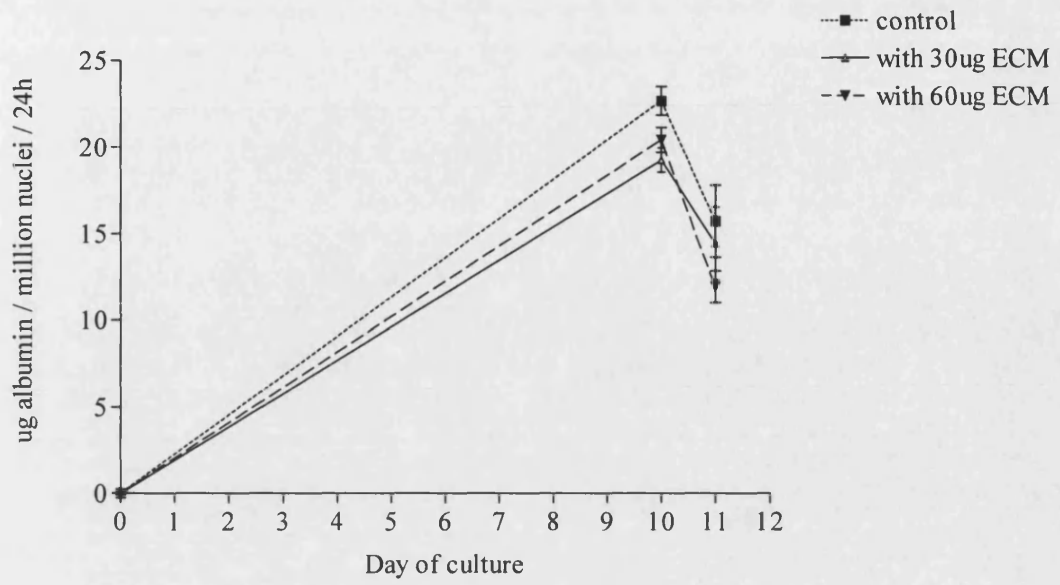


Fig. 65 Albumin secretion 3-D cultures with EHS gel ECM in alginate suspension and culture medium. Mean values ± S.D. n=4 for each time point.

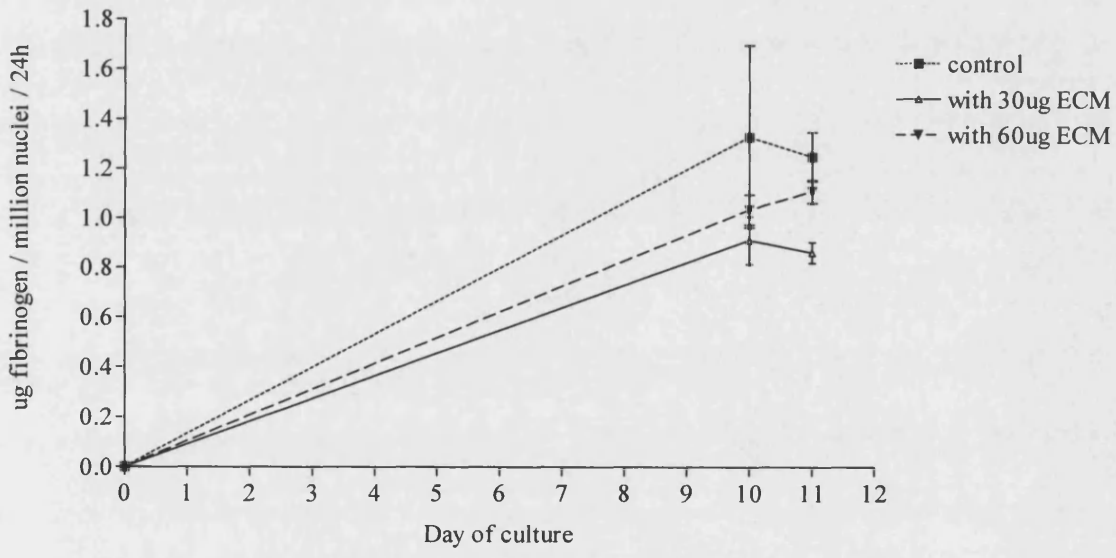


Fig. 66 Fibrinogen secretion 3-D cultures with EHS gel ECM in alginate suspension and culture medium. Mean values ± S.D. n=4 for each time point.

5.6 Discussion

This chapter has explored the role of the cell cycle in HepG2 cells in 3-D culture alone, and the ability of exogenous ECM added to 3-D culture, to affect both proliferation and performance of this culture system. Cell performance in monolayer and 3-D HepG2 culture was assessed by protein synthesis as a measure of liver specific function and cell number. As discussed, function can be improved by increasing function per cell at later times of 3-D culture when cell numbers are greater, and/or increase cell number at times of peak function i.e. at Days 8-10.

Khalil (2000) showed that the addition of many differentiating agents to 3-D culture preparations and medium, such as DMSO, sodium butyrate, and retinoic acid, at optimal concentrations of each of the agents, did not maintain function after Day 11, even though cell growth was inhibited dramatically in some cases e.g. 2 % DMSO and 2 mM sodium butyrate. A decline in biosynthetic function was still evident with extended culture.

In addition to chemical differentiating agents, mechanical systems have been developed which can increase cell proliferation and improve function. The rotary cell culture system (RCCS) (Khaoustov *et al.* 2001), can culture cells in a 3-D spheroidal form, but unlike the static 3-D culture system, it provides an environment in which the cells are cultured in a unique microgravity environment of low shear force and high-mass transfer, allowing better membrane oxygenation and nutrient availability. Work utilising this culture system is currently in progress in the group. In order to investigate cell performance further, the role of the cell cycle in 3-D culture and compared to monolayer HepG2 culture was assessed using a protein array analysis of cell cycle proteins expressed, when performance is enhanced and diminished. Cells were also quantified in the growth fraction and specifically in DNA synthesis. Finally it was attempted to manipulate cell performance by the addition of ECM.

Powerblot analysis of cell cycle proteins

The major group of proteins represented were cell cycle proteins. After comparing the Day 8 cultures in 3-D versus monolayer HepG2 cultures, and then comparing further Day 15, 3-D cultures compared to Day 15, 3-D cultures, as expected it was found that the majority of the cell cycle proteins showed a decrease in expression. This was expected as the doubling time of HepG2 cells in monolayer decreased from 24 hours to 3-4 days when proliferating in 3-D culture. Cyclin B, when newly synthesised, produces active Maturation Promoting Factor required for mitosis; the decrease in cyclin observed in Day 8 3-D culture compared to monolayer HepG2 culture, supports the faster doubling time observed in monolayer HepG2 culture. The increase observed for KAP-27, a CDK associated phosphatase also supports the decrease in cell proliferation rate demonstrated in 3-D culture compared to monolayer culture. KAP binds to cdc2 and CDK2 in mammalian cells, indicating its role in cell cycle control (Hannon *et al.* 1994). CDK2 cyclin dependent kinases are delayed response proteins and are present at a constant level in exponentially growing cells but disappear when cells enter G_0. CDK2 participates in control of the G_1 and S phase (Bartek and Lukas 2001). Cyclins are key molecules in cell cycle control because of their specific and periodic expression during cell cycle progression.

In Day 15 vs. Day 8 cultures, there was an increase in Rb (Retinoblastoma protein). HepG2 cells contain normal Rb, which is a tumour suppressor, and plays a central role in the control of cell cycle progression, by acting as a brake on cell cycle progression to regulate transcription and control cell growth (Radulescu *et al.* 2000;Zauberman *et al.* 1997). The D-type cyclins (cyclins D1, D2 and D3) complex with cdk4 and cdk6 to regulate transition from the G1 phase into the S phase by phosphorylation and inactivation of Rb (Suzui *et al.* 2002). Therefore, the increase in Rb protein could be supported from the finding that cyclin D proteins or cdk4 and cdk6 were not upregulated in the culture conditions.

All other proteins associated with cell cycle progression showed a decrease in expression in both comparative conditions, demonstrating that HepG2 cell proliferation is more controlled in 3-D culture than in monolayer culture.

Assessing cell proliferation in 3-D HepG2 cultures and with additional ECM

Conventional nuclei counts give an indication of total cell number but by incorporating measurement of BrdU and Ki-67 staining, an indication of the localisation and pattern of proliferation in spheroidal culture was obtained. Similar percentage increases of cell number were seen with all manipulations of ECM. BrdU and Ki-67 showed positively labelled cells throughout the spheroid indicating that even at the centre of the spheroids; there was maintenance not only of viability but also the capacity to proliferate. This demonstrates a population of actively proliferating cells throughout the spheroid and suggests that the diminished function observed in later times of 3-D culture was not as a result of non-viable cells within the centre of spheroids, and not due to a lack of nutrients or oxygenation, particularly in the centre of larger spheroids at later times of culture, which could have lead to loss of function. In fact similar levels of positive incorporation were observed in small and large spheroids as shown in Fig.50 and Fig.54. The pattern of staining revealed by BrdU and Ki-67 labelling also supports the evidence obtained with PI/FDA staining.

In one study, which investigated BrdU incorporation and Ki-67 expression during rat liver regeneration after a standard two-thirds partial hepatectomy, the expression pattern of Ki-67 labelling correlates with the labelling pattern obtained by BrdU incorporation (Gerlach *et al.* 1997). At Day 8 and Day 15, 3-D HepG2 culture, approximately 50% of cells were in the active phases of the cell cycle.

Role of ECM

Selden *et al* 2000 showed that ECM expression was increased in 3-D cultures compared to monolayer HepG2 cultures. The following ECM proteins were detected immunohistochemically:- collagens I, III, V and VI, the glycoproteins fibronectin, tenascin and vitronectin, and the basement membrane protein laminin. In 3-D cultures, all proteins except tenascin were strongly expressed, as compared with weak or undetectable expression in monolayer cultures, even with 10-fold increases in the antibody concentration used. In addition, various integrin chains

on the cell surface which bind ECM proteins were also upregulated in 3-D culture, such as α2, α5, αVβ5 which binds laminin, and β1 and demonstrated that the 3-D environment created by alginate encapsulation of cell lines leads to cell behaviour mimicking that *in vivo.* Therefore, it was anticipated that the addition of ECM substratum at Day 0 of 3-D culture might mimic the improved function earlier on, which was observed later on at times of 3-D culture i.e. from Day 8 and maintain and improve this already upregulated function, particularly from Day 11 of 3-D HepG2 culture when cells are still proliferating but function per cell starts to diminish.

ECM and its role in hepatocytic differentiated function

Components of ECM contribute to the development and normal functioning of all cell types, together with growth and differentiation factors, and cell adhesion molecules. A specialised ECM is a prerequisite for the existence of multicellular organisms. It maintains tissue architecture and cellular polarisation and is important for cell migration, morphogenesis, and differentiation and wound healing. ECM is made of structural components, such as collagen, noncollagenous structural proteins, elastin, proteoglycans, glycosaminoglycans (Rosso *et al.* 2004).

Hepatocytes cultured on plastic attach poorly, function badly, and soon die (Gomez-Lechon *et al.* 1998;Sanchez *et al.* 2000;Yin *et al.* 2003). Modifications to conventional culture conditions have resulted in dramatic improvements in the maintenance of hepatic function and the use of complex ECM substrata e.g. Matrigel (mouse sarcoma derived matrix led to preservation of liver specific functions such as CYP450 expression (Davila and Morris 1999), and sustained higher expression of liver enriched transcription factors (Sugimoto *et al.* 2002).
In contrast to the flattened cells with prominent intracellular microfilaments on thin collagen, on EHS (matrigel) hepatocytes cluster in multicellular aggregates maintaining a rounded shape and exhibiting prominent endoplasmic reticulum (Lindblad *et al.* 1991).
However, in this study, the addition of ECM enhanced cell numbers both in

monolayer HepG2 culture and 3-D HepG2 culture, particularly at Day 11, when function starts to diminish, but did not improve function per cell measured by protein secretion. Previous work in the group, using human placental ECM demonstrated improvements in both proliferation of spheroids and function per cell. However, this work was carried out 3 years ago and ECM is a biological preparation that varies from batch to batch. These findings were unfortunately not reflected in my investigations.

The data suggests that loss of function at Day 15, 3-D culture is not as a result of dead, non-viable cells in the centre of spheroids, and this is supported by downregulation of proteins such as Bid 1.7 and Nip-1, both pro-apoptotic proteins (Kim *et al.* 2000;Zhang *et al.* 2003).

With respect to the increased proliferation observed, further work could involve looking at ECM signalling pathways and molecules in these signalling pathways, such as ERK1 and 2, which are activated in response to increased cell proliferation (Agarwal and Glasel 1999;Neuhaus *et al.* 1996). To demonstrate initially whether this signalling pathway is activated, the levels of phosphorylated kinases, such as ERK1 and ERK2 in this pathway can be compared in spheroids with and without exogenous ECM. In addition, by blocking this pathway with an MEK inhibitor such as PD184352 (Allen *et al.* 2003), and observing the effects, one could determine whether the increase in proliferation observed with the addition of endogenous ECM, was attributed to the activation of this pathway.
Many integrins, which provide receptors for molecules of the ECM, have shown to be upregulated in 3-D culture (Khalil 2000). By investigating the levels of expression of these integrins over the 3-D culture period, particularly at times of peak and diminished function, with and without the addition of exogenous ECM, it could be demonstrated whether expression of these integrins could be manipulated to enhance function per cell as well as increasing cell proliferation (Wilson *et al.* 2003).

Chapter 6

Overall Discussion

6.0 Overall Discussion

Summary of work

This investigation has shown that liver specific functions approaching *in vivo* levels can be achieved using a 3-D HepG2 culture system, compared to monolayer culture, in which differentiated function is considerably less. Function in 3-D culture is optimal between Days 8-10, and thereafter there is a decline in function in spite of continued proliferation and viability. For cells to be used therapeutically, to replace function in a BAL, it is necessary to increase function per cell either at later time points of 3-D HepG2 culture when cell numbers are greater, and/or increase cell number at times of peak function i.e. Days 8-10 of 3-D culture.

The initial hypothesis that changes in the levels of or presence of different transcription factors were either responsible for optimal or diminished function could not be elucidated from the RNase protection assay studies alone and sufficient information to support this hypothesis could not be obtained from microarray analysis.

However, Microarray analysis did provide considerably more information on gene and protein expression between the HepG2 culture conditions and led to the hypotheses that a) genes and proteins were expressed upon activation of stress factors, which may be turned on as an adaptive response as a consequence of increased metabolic activity. These stress responses early on may reflect increased function of healthy cells i.e. at Day 8, 3-D culture (Nilsson *et al.* 2002), and b) the diminished function observed is attributed to stress factors such as hypoxia and/or oxidative stress, and genes and proteins are expressed to modulate their environment in response to these stresses.

Hypoxic stress was initially investigated as a possible cause for the downregulation of function observed, and although evidence for this stress was evident, manipulating the 3-D culture environment to overcome this was unsuccessful, with respect to enhancing cell performance. Hypoxic stress can trigger a signalling cascade, constituting many of the MAPKinases investigated i.e. MEKK3 and MKK3b, which could also be activated by oxidative stress

factors.

Evidence of increased expression of these proteins and increased levels of lipid peroxidation and protein oxidation at times of diminished function at Day 15, 3-D culture compared to peak function at Day 8, 3-D culture, also suggested that oxidative stress was present. However, attempts to diminish this stress in order to improve cell performance were ineffective.

The role of the cell cycle was investigated in 3-D cultures alone with Powerblot analysis of proteins, to demonstrate cell cycle related proteins, conventional nuclei counting and immunostaining in 3-D cultures alone initially, and then proliferation studies were carried out after the addition of ECM, which was used to enhance cell performance. Although ECM increased cell number at times of peak function, it was not at adequate levels to replace function in a BAL. The results nevertheless did demonstrate that there was active proliferation throughout the spheroid, and the diminished function observed was not as a result of necrosis at the centre of spheroids, but could be due to other environmental changes.

There are a number of strategies for future work which could have been carried out, in order to enhance cell performance.

Gene expression at the transcriptional level and Microarray Studies

Methods to measure gene expression with respect to investigating the presence and levels of transcription factors were carried out and alternative methods, which were not readily available during the course of the research, but are now, have been described in the discussion of chapter 3. If certain genes were found to be upregulated at times of diminished function, then using either antisense oligonucleotides (AS ONs) or short interfering RNAs (siRNAs), specific inhibition of gene expression could have been mediated via binding to complementary regions on the mRNA molecules encoded by these target genes (Wall and Shi 2003). Gene expression may have been manipulated and for example, downregulating these genes could enhance cell function at later times of 3-D culture.

However, the practical application of siRNA technology has been limited because

these molecules can also generate a number of less specific and undesired side effects within a cell. It is difficult to distinguish between the true effects of gene inhibition and those generated by other mechanisms. Finding ways to better control siRNA specificity to reduce these side effects is absolutely critical.

Manipulating "stress response" signalling pathways to improve cell performance

Western blotting for "key" stress related proteins demonstrated that the JNK stress activated pathway was switched on in Day 15, 3-D HepG2 culture and in monolayer HepG2 cell culture, where differentiated function is diminished or low, as shown by increased phosphorylation of JNK/SAPK1/MAPKp49 protein. These results, along with increased levels of protein oxidation and lipid peroxidation in these culture conditions, suggested that oxidative stress was causing the diminished function observed. However, the supplementation of additional anti-oxidants did not increase cell numbers or increase synthetic function per cell, therefore suggesting that any evidence of oxidative stress may be a result of the natural "ageing" process in the cells (Rafique *et al.* 2004;Tahara 1993).

Nevertheless, JNK can be activated by other stressors and mediates apoptosis, as well as causing downregulation of function in various culture systems (Mielke *et al.* 1999;Takagi *et al.* 2004) and therefore, may have a role in downregulation of function observed in this culture system, so by the addition of chemical inhibitors of JNK, one could observe whether blocking the activity of JNK could improve cell performance (Tong *et al.* 2001;Vivo *et al.* 2003).

It was also shown that diminished function at Day 15 was not as a result of necrosis at the cell centre as cell viability was shown by PI/FDA staining and positively labelled BrdU and Ki-67 stained cells throughout the spheroids. Various differentiating agents were supplemented in the medium i.e. retinoic acid, DMSO and did not improve function (Khalil 2000).

The role of ECM and other differentiating agents to enhance cell performance

The addition of ECM however did enhance cell number at Day 11, when function starts to diminish. Many integrins have been shown to be upregulated in 3-D HepG2 culture (Khalil 2000) and as discussed in chapter 5, the levels of integrins at times of peak and diminished function could be investigated and this would also help determine which ECM molecules they are binding to, with and without the addition of exogenous ECM and manipulating the expression of these ECM molecules to enhance cell function.

Paradoxically, ECM proteins may be detrimental to cell performance as it has certain negative effects, which may explain why an increase in cell proliferation was observed and not synthetic function per cell. Fibronectin, for example, operates through its $\alpha_5\beta_1$ integrin receptor and induces RNase activity and cell spreading which results in the loss of both CYP2C11 and CuZnSOD mRNAs. Therefore, fibronectin at certain concentrations, either in 3-D cultures alone, or with addition of exogenous ECM, could be inhibiting cell performance. It is also important to determine levels of ECM proteins at different time points of 3-D HepG2 cell culture and in monolayer HepG2 cell culture. If fibronectin was upregulated at times of diminished function, and could be contributing to the loss of function observed, then using genistein, a tyrosine kinase inhibitor, which could block the activity of the $\alpha_5\beta_1$ integrin and possibly inhibit the function of fibronectin, could prevent this loss of function (Hodgkinson *et al.* 2000;Paine and Andreakos 2004;Xu *et al.* 2003).

Other differentiating agents, which could been used, include a compound called trichostatin A (TSA) that is known to affect histone proteins that regulate transcriptional promoters by acting as a potent and specific inhibitor of mammalian histone deacetylase (HDAC) both *in vivo* and *in vitro*. TSA inhibits the eukaryotic cell cycle and induces morphological reversion of transformed cells (Hwang *et al.* 2004;Liu *et al.* 2003). TSA has been shown to induce hepatocyte differentiation in human hepatoma cell lines.

In this project, manipulation of the 3-D HepG2 culture system with TSA may have been a useful experimental approach, as Yamashita et al 2003 demonstrated

that TSA increased the expression of many liver specific genes. For example, C/EBPα, HNF-1α, HNF-3α and HNF-4α were upregulated from 1.5-to 3.0-fold by TSA (Yamashita *et al.* 2003), and in both the HepG2 and Huh-7 cell lines, TSA significantly decreased ammonia concentrations and has been shown to increase function in HepG2 cells under reduced serum conditions (Herold *et al.* 2002).

In addition, TSA not only has an effect on cell lines, as Henkens Tom et al showed in a presentation their work at the Hepatocyte Users Group meeting in 2003 that primary cultures of rat hepatocytes treated with 25μM TSA which became morphologically more differentiated compared to control cultures, shown by the *in vivo* like cuboidal shape, presence of bile canaliculi and secretion of higher levels of albumin. Nevertheless, at the concentration used, phase I and II biotransformation activities were not significantly affected.

Nevertheless, Yamashita et al 2003 did show that TSA did not reactivate expression when the genes were unexpressed so the basis for gene selectivity of TSA is not known and needs to be examined.

Alternative approaches

Increasing mass transfer

Another approach to manipulating the 3-D culture environment, is to improve culture conditions to allow for sufficient mass transfer, which is not provided in the static cell culture system in which 3-D spheroids are maintained. As previously described, the group is working on providing an environment for spheroids, allowing high-mass transfer and providing membrane oxygenation. In static experimental culture, nutrient depletion and toxin build up become more likely as the encapsulated cell population becomes denser within the bead. In a bioreactor system where the culture medium in constantly replenished, the function of the cells may improve. Although, oxygen concentration in the 3-D culture environment was increased and had no effect in enhancing performance, in a continuous culture system, rather than static culture, this may have proved beneficial.

Increasing the number of spheroids/cells within the alginate bead would reduce

the volume of "dead space" which exists, although some "dead space" is required to allow for sufficient oxygen transport and nutrient diffusion.

Other forms of spheroidal culture

Alternative approaches for inducing spheroidal culture have been demonstrated by Liver spheroidal cultures have been prepared by plating cells into 6 well plates and placing them on a gyrotatory shaker (Ma *et al.* 2003). After 6 days from initial culture, spheroids were morphologically classified as mature.

Interestingly, Purcell's group recommended the period from Day 6 to Day 15 for functional and toxicological tests. This recommendation is strongly supported by the kinetic change in albumin secretion. The culture period used for assessment by Purcell's group parallels the culture time period used to assess liver specific function in the 3-D culture system described here. In addition, up to Day 15, 3-D culture, certain liver specific functions i.e. albumin secretion, although much diminished, is still greater in Day 15, 3-D culture than monolayer HepG2 culture. They also characterised and compared cytotoxicity endpoints using liver spheroids and HepG2 spheroids as *in vitro* models and concluded that all model hepatoxins, which included propranolol and paracetamol, caused *in vitro* toxicity in both rat liver spheroid and HepG2 spheroid models, so providing reliable models for hepatic cytotoxicity screening (Xu *et al.* 2003).

Manipulation of HepG2 cells and other cell sources for potential use in a BAL

The main advantage of a cell line over primary cells, which otherwise would be the ideal cell component for use at the biological component of a BAL, is their ready availability and their proliferative capacity. The HepG2 cell line described fulfils some of the criteria required for use in a BAL, including the retention of both replicating capacity and highly differentiated hepatocyte-specific functions, and being of human origin. However, although cell performance in 3-D culture is better than in monolayer culture, many liver specific functions such as urea synthesis are absent due to the absence of genes such as OTC, the rate-limiting enzyme in the urea cycle (Wilcken 2004). Currently others in the group are working on overexpressing OTC in HepG2 cells by transfection and cloning, and

HepG2 cells could also be transfected with a mixture of other genes, such as cytochrome P450s, and the main liver enriched transcription factors such as HNF-4, which OTC and cytochrome P450 are both transactivated by, in order to maintain differentiated function long term.

A possible indefinite source of hepatocytes for a BAL could be stem cells. However at present, the stable pluripotent stem cell lines established show heterogeneous differentiation, producing a mixture of cells, instead of a pure population of a defined cell type. As stem cells have the ability to differentiate into multiple cell types, by manipulating stem cells towards a specific differentiated pathway, progress would be made (Stockmann and IJzermans 2002).

Until then, immortalised cell HepG2 spheroidal culture in alginate is a promising and reliable basis for the biological component of a BAL.

APPENDIX

ROYAL FREE HOSPITAL AND MEDICAL SCHOOL ETHICS COMMITTEE

APPLICATION FORM

EC REF..... (Leave blank)

SHORT TITLE OF PROJECT
(or your reference number)

Liver cell studies

FULL TITLE

Culture of human liver cells obtained at surgery

CONTACT NAME & ADDRESS
(to be used in all correspondence relating to this application)

Prof HJF Hodgson
Hepatology – Dept of Medicine
Upper 3rd Floor Medical School
Royal Free Campus
Rowland Hill Street
NW3 2PF

When completing this form on a word processor you may expand any section but please ensure that you retain the page breaks so that each section starts at the top of a new page. Handwritten applications will not be accepted.

ROYAL FREE HOSPITAL
POND STREET
LONDON NW3 2QG
TELEPHONE 020 7794 0500
FAX 020 7830 2961

THE ROYAL FREE HAMPSTEAD NHS TRUST

25 F

CHAIRMAN JOHN CARNER
CHIEF EXECUTIVE BRIAN ELSE
REPLY TO EXTENSION 5628

24 February 2000

Professor H Hodgson
Hepatology – Department of Medicine

Dear Professor Hodgson

Re: CULTURE OF HUMAN LIVER CELLS OBTAINED AT SURGERY

I am pleased to be able to inform you that your recent submission to the Royal Free Hosp Medical School Local Research Ethics Committee has now received approval by Chair Action. This approval will be formally documented at the next meeting of the full commit

This approval does not mean that the study may commence. The study may only following approval by the Trust through the office of the Director of Research & Develo (please contact Sadaf Zaidi on extension 8304).

Please note the code number (38-2K) that the submission has been given and quote this correspondence.

Yours sincerely

[redacted]

Maureen Carroll
Secretary
Royal Free Hospital & Medical School
Local Research Ethics Committee

cc Mr J Farrell, Head of Pharmaceutical Services
Ms S Zaidi, Research & Development

Royal Free Hampstead NHS
NHS Trust

Royal Free Local Research Ethics Committee
Chief Executives office
Pond Street
London
Hampstead
London
NW3 2QG

11 March 2002

Professor Humphrey Hodgson
Centre of Hepatology (U3)
Royal Free Hampstead NHS Trust
Pond Street
Hampstead
London
NW3 2QG

Dear Professor Hodgson

Culture of human liver cells obtained at surgery.

Ethics Reference: [redacted]

Thank you for your letter dated 27th February 2002 enclosing the amendment to the consent form on the above study.

Your request to continue with the trial has been approved by Chairman's Action and will be fully documented at the next committee meeting.

Therefore, you are free to continue with your project.

Yours sincerely

[redacted]

Rosemary Brown
Ethics Committee Secretary
Royal Free Local Research Ethics Committee

ROYAL FREE and UNIVERSITY COLLEGE MEDICAL SCHOOL
University College London
ROYAL FREE HAMPSTEAD NHS TRUST

CENTRE FOR HEPATOLOGY
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Royal Free Campus
Rowland Hill Street
London NW3 2PF

FACSIMILE COVER SHEET

Date:

To: J

From: (

Number

Please ☎ ..(

B.1 Preparing cRNA targets for microchip analysis

Synthesis of Double-Stranded cDNA from Total RNA

B 1.1 Materials

- T7- $(dT)_{24}$ primer (HPLC purified)
- DEPC treated water
- Superscript Choice System, (Invitrogen, Life Technologies P/N 18090-019)
- Phase Lock Gel, Eppendorf-5 Prime, Inc., P/N pl-188233
- Phenol/chloroform/isoamyl alcohol. Ambion P/N 9732

B 1.2 Method

Starting with 5.0 to 40µg of high quality RNA, the appropriate amount of Superscript II Reverse Transcriptase enzyme to be added was calculated and DEPC treated water to make a total volume of 20µl.

B 1.2.1 First strand cDNA synthesis

	Reagents in reaction	
Step 1: primer hybridisation Incubated at 70°C for 10 minutes. Quickly spun and placed on ice.	DEPC-H_2O (variable) RNA (variable 5.0 - 40µg) T7-$(dT)^{24}$ primer (100pmol / µl)	x µl y µl 1 µl
Step 2: temperature adjustment Incubated at 42° C for 2 minutes.	5X First Strand Buffer 0.1M DTT 10mM dNTP mix	4 µl 2 µl 1 µl
Step 3: first strand synthesis Mixed well. Incubated at 42° C for 1 hour.	SSII RT	z µl
	Total Volume	20µl

B 1.2.2 Second strand cDNA synthesis

First strand reactions were placed on ice and then centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany). The following reagents were added to the First Strand synthesis tube.

Component	Volume (µl)	Final concentration or amount in reaction
DEPC-treated water	91µl	
5X Second Strand Reaction Buffer	30µl	1X
10mM dATP, dCTP, dGTP, dTTP	3µl	200µM each
10U/µl DNA Ligase	1µl	10U
10U/µl DNA Polymerase I	4µl	40U
2U/µl RNase H	1µl	2U
Final Volume	150µl	

The tube was gently tapped to mix, centrifuged for 1 minute at 10000g (Eppendorf 5402, Germany) to remove condensation and incubated at 16° C for 2 hours in a cooling waterbath. T4 DNA, 2µl (10U) polymerase was added thereafter. This was incubated for 5 minutes at 16° C. 10µl of 0.5M EDTA was added and the double stranded cDNA was cleaned up using Phase Lock Gels (PLG)- Phenol/Chloroform Extraction. PLGs form an inert sealed barrier between the aqueous and organic phases of phenol-chloroform extractions. The solid barrier allows more complete recovery of the sample (aqueous phase) and eliminates possible contamination of the sample. Briefly, an equal volume of phenol:chloroform:isoamyl:alchohol (saturated with 10mM Tris-HCL pH 8.0/1mM EDTA) was added to the final cDNA synthesis preparation and vortexed. The entire cDNA-phenol/chloroform mixture was transferred to the PLG tube. Without vortexing, the tube was centrifuged at full speed 12000g for 2

minutes. The aqueous upper phase was transferred to a fresh 1.5 ml tube.

To concentrate the cDNA, the solution was precipitated with ethanol. To the sample, 0.5 volumes of 7.5M NH_4Ac and 2.5 volumes of absolute ethanol (stored at -20°C) were added, vortexed and immediately centrifuged at ≥ 12 000g in a microcentrifuge at room temperature for 20 minutes. The supernatant was removed and the pellet washed with 0.5ml of 80% (ethanol stored at -20°C). The sample was centrifuged again at ≥ 12000g at room temperature for 5 minutes. The ethanol was removed very carefully and the ethanol wash was repeated again. The pellet was air dried and resuspended in 12µl of RNase-free water.

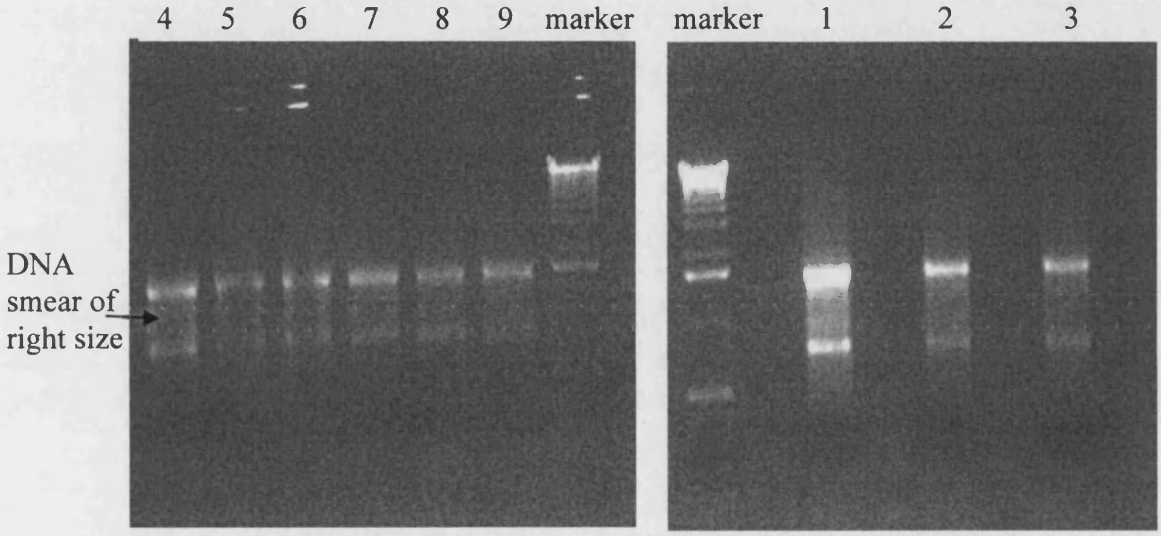

Fig.67 Double stranded cDNA after 2nd strand cDNA synthesis

B 2 RNA transcript labelling

Using a labelling kit, large amounts of hybridisable biotin labelled RNA targets can be produced by *in vitro* transcription using T7 RNA polymerase and biotin labelled nucleotides. RNA-DNA hybrids have a higher melting temperature than corresponding DNA-DNA hybrids and single stranded RNA targets offer higher target avidity and greater sensitivity than DNA probes.

Biotin labelled RNA targets can hybridise to arrays of DNA probes on chips and detected by a reporter molecule linked to streptavidin, by excitation of a fluorophore conjugated to streptavidin.

B 2.1 Materials

- Enzo BioArray RNA Transcript Labelling Kit P/N 900188 (Enzo Diagnostics, NY, USA)
- RNeasy Mini Kit (Qiagen Cat No. 74104

Reagent	Volume
Template double stranded DNA	variable-1μg of cDNA
Distilled water	variable- to give a final reaction volume of 40μl
10 X HY Reaction Buffer	4μl
10 X Biotin Labelled Ribonucleotides	4μl
10 X DTT	4μl
10 X RNase Inhibitor	4μl
20 X T7 RNA Polymerase	2μl
Total Volume	40μl

B 2.2 Methods

Tubes were incubated in a waterbath at 37° C for 4-5 hours, mixing gently 4-5 times during the incubation.

The *in vitro* transcription reaction was treated to remove unincorporated nucleotides. A small sample of product was saved for analysis by gel electrophoresis. The sample of product was saved for analysis by gel electrophoresis. The sample was cleaned using Qiagen RNeasy spin columns. The cRNA (the *in vitro* transcription product) was quantified by measuring its absorbance at 260nm and the purity measured by the ratio of its absorbance at 260nm and 280nm. A good quality sample should have a ratio of approximately 2.0. Ethidium bromide gel electrophoresis of the IVT product was carried out to

estimate the yield and size distribution of labeled products. Parallel gels of unpurified and purified IVT product can help determine the loss of sample during the clean up process.

1% of each sample was analysed by gel electrophoresis though a 1% agarose gel.

Quantification of RNA after concentration of IVT reactions – used to determine level of hybridisation target to be used on chip

The cRNA was generated with T7 RNA polymerase and labelled with biotin ribonucleotides. Ethidium bromide gel electrophoresis of the IVT product was carried out to estimate the yield and size distribution of labelled products.

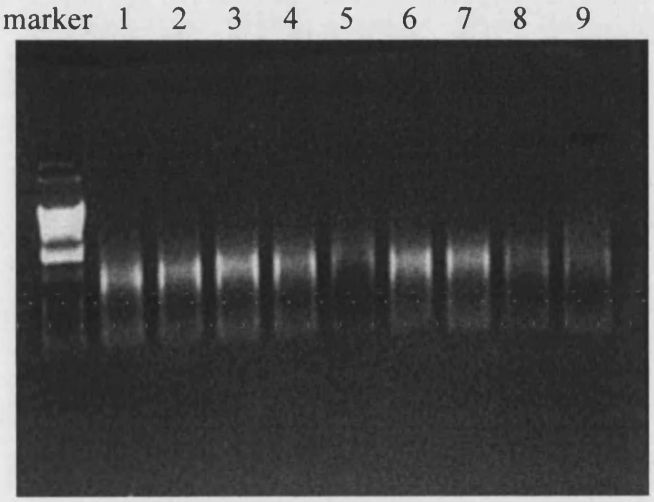

Fig. 68 cRNA in the samples from different culture conditions showing a good 28S:18S ratio

B 3 Fragmenting the cRNA for target preparation

This procedure produced a distribution of RNA fragment sizes from approximately 35 to 200 bases. The cRNA had to be a minimum concentration of 0.6µg/µL. cRNA was fragmented with 5X Fragmentation Buffer and RNAse free water made up to a final volume of 40µL. The sample was incubated at 94°C for 35 minutes, and then placed on ice. A small sample was saved for gel analysis.

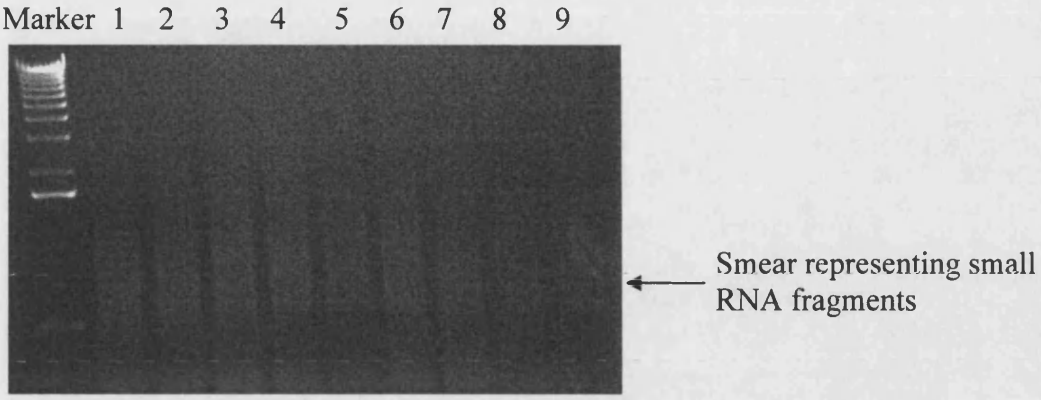

Fig. 69 cRNA after fragmentation for target preparation from culture conditions

B 3.1 Preparing the hybridisation target

For each target, the following reagents were added;

Component	**Volume**	**Final concentration**
Fragmented cRNA	10µg	0.05µg/µl
Control Oligonucleotide B2 (3nM)	3.3µL	50pM
20X Eukaryotic Hybridisation Controls (BioB, BioC, BioD, cre)	10µL	5.25pM
Herring Sperm DNA (10mg/mL)	2µL	0.1mg/mL
Acetylated BSA (50mg/mL)	2µL	0.5mg/mL
2X Hybridisation Buffer	100µL	1X
H_2O	Up to 200µL	
Final volume	200µL	

The probe array was equilibrated to room temperature before use and the hybridisation cocktail was heated for 5 minutes at 99°C in a heat block. Meanwhile, 160µl of 1X Hybridisation buffer was placed in the array to wet it and the probe array was incubated at 45°C for 10 minutes with rotation.

After the hybridisation cocktail was heated at 99°C for 5 minutes, the hybridisation control was transferred to a 45°C heat block for 5 minutes. The cocktail was spun at maximum speed in a centrifuge for 5 minutes to remove any insoluble material from the hybridisation mixture. The buffer solution was removed from the probe array cartridge and the purified hybridisation replaced it in the array. The probe array was placed in a rotisserie box in 45°C oven. The probes were hybridised for 16 hours.

B 3.2 Washing and staining of arrays
B 3.2.1 Materials

SAPE (Streptavidin Phycoerythrin) Stain Solution-
For 1200µl solution

600µl of 2X Stain Buffer
510µl of water
48µl of 50mg/mL acetylated BSA (final concentration of 2µg/µL)
12µl of 1mg/mL SAPE (final concentration of 10µg/mL)

Antibody solution-
For 600µL solution

300µl of 2X Stain Buffer
266µl of water
24µl of 50mg/ml acetylated BSA (final concentration of 2 mg/mL)
6.0µl of 10mg/mL normal goat IgG (final concentration of 0.1mg/mL)
3.6µL of 0.5mg/mL biotinylated antibody (final concentration of 3 µg/mL)

5% (w/v) Antifoam Stock Solution

Stringent Wash Buffer
For 1000mL

83.3 mL of 12 X MES Stock Buffer
5.2 mL of 5M NaCl
1.0 mL of 10% Tween 20
910.5 mL of water
Filtered through a 0.2µm filter

Non-Stringent Wash Buffer
For 1000mL

300 mL of 20X SSPE (3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA)
1.0 mL of 10% Tween 20
698mL of water
Filtered through a 0.2μm filter. After filtering added 1.0 mL 5% antifoam.

2 X Stain Buffer
For 250mL

41.7mL 12X MES Stock Buffer
92.5mL 5M NaCl
2.5mL 10% Tween 20
112.8mL water
Filtered through a 0.2μm filter. After filtering, added 0.5mL of 5% Anti-foam.

10mg/mL Goat 1gG Stock

Resuspended in 50mg in 5 mL PBS. Store at 4°C.

B 3.2.2 Method

The probe arrays were placed in a fluidics station in which the staining and washing took place. A microcentrifuge tube containing 600μL SAPE solution was placed into the sample holder. When the LCD window on the fluidics station indicated the microfuge tube containing the SAPE solution was replaced with a microfuge tube containing 600μL of antibody stain solution into the sample holder. When the LCD window indicated again, the microfuge tube was replaced again with the second 600μl SAPE solution. When the washing/staining protocol was complete the probe array was removed and as long as there were no large bubbles on the array, the probe was scanned. If they were not scanned immediately, they were stored at 4°C, in the dark.

B.4 Analysis

The cRNA from the culture conditions was hybridized to arrays of DNA probes on Affymetrix Human Gene Chips U95Av2, n=3.

For each sequence, a probe set contains Perfect Match (PM) and single base

mismatch (MM) sequences complementary for each probe. When the intensity of the MM probe cell is significantly greater than the PM probe cell, the probe pair is termed **Negative.**

A probe pair is considered to **Increase** if the intensity difference between the PM and the MM probe cells in the experimental is significantly *higher* than in the baseline sample.

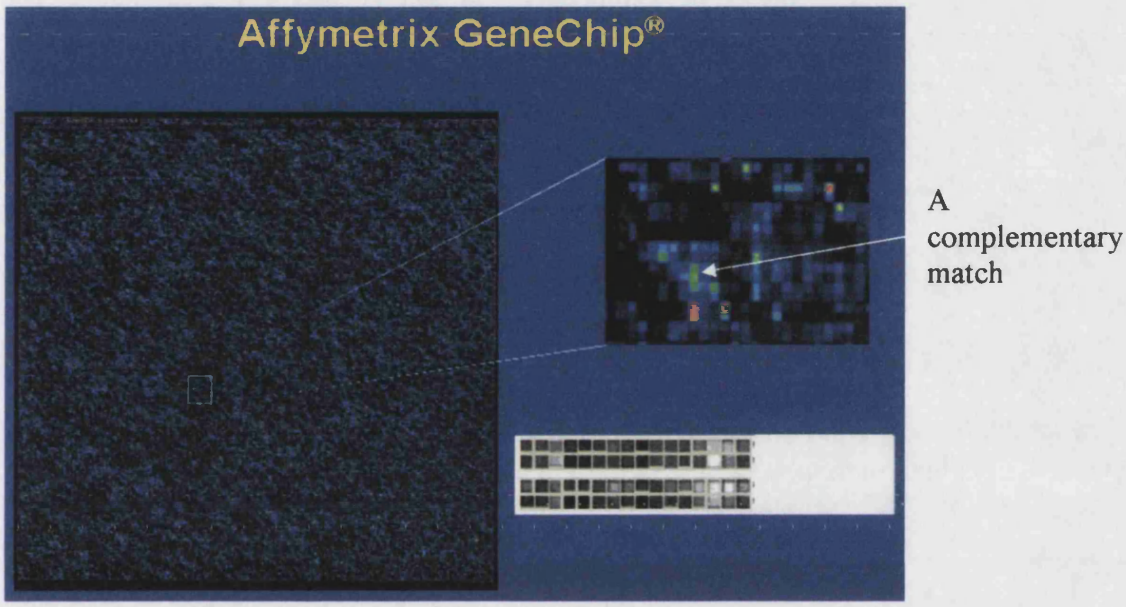

Fig. 70 Detection by fluorescence on Affymetrix Chip

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