FORMATION, MORPHOLOGY, AND FUNCTION OF A 3D HEPATOCYTE – HEPATIC STELLATE CELL CO-CULTURE SYSTEM

ROBERT JAMES THOMAS

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

March 2006

Abstract

In these studies the ability of a three-dimensional hepatocyte-stellate cell co-culture system to preserve some key aspects of differentiated hepatocyte function in vitro is demonstrated. A poly(DL-lactic Acid) surface allows dynamic and rapid interaction of hepatocytes and stellate cells to form co-culture spheroids in a complex multistage process (shown by time lapse microscopy). After five days the spheroids have developed a substantial extracellular matrix support and hepatic ultra-structure including bile canaliculi, tight junctions, desmosomes and lipid storage. The distribution of the stellate cells in the final structure is related to their motile and aggregating role in spheroid formation, i.e. mainly central and peripheral, and provides a unique and generically applicable insight into the dynamics of multicellular spheroid formation where aggregation is induced by one cell type and imposed on another.

The spheroid morphology supports enhanced cell viability relative to hepatocytes in a mono-culture mono-layer. Co-culture spheroids also have superior cytochrome P450 3A and 2B function, and increased inducibility of 2B function, relative to a range of hepatocyte monoculture techniques (HPLC detection of testosterone metabolites). Increased function in co-culture is supported by greater expression of cytochrome P450 3A23, 1A2, and 2E1 mRNA relative to monoculture (RT-QPCR). Also, high hepatocyte growth factor mRNA expression in co-culture suggests a post-traumatic, or possibly

regenerative, environment. A preliminary study of human hepatocytes co-cultured with rat stellate cells demonstrated prolonged function of cytochrome P450 3A4, 2C19 and 2C9. The co-culture spheroids are also shown to maintain a low level of sensitivity to hepatotoxins DDC and amiodarone after seven days in culture.

This study shows that stellate cells facilitate spheroid formation, influence spheroid architecture, and are an effective method of preserving some aspects of hepatocyte function in the early stage of culture.

List of Publications

Robert J Thomas, Rena Bhandari, David Barratt, Andrew Bennett, Jeffrey Fry, Desmond Powe, Brian Thomson, Kevin M Shakesheff. (2005) The effect of three-dimensional co-culture of hepatocytes and hepatic stellate cells on key hepatocyte functions in vitro. *Cells, Tissues, Organs*. 181(2):67-79.

Thomas RJ, Bennett A, Thomson B, and Shakesheff KM. (2006) Hepatic Stellate Cells On Poly(DL-lactic Acid) Surfaces Control The Formation Of 3D Hepatocyte Co-Culture Aggregates *In Vitro. European Cells and materials..* 11:16-26.

Acknowledgements

I would like to acknowledge the help and advice of my supervisors Kevin Shakesheff, Brian Thomson, Andrew Bennett, and Dave Barrett. I would also like to thank Des Powe and Jeff Fry for their expert input and assistance.

Finally, I would like to acknowledge the support of family and friends who have provided 3 years of mentoring and encouragement.

List of Contents

List of Figures

List of Tables

Abbreviations

BEC – Biliary epithelial cell

BMP - Bone morphogenetic proteins

CCAAT/enhancer binding protein (C/EBP

DDC – N,N-Diethyldithiocarbamide

ECM - Extracellular matrix

EGF - Epidermal growth factor

FGF – Fibroblast growth factor

GFAP - Glial fibrillary acidic protein

HGF - Hepatocyte growth factor

HNF – Hepatocyte nuclear factor

HPLC - High performance liquid chromatography

HSC – Hepatic stellate cell

IGF – Insulin growth factor

IL - Interleukin

ILGF – Insulin like growth factor

KC – Kupffer cell

LD50 – Lethal dose required to elicit 50% cell death

LDH – Lactate Dehydrogenase

MEGX - Mono-ethyl-glycine-xylidide

MMP - matrix metalloproteinase

NO – Nitric Oxide

P450 - Cytochrome P450 enzyme

PAR – Proline acid rich

$P_{DL}LA$ - Poly(DL-lactic acid)

PDGF – Platelet derived growth factor

ROS – Reactive oxygen species

RT-QPCR – Reverse transcriptase quantitative polymerase chain reaction

SCF – Stem cell factor

SEC – Sinusoidal endothelial cell

αSMA – Alpha smooth muscle actin

TCP – Tissue culture plastic

TEM – Transmission electron microscopy

TNF – Tumour necrosis factor

TGF – Transforming growth factor

CHAPTER 1

Introduction

1.1 Introduction

The work in this thesis was designed to characterise the formation, morphology and P450 function of a 3D hepatocyte-stellate cell co-culture system. This introduction initially describes the structure and function of the liver and the roles and regulation of parenchymal and non-parenchymal hepatic cell types. Current tissue engineering strategies are then discussed and related to the knowledge of liver homeostasis in vivo.

1.2 The liver

The liver is the largest gland in the human body and carries out a wide array of homeostatic functions. It receives blood directly from the gut and has an important protective role in foreign particle phagocytosis and in degradation of xenobiotics via oxidation and conjugation. It brakes down nutrients and endogenous elements such as hormones, produces numerous blood proteins such as albumin and proteins of the clotting cascade, and is responsible for the production of bile to help in the digestion of fats. The liver is also an important storage site with reserves of glycogen, iron and vitamins A, D, and B_{12}. The functions of the liver are reviewed in more detail under each individual hepatic cell type (section 1.2.2)

1.2.1 The structure of the liver

The macro structure of the liver comprises four lobes. Each lobe comprises numerous hexagonal functional units called lobules. The lobes and lobules vary greatly between species in size and definition; lobules in the porcine liver have a well defined extracellular matrix (ECM) boundary whilst there is little visible distinction between human lobules. Each lobule has a portal triad at its six peripheral points that it shares with two other tessellating lobules. The liver receives a dual blood supply from the portal vein and hepatic artery that enters the lobule via branches of these vessels in the portal triad. The blood filters through sinusoids that separate parenchymal plates (or chords) of hepatocytes and drains into a central vein. The sinusoidal endothelium is discontinuous and fenestrated on a basement membrane and is separated from the hepatocytes by a gap known as the space of Disse. The hepatocytes excrete bile salts into depressions between abutting cells known as bile canaliculi. These drain in the opposite direction to the blood flow to a collecting bile ductule (Herings canal), which is the third vessel of the portal triad. The organisation of the liver lobule is shown in figure 1.1.

Figure 1.1

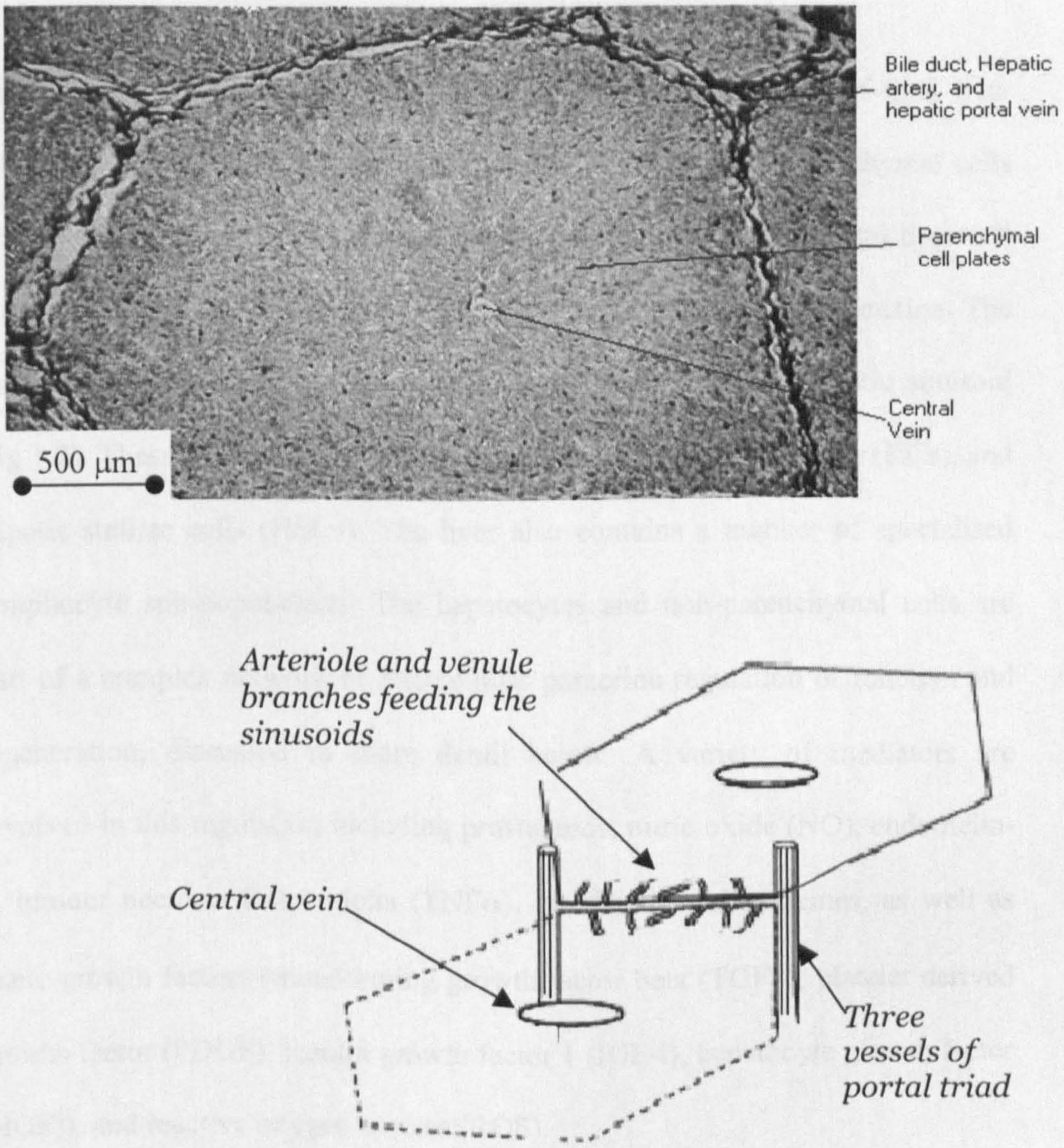


Figure 1.1: A micrograph section and schematic section showing the lobular organisation of the liver and associated blood flow.

1.2.2 The cell types of the liver: Function and paracrine communication

Hepatocytes, the parenchymal cell of the liver, constitute 80% of total liver volume and carry out the majority of hepatic function. Non-parenchymal cells occupy only 6.5% of total liver volume but represent 40% of the total liver cell number and have complex roles in paracrine regulation of hepatic function. The three major non-parenchymal cell types line the walls of the hepatic sinusoid (fig 1.2). These are sinusoidal endothelial cells (SECs), Kupffer cells (KCs), and hepatic stellate cells (HSCs). The liver also contains a number of specialised lymphocyte sub-populations. The hepatocytes and non-parenchymal cells are part of a complex network of intercellular paracrine regulation of function and regeneration, discussed in more detail below. A variety of mediators are involved in this regulation including prostanoids, nitric oxide (NO), endothelin-1, tumour necrosis factor alpha (TNFα), interleukins, chemokines, as well as many growth factors (transforming growth factor beta (TGFβ), platelet derived growth factor (PDGF), Insulin growth factor 1 (IGF-I), hepatocyte growth factor (HGF)), and reactive oxygen species (ROS).

Figure 1.2

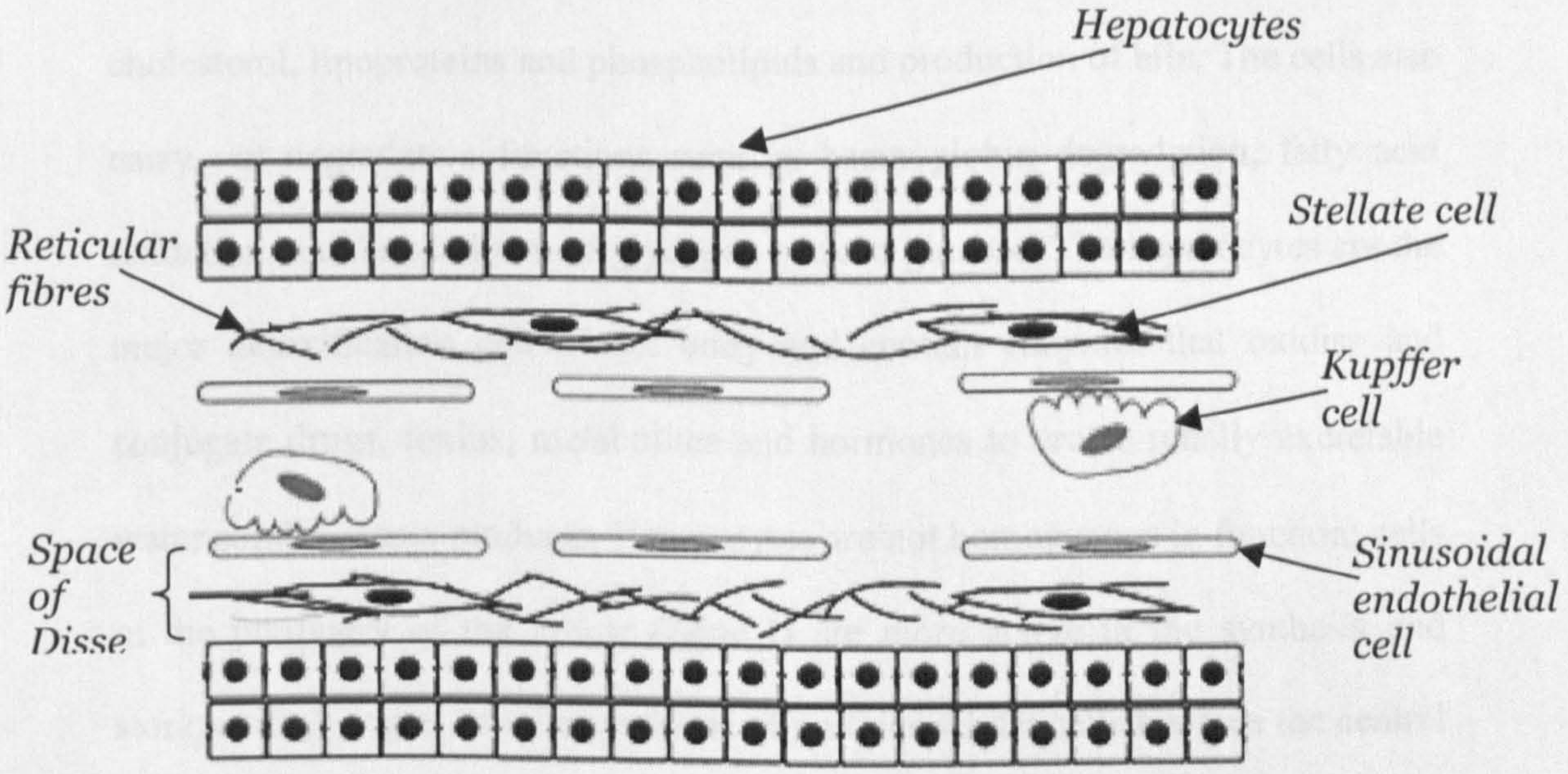


Figure 1.2: A schematic of the liver sinusoid and the juxtaposition of associated parenchymal and non-parenchymal cell types.

1.2.2.1 Hepatocytes

Hepatocytes have a diverse range of synthetic, degradative and regulatory functions. Synthetic functions include production of most plasma proteins, interconversion of amino acids, production of glucose from lipids and amino acids, formation (and storage) of glycogen from glucose, synthesis of cholesterol, lipoproteins and phospholipids and production of bile. The cells also carry out degradative functions such as haemoglobin degradation, fatty acid oxidation, and break down of glycogen back to glucose. The hepatocytes are the major detoxification cell of the body and contain enzymes that oxidise and conjugate drugs, toxins, metabolites and hormones to create renally excretable water soluble waste products. Hepatocytes are not homogenous in function; cells in the periphery of the lobule (Zone I) are more active in the synthesis and storage of glycogen and the synthesis of proteins whilst cells towards the central vein are more active in detoxification (Zone III). Reverse perfusion of the liver and perfusion systems have respectively demonstrated reversed patterns of lobule function and gradients of hepatocyte function indicating hepatocytes are not committed to a zone phenotype (Den Otter and Tuit, 1972; Allen *et al,* 2005). The inference is that the specific hepatocyte phenotype is determined by microenvironment factors such as oxygen tension, nutrient status, and differences in ECM composition or sequestered factors.

1.2.2.1.1 The cytochrome P450 mixed function oxidase system

The P450 mixed function oxidase (monooxidase) system is one of the most diverse enzymatic systems in nature catalysing a vast array of reactions in animals, plants, fungi and bacteria. Thousands of different enzymes have been identified - humans have over 50 in 18 families and rats over 80. In mammals the enzymes are concentrated in the liver, but are also present in many other tissues such as lung and kidney. P450 enzymes are central in many hepatic functions; the major roles of the enzymes in mammals include steroid synthesis, xenobiotic metabolism, arachidonic acid metabolism, fatty acid metabolism and synthesis of products such as thromboxane, prostacyclins, cholesterol and bile acids. The most important reaction carried out in mammals involves the insertion of a single molecular oxygen atom into the substrate, the other being used to generate water. In the case of xenobiotic metabolism this mechanism often introduces a hydroxyl group to the substrate that is a chemical handle for subsequent conjugation.

The P450 enzyme families are specialised for their different roles and are not equally abundant. This results in concentration of research on certain isoforms. For example, P450 3A is by far the most abundant isoform in human liver (Waxman, 1999) and is the most important drug metabolising family, oxidising about 50% of known oxidised pharmaceuticals. P450 2D6 is also responsible for a large proportion of xenobiotic and pharmaceutical metabolism. P450 2E1 is a xenobiotic metabolising enzyme of particular importance due to being induced by ethanol. Significant species differences exist in types and quantities of P450

enzymes, but also polymorphisms and sex specific differences occur within species.

1.2.2.2 Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells constitute the lining or wall of the hepatic sinusoid. They have small fenestrations that allow free diffusion of many substances between the blood and the hepatocyte surface and also perform endocytosis of many ligands including glycoproteins, components of the ECM, immune complexes, transferrin and ceruloplasmin. SECs may have a role in immune tolerance. They secrete cytokines, eicosanoids (i.e., prostanoids and leukotrienes), endothelin-1, NO, and some ECM components.

1.2.2.3 Kupffer Cells

Kupffer cells are macrophages located within the sinusoids that perform endocytosis and phagocytosis on particles from the gut, soluble bacterial products, and such endogenous waste as senescent or damaged erythrocytes. Hepatic macrophages play an important part in early phase liver inflammation through secretion of potent inflammatory mediators (ROS, eicosanoids, NO, carbon monoxide, IL-1, TNFα, TGFβ, and other cytokines). High exposure to bacterial products, especially endotoxin (LPS), can initiate this process and ultimately lead to hepatocyte and liver injury. In this process KCs also release enzymes involved in ECM remodelling. Inflammatory mediators stimulate glucose release from hepatocytes via prostaglandins released from KCs and

thereby have a role in regulating hepatocyte glucose metabolism. A further role of liver macrophages is modulation of the immune response and tolerance to certain antigens via complex paracrine interactions involving T-cells, SECs, and modulatory cytokine release.

1.2.2.4 Hepatic stellate cells

Hepatic stellate cells (HSCs) are located in the perisinusoidal space. They have been known variously as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells. They are characterized by an abundance of cytoplasmic fat droplets, branching cytoplasmic processes and co-localisation of a number of key immunological markers such as glial fibrillary acidic protein (GFAP) and desmin (species dependent). In the normal liver HSCs store vitamin A, produce ECM components such as collagen (Lewindon *et al,* 2002), are potentially involved in vascularisation of new tissue (Jung *et al,* 2003), and also regulate the contractility, and therefore blood flow, of sinusoids via α-smooth muscle actin (αSMA) contraction. Acute damage to hepatocytes activates transformation of quiescent HSCs into myofibroblast-like cells that play a key role in the development of the inflammatory fibrotic response. Stellate cells are thought to have an important cell-cell signalling role in liver homeostasis *in vivo* and are highly active in liver regeneration (Mabuchi *et al,* 2004). HSCs and hepatocytes bilaterally modulate proliferation: HSCs release cytokines such as HGF and TGFβ that respectively positively or negatively modulate hepatocyte proliferation, and hepatocytes release nerve growth factor (NGF) that is thought to cause stellate cell apoptosis (Oakley *et al,* 2003).

A range of autocrine and paracrine factors regulates HSC function. Mediators that affect constriction or relaxation of HSCs derive from various sources such as hepatocytes (carbon monoxide, leukotrienes), SECs (endothelin, NO, prostaglandins), KCs (prostaglandins, NO), and HSCs themselves (endothelin, NO). In the fibrogenic response, TGFβ, and the related bone morphogenetic proteins (BMPs), are pivotal HSC and KC derived cytokines involved in the fibrogenic aspect of stellate cell activation and have similar effects both in vivo and in vitro (Liu X *et al*, 2000; Malik *et al*, 2002). TGFβ is secreted in latent form and activated by hepatocytes. It significantly inhibits stellate cell proliferation (Shen *et al*, 2003), up regulates matrix metalloproteinase 2 (MMP-2) and activates MMP-2 dependent and independent migratory pathways (Yang *et al*, 2003). In vivo, inhibition of TGFβ promotes hepatocyte regeneration, reduces ECM accumulation in the space of Disse, and leads to an increased proportion of HSCs that have a fat droplet rich "quiescent" morphology (Nakamura *et al*, 2000). Another key HSC proliferative cytokine produced by both HSCs and KCs is PDGF. It shares the MMP-2/integrin α1/2 mediated pro-migratory activity of TGFβ (Yang *et al*, 2003), and is also involved in the activation of stellate cells via promotion of mRNA for type 1 and 3 procollagens and PDGF itself (Liu *et al*, 2000(b)). Interleukin 6 (IL-6), produced by HSCs, KCs and SECs is another major cytokine thought to be involved in the fibrogenic and mitogenic aspects of HSC activation. Other cytokines with mitogenic activity on stellate cells include TGFα, IL-1, TNFα, and ILGF (Tsukamoto *et al*, 1999).

Stellate cells are also an important paracrine regulator of hepatocytes. HGF, extracellular heparan sulphate (EHS) and heparan sulphate proteoglycan (HSP) are stellate factors that promote hepatocyte proliferation in stellate-hepatocyte co-culture in vitro (Skrtic *et al,* 1999; Uyama *et al*, 2002). HGF production by stellate cells is stimulated via hepatocyte produced insulin-like growth factor (ILGF-1) (and possibly other factors) (Skrtic *et al*, 1999). Activated stellate cells begin to express the HGF receptor (c-met) and the HGF increases the production of profibrogenic, anti-proliferative TGFβ in these cells creating a paracrine loop (Ikeda *et al,* 1998). IL-6 is also important in the proliferative response of hepatocytes following partial hepatectomy.

1.2.3 The extracellular matrix environment and liver function

The ECM is an important regulator of differentiation and proliferation in many tissue types (Roskelley 1995; Maher and Bissell 1993). The ECM structure of the liver is not homogenous, but varies across the lobule, and the specific composition is thought to influence the phenotype of the hepatocytes (Reid *et al,* 1992; Martinez-Hernandez and Amenta, 1993). The space of Disse contains mainly type III reticular collagen, but also some type I and IV. Around the portal triad ECM is predominantly type IV collagen, fetal laminin and fetal chondroitin sulphate proteoglycans, similar in composition to fetal liver ECM. In the portal triad region gene expression is correspondingly weighted towards early genes such as α-fetoprotein and albumin. In Zone 2, the composition changes towards mixtures of type IV collagen and fibrillar collagens, mixtures of laminin and fibronectin, and proteoglycans. PEPCK, IGF-1 and connexin 26

gene expression are enriched in hepatocytes in this area. Around the central vein fibrillar collagens, fibronectin, and highly sulphated forms of heparin sulphate proteoglycans predominate and developmentally late gene expression such as connexin 32, CYP 3A and 2E is stronger (Reid *et al*, 1990, 1992; Sigal *et al*, 1992). ECM regulation of tissue specific gene expression, such as HNF-3α through ECM modulation (Dipersio *et al,* 1991) and gap junction expression through glycosaminoglycan presentation (Spray *et al,* 1987; Fujita *et al,* 1986), can also be achieved in vitro.

1.2.4 The transcriptional control of hepatocyte function

Expression of mRNA for proteins involved in liver specific function is under the control of a complex network of transcription factors. Most of these are liver enriched transcription factors that are more prolific in, but not specific to, hepatocytes. A few key families of liver enriched transcription factors predominate in the regulation of liver specific function through a non hierarchical control network. Each transcription factor gene has at least two regulatory elements, a promoter and far-upstream enhancer. Hetero and homo dimerisation, transactivation and multiple enhancer binding regions between members of a given transcription family add an additional layer of complexity and fine control. This combinatorial control is an efficient mechanism to achieve both diversity and stringency in control of gene expression (Reviewed in: Cereghini, 1996).

The family of Hepatocyte Nuclear Factor 1 (HNF1) transcription factors has been relatively extensively studied. Recognition sites for this transcription factor have been identified in more than 30 liver specific genes including the albumin, β-fibrinogen and connexin 32 promoters (Piechocki *et al.* 2000) (Reviewed in: Tronche and Yaniv, 1992) and HNF1 expression correlates with the mature hepatocyte phenotype of the cell (Baumhueter *et al.* 1988). The less studied HNF3 family has been shown to participate in expression of several liver specific genes, including transthyretin and the α1-antitrypsin gene (Costa *et al,* 1989) but it is now thought to have a predominantly developmental role (Ang *et al,* 1993).

CCAAT/enhancer binding protein (C/EBP) has recognition sites in a large number of liver specific genes including albumin, C-reactive protein, factor IX and transferrin (Review: Cereghini, 1996). C/EBPα, β, and γ have been characterized and can all form homo and heterodimers to regulate responses. In the liver C/EBPα protein is only found in differentiated hepatocytes and adipocytes (Johnson *et al*, 1994). High levels of C/EBPα are associated with highly differentiated cells and reduced C/EBPα in both culture and during liver regeneration suggests a role in the maintenance of the differentiated state of hepatocytes (Umek *et al*, 1991; Mischoulon *et al*, 1992).

The role of the proline and acid-rich subfamily of bZip transcription factors in liver function is comparatively understudied (Review: Cereghini, 1996). Factor binding to albumin-D element (DBP) interacts with liver specific genes including the albumin gene and the gene encoding 7α hydroxylase (C7αH – the

rate limiting enzyme in conversion of cholestrol to bile acid). The activity of C7αH follows a similar circadian rhythm to this transcription factor suggesting a role in cholesterol homeostasis (Lavery and Schibler, 1993). In common with other factors discussed, it is down regulated during liver regeneration. PAR proteins bind to a subset of C/EBP recognition sites with greater specificity than C/EBP proteins

A further set of transcription factors is the subfamily of the nuclear receptor super family and includes the orphan receptors HNF4, Chicken ovalbumin upstream promoter transcription factor/Ear3 (CoupTF1/Ear3), and apolipoprotein regulatory protein 1/CoupTF II (Arp-1/CoupTF II). HNF4 interacts with regulatory regions in promoters and enhancers of genes whose products are involved in diverse functions such as cholesterol and amino acid metabolism, gluconeogenesis and coagulation. The recognition sequence for HNF4 also interacts with the closely related orphan receptors CoupTFI/Ear3, Arp-1 (AKA CoupTFII), EarII as well as a range of retinoic x receptor (RXR) and retinoic acid receptor (RAR) homo and heterodimers (Review: Cereghini, 1996)(Nakshatri and Chambon, 1994). HNF1 and α1 antitrypsin promoters only bind HNF4. Coup-TFs are negative transcription factors that compete for some of their binding sites with other positive transcription factor members of the family such as the vitamin D receptor, thyroid hormone receptor, RAR, RXR (Hatzis and Talianidis, 2001), the peroxisome proliferation activated regulator, and HNF-4 (Qiu *et al,* 1996), the balance determining the level of transcription. The maintenance of liver specific function by dexamethasone is probably attributable to its upregulating effect on RXR (Wan *et al,* 1994). RAR/RXR

heterodimers also bind the HNF3α promoter (Jacob *et al,* 1999), induce α-fetoprotein a marker of hepatocyte differentiation and maturation (Li *et al,* 1996) as well as binding the Coup-TF promoter, presumably in a complex negative feed back loop.

1.2.5 The in vivo – in vitro divide

The complexity of hepatic function and its regulation are evident from the information above. A precise micro-structure, paracrine regulatory network, and specific ECM and physical environment, all combine to influence a complex web of hepatocyte enriched transcription factors to produce a given hepatic function. Although much is known, the mechanisms responsible at each level of this control are not fully understood. However, their regulation is key to differentiated function in vivo and the current understanding of their control is the best source to provide direction for liver engineering strategies in vitro.

1.3 Tissue engineering

The simple concept of tissue engineering involves the removal of a cell from a host or donor, the multiplication of that cell to a functional unit, and then the return to the host. The field also offers the potential to develop in vitro organ assays and disease models as well as tissue based therapeutic solutions. Tissue engineering is a multidisciplinary area requiring expertise in cell biology, material science, and beyond. Its aims are to manipulate tissue growth and function through design and optimisation of novel culture techniques, surfaces and scaffolds. Demand is rapidly growing for this technology across a wide range of therapeutic areas. Significant progress has been achieved in skin, cartilage, and even nerve tissue engineering. More complex tissues such as the pancreatic beta cell islets or the liver pose greater challenges.

1.3.1 The need for tissue engineered liver

There is an increasing urgency for hepatocyte culture systems that sustain hepatocyte function in vitro. Such systems could contribute to the development of solutions to a variety of industrial and therapeutic problems. In industry, high throughput metabolism and toxicology screens are required that will improve accuracy and reproducibility of developmental compound screening and also help comply with the pressure to 'reduce, refine and replace' animal testing in areas of research that historically have relied heavily on animal based methods. An example of this is recent EU legislation (2003/15/EC) that prohibits cosmetics firms selling products tested on animals within the EU after 2009

resulting in a rush to validate alternative in vitro systems for a range of tissues and assay types. In therapeutic terms, the need for liver assist or replacement technology is also urgent: the waiting list for liver transplant stands at nearly 18 000 in the US with over 20% of patients having waited more than 5 years (The organ procurement and transplantation network, October, 2005). In vitro culture systems offer the potential of hepatic dialysis to allow regeneration of a patients own liver, or alternatively of a seeding system to repopulate a damaged liver. A further application for in vitro liver culture is the study of liver disease where improved models of conditions such as fibrosis, cirrhosis, and viral infection and replication are required. This is of particular importance as Hepatitis C has an estimated infection rate of just less than 1% in the UK population and a global incidence estimated at 2.2% (The global burden of Hepatitis C working group, 2004). Future levels of cirrhosis and liver cancer are predicted to be correspondingly high and it is therefore important to improve the currently limited research capabilities into the pathology of these conditions.

1.3.2 The challenge of hepatic tissue engineering

Creating a tissue engineered liver model should be relatively easy. The liver exhibits amazing regenerative capacity; after two-thirds hepatectomy liver mass and function are rapidly recovered over a matter of weeks. However, once isolated, hepatocytes cultured on tissue culture plastic rapidly de-differentiate and die. The cells progressively lose their liver specific attributes, such as xenobiotic metabolism, production of blood proteins, and polarised membrane structure. The time course of deterioration for each function differs; rapid loss of

some major P450 enzymes occurs within the first day of culture, and production of proteins such as albumin lasts longer (Guillouzo, 1998). This functional instability presents a barrier to the development of robust *in vitro* cell culture models.

Liver tissue engineering is further challenged by the low availability of human cells. Currently, cells are used from hepatic resections or from organs unsuitable for transplant. Other species can be used but there is significant inter-species functional variation such as in the prevalence of various P450 enzymes and in the manner of dedifferentiation. Furthermore, any successful human hepatocyte culture system would create a demand that would be impossible to meet through current sources. Attempts to address this issue are being made through stem cell or progenitor cell differentiation research, perpetual culture systems, or, in the case of therapeutic support or seeding systems, through use of a patients' own cells.

The cause and nature of the changes in hepatocytes that occur in culture are disputed; even the terminology, dedifferentiation or adaptation, is contentious. Transcriptional changes are variously attributed to the loss of an ECM support, paracrine signals or other endogenous regulators. This is supported by the benefits of media supplements (Washizu *et al*, 2000) and also by the requirements of an ECM to maintain functions such as P450 inducibility (Schuetz et al, 1988). Alternatively, it is suggested that loss of mRNA is not solely due to loss of endogenous signals, but an active response to the cells new environment (Wang *et al*, 1997). For example fibronectin reportedly mediates

RNAse induction via integrin receptors contributing to loss of P450 2C11 (Hodgkinson *et al*, 2000). The nature of the change of cell transcription *in vitro* has implications for strategies to prevent it.

1.3.3 Techniques in liver tissue engineering

The functional and longevity limitations of traditional hepatocyte culture have driven the development of a plethora of techniques designed to reproduce the essential stimuli required to maintain the in vivo phenotype of hepatocytes in vitro (Powers *et al,* 2002, Bhatia *et al,* 1998, Michalopoulos *et al,* 1975). Strategies are diverse and include growth factor or nutrient media supplementation, incorporation of ECM components in the culture environment, promotion of three-dimensional structure formation, and co-culture with a second cell type. These techniques are not mutually exclusive; co-culture may mediate an effect through soluble mediator or ECM production by the second cell type, and some ECM based methods have a three dimensional aspect and develop cell polarity.

1.3.3.1 Media supplementation and long term hepatocyte culture

Early attempts to prolong hepatocyte function in vitro involved variation of the culture media composition. Some supplements have become common additives to hepatocyte culture medium with relatively well defined effects. Dexamethasone is often used to augment matrix gene transcription (fibronectin and collagen), tyrosine aminotransferase and other liver enriched activity

(Jefferson *et al,* 1985). However, it has also been shown to inhibit spheroid formation (Abu-Absi *et al,* 2005) and may therefore interfere with development of morphology beneficial for long-term culture. Nicotinamide can be used to stimulate proliferation in cultured primary hepatocytes (Sato *et al,* 1999) and nicotinic acid to facilitate small colony formation, the latter reportedly without any effect on proliferation. Insulin and epidermal growth factor (EGF) can be used as in vitro hepatocyte growth factors (Nakamura and Ichihara, 1985), whilst EGF combined with HGF supplementation allows culture of hepatocytes for up to 5 weeks with well-preserved morphology and expression of a number of in-vivo like markers, liver specific proteins and liver-enriched transcription factors (Runge *et al,* 2000). A balance of additives such as these can be used to help promote the desired phenotype, i.e. proliferative or functional, for the purpose of the culture.

1.3.3.2 The role of extracellular matrix in liver tissue engineering

A complex and graduated ECM network surrounds hepatocytes in vivo and is thought to influence hepatocyte phenotype. Fibronectin is the predominant component but there is also some collagen type I, minor quantities of types III, IV, V, and VI as well as other proteins (see section 1.2.3, Reviewed in: Martinez-Hernandez and Amenta, 1993). Hepatocytes have also been demonstrated to respond to ECM stimuli in vitro. Culture surfaces coated with ECM components such as fibronectin, collagen, laminin, vitronectin or commercial products such as Matrigel™ are claimed to improve hepatocyte adherence, prolong survival and improve functionality in the short term.

Increasing levels of cell deposition and cell organisation on these surfaces (Clement *et al.* 1984), as well as ECM dependent cytokeratin organisation (Baffet *et al.* 1991), correlate well with higher hepatocyte specific function. Remodelling of the ECM environment involving the cell-cell interaction dependent activation of MMP-2 has also been observed in culture (Theret *et al.* 1997).

Fibronectin is a comparatively well studied ECM component due to its prevalence in vivo. A culture surface presenting the integrin-binding motif of fibronectin (the arginine-glycine-aspartic acid sequence (RGD)) reportedly enhances differentiated function and maintains the round *in vivo* like morphology of hepatocytes in vitro (Bhadriraju and Hansen, 2000). Compared with collagen type I or entactin-collagen IV-laminin (ECL), fibronectin is also reported to be the only ECM protein to support in vitro formation of chords reminiscent of hepatocyte plate organization in vivo with expression of CK18, albumin, α-fetoprotein, and associated transcription factors HNF4 and HNF1α (Sanchez *et al,* 2000).

Matrigel™ (a protein mixture from a murine fibroblastic cell line) is a popular ECM blend used as a hepatocyte culture substratum that, in the short term, supports hepatocyte survival and function including a number of P450 activities (Schuetz *et al,* 1988, Liu *et al,* 1991). It is associated with general higher expression of liver specific proteins and supports expression of the gap junction protein connexin 32 and EGF receptor specific epitope, none of which are observed with type I collagen (Moghe *et al.* 1996). It is possible that the

properties of Matrigel™ (predominantly type IV collagen, laminin, and heparan sulfate proteoglycan (HSP)) are attributable to its incorporation of several basal lamina components. A study attempting to isolate the effects of individual ECM components has suggested that matrix rich in laminin, a Matrigel™ constituent, provides the most convincing maintenance of ECM supported phenotype in vitro (Schuetz *et al*, 1988). Matrigel™ also contains glycosaminoglycans and proteoglycans that independently demonstrate potent concentration dependent induction of hepatocyte gap junction expression and function and increase the expression of liver enriched mRNA (Fujita *et al.* 1987). In addition, these mediators often cause contraction of the cell sheet and consequent increase in cell packing density, a phenomenon frequently associated with differentiated function.

Although the majority of evidence supports ECM promotion of in vitro hepatocyte function, alternative reports suggest that whilst hepatocytes adhere well to ECM coated surfaces this is associated with flattening of the cells and loss of function (Michalopoulos *et al*, 1975; Rojkind *et al*, 1980). Furthermore, loss of certain hepatocyte specific gene expression and cellular spreading, mediated via a specific integrin receptor, has been attributed to fibronectin *in vitro* (Hodgkinson *et al,* 2000). However, fibronectin clearly does not cause dedifferentiation in vivo, presumably due to a moderating factor in the ECM or from nearby cells. These conflicting studies demonstrate the difficulty of viewing various ECM components in isolation. Some of the variation may also be accounted for by differences in culture morphology, poorly defined cell populations and variable culture conditions between laboratories.

1.3.3.3 Three dimensional culture structures in liver tissue engineering

The composition of the support matrix surrounding the hepatocytes is only one of the factors determining cellular phenotype. The morphology and juxtaposition of the cells is also closely linked to gene expression. A wide variety of 3D culture techniques have been developed including spheroid formation, sandwich culture, micro spheres and polymer scaffolds amongst others (see section 5.1). Advances in 3D cell culture techniques have produced benefits that include preservation of membrane polarity and cell structure, maintenance of functional attributes such as albumin production, suppression of markers of de-differentiation such as α-fetoprotein, and maintenance of EGF and HGF receptor expression (Ringel *et al*, 2005; Dunn *et al*, 1989; Powers *et al*, 2002, Engl *et al*. 2004, Hanada *et al*. 2003, Kudryavtseva *et al*, 2003).

A relatively common method of producing 3D cell constructs involves cell culture on non-adherent surfaces to produce aggregates or spheroids. These develop tight junctions between cells, have microvilli lined channels and bile canaliculi with active secretion (Koide *et al*, 1990), and have polarised distribution of basal and apical membrane proteins (Abu-Absi *et al*, 2002). Hepatocyte spheroids also demonstrate improved base line and inducible tissue-specific gene expression (Tamura *et al*, 1995). However, spheroid aggregates suffer from limitations such as consolidation to larger aggregates and the formation of a barrier layer. Central necrosis after approximately a week, presumably due to nutrient deprivation or waste build up, and reduced toxicity

sensitivity are both reported in some spheroid models (Battle T *et al,* 1999; Torok E *et al,* 2001).

1.3.3.4 Co-cultures and liver tissue engineering

Co-culture of hepatocytes with a second cell type has been shown to prolong a differentiated hepatocyte phenotype *in vitro*. In early studies human fibroblasts were used as the second cell type and promoted better hepatocyte attachment and cyclophosphamide metabolism (Kligerman *et al*, 1980). This was followed by a number of studies using fibroblast cell lines, such as 3T3, that demonstrated prolonged P450 function (Riccalton Banks *et al*, 2003(b); Washizu *et al*, 2001).

The early work with fibroblasts was followed by co-culture of hepatocytes with liver epithelial cells. This is probably the most extensively studied system; it improved hepatocyte acute phase protein production and P450 activity (Guillouzo *et al*, 1984; Begue *et al,* 1984, Akrawi *et al*, 1993), taurocholate uptake (Foliot *et al*, 1985), liver enriched mRNA expression (Fraslin *et al*, 1985), coagulation factor production and urea production (Auth *et al*, 2005). Some of these benefits, such as xenobiotic metabolism, were also demonstrated with epithelial-like cell line co-cultures offering a system easier to work with than one involving non-continuous epithelial cells (Donato *et al*, 1990; Donato *et al*, 1991).

Sinusoidal endothelial cells have been used in various forms as hepatocyte co-culture partners. A hepatic derived sinusoidal endothelial cell line, a pulmonary

endothelial cell line, and aortic endothelial cells amongst others have variously been shown to reduce α-fetoprotein expression and prolong production of albumin and other liver associated proteins (Morin and Normand, 1986; Talamini *et al*, 1998; Harimoto *et al,* 2002).

The remaining cell type of the sinusoidal compartment, the stellate cell, has also been trialled as an in vitro co-culture component. Due to the pivotal role of this cell in fibrosis, these studies have often been directed at ECM deposition (Loreal *et al*, 1993), or associated disease related responses such as alcohol induction of procollagen expression (Fontana *et al*, 1997). In terms of improved hepatocyte function, co-culture with the stellate cell line Li90 supports improved P450 function but not urea production (Okamoto *et al*, 1998). However, co-culture with stellate cell line CFSC-2G reduces functionality in terms of albumin content and metabolic rate, as well as reducing proliferation (Arnaud *et al*, 2003). Primary stellate cells are also reported to maintain hepatocyte numbers in mixed co-culture, relative to a 25% drop in mono-culture, and increase hepatocyte numbers in separated co-culture, suggesting a complex regulatory role in hepatocyte proliferation (Uyama *et al*, 2002).

Other functional co-cultures have included hepatocytes with bone marrow cells that form functional aggregates (Ijima *et al*, 2005), and hepatocytes with pancreatic islets, where improved function is attributed to insulin and glucagons (Kaufmann *et al*, 1999). A number of studies have considered Kupffer cells and other macrophages as co-culture components but usually with the aim of studying Kupffer mediated toxicity in response to factors such as endotoxin and

not prolonged function (Hoebe *et al*, 2001). The related field of foetal hepatocyte differentiation has also used co-culture with liver derived cells to direct differentiation of stem cells and various progenitor cells.

The complexity of cross regulation of all these cell types in vitro, as outlined in section 1.2.2, may limit the functional potential of a system involving only two cell types. This view is supported by a particularly successful hepatocyte culture model involving pre-culture of mixed stromal cells on a mesh followed by seeding of hepatocytes that results in long and diverse function over several months (Naughton *et al*, 1995). The nature of the stromal cell population is not defined however and an alternative study suggests that stellate cells and kupffer cells predominate over endothelial cells in mixed non-parenchymal cell culture with associated benefits similar to those previously reported (Ries *et al*, 2000).

The development of the co-culture field has led to a desire to control and understand the interactions between different cell types in vitro. Techniques have been developed to control the spatial arrangement of cells on culture surfaces i.e. photosensitive surfaces that allow micro patterning through UV light and photomasks (Kang *et al,* 2004), magnetic nanoparticle containing cells that can be controlled using magnetic forces (Ito *et al*, 2004), and thermo responsive polymers that can regulate attachment and patterning (Tsuda *et al*, 2005). Such technologies contribute to further understanding of cellular interactions and the basis of the co-culture effect.

The basis for the co-culture effect probably varies depending on the co-culture component, and in a number of cases has been shown to be relatively non-specific. For example, hepatocyte albumin and urea production are proportional to cadherin presentation by a co-cultured non-functional transfected cell line (Brieva and Moghe, 2001), support of hepatocyte differentiated function in organotypic islands formed by co-culture with various cell lines is reportedly totally dependent on the ability of the cell line to produce a 3D collagen network (Kudryavtseva, 2003), and functional benefits in an epithelial cell co-culture system are dependent on cell contact, but not on junctional communication (Mesnil *et al*, 1987). In heterotypic culture with 3T3 cells, micro-structure control has demonstrated enhanced liver specific function specifically in hepatocytes with a heterotypic interface (Bhatia *et al*, 1998), but this is countered by the claim that TGFβ production is responsible for hepatocyte function in 3T3 co-culture (Chia *et al*, 2005). Gene array technology may provide the most illuminating insight into the mechanisms of hepatocyte function support by co-culture. The technique has been used to identify 17 communication related genes, such as N-cadherin, that are upregulated in a range of different fibroblast co-cultures and are therefore potentially involved in hepatocyte modulation in co-culture (Khetani *et al*, 2004).

1.3.3.5 Bioreactors in liver tissue engineering

The most advanced and complex liver culture involves the use of bioreactors. These are designed to allow control of elements of the culture system environment. The drive to develop complex bioreactors is provided by evidence

that physical parameters such as perfusion/shear flow (Kan *et al*, 1998, Kan *et al*, 2004) or oxygenation (Tilles *et al*, 2001) influence hepatocyte function and longevity. Bioreactors often involve a combination of known functionally beneficial factors, such as co-culture or ECM support. Functionally beneficial systems have included perfusion of human 3D non-parenchymal cell hepatocyte co-culture aggregates with integral oxygenation (Zeilinger *et al*, 2002), perfusion of hepatocyte BEC co-culture in collagen gel (Auth *et al*, 1998), hepatocyte SEC co-culture in a flow bioreactor (Pollok *et al*, 1998), and hepatocyte, BEC and SEC co-culture in a rotary low shear force bioreactor (Yoffe *et al*, 1999). These systems have variously shown improvements in the cell parameters measured e.g. P450 activity, albumin production, or in vivo like organisation. Detailed analysis of a channelled bioreactor has also revealed zonation of hepatocyte function from the entrance to the exit channel (Allen *et al*, 2005). Porcine cells in a model of extracorporeal liver support have been used to reduce ammonia levels and maintain P450 metabolism in toxic plasma circulated through an oxygenated bioreactor for 6 hours (Papadimitriou *et al*, 2004) and, in an alternative model, maintain ammonia detoxification and P450 activity for two weeks in a perfused high density sandwich between semi-permeable membranes (De Bartolo and Bader, 2001).

The development of a culture system with flow is attractive as it provides potential for an extracorporeal BAL support device, and, in this way, bioreactors are the link between in vitro culture systems and a clinically useful BAL support system. Most of these bioreactors show promising qualities for an extracorporeal BAL support device. However, analysing cause and effect in systems this

complex is extremely difficult, with individual factors hard to isolate. For example, SEC and nonparenchymal cell co-culture on 3D polymers under flow is reported, contrary to what the majority of the literature would lead us to suspect, to have no effect on albumin secretion or urea synthesis (Kaihara et al, 2000). Such anomalies are most likely due to different bioreactor designs resulting in multiple different variables between groups other than those described as the target of the experiments.

1.3.3.6 Alternative strategies to mature hepatocyte isolation and culture

The culture of mature hepatocytes in vitro is an important field in the search for in vitro screening systems and extracorporeal BAL systems. However, there are related fields that have also shown progress towards the same goals. Most of these techniques offer alternatives that circumvent the shortage of human tissue.

Cell lines are a simple solution promoted for in vitro testing due to their availability and ease of use and validation. However, older cell lines such as HepG2 cells are woefully inadequate for studying a variety of hepatocyte functions (Wilkening *et al*, 2003) and, whilst newer cell lines express higher levels of liver specific proteins e.g. P450 enzymes (Degawa *et al*, 2003), these are not as high as primary hepatocytes, and not sufficiently *in vivo* like in their responses.

Transgenic animals offer the possibility of metabolism and toxicology tests in animals with a humanised liver phenotype, or of growing transgenic

immunologically compatible human organs in animals for xenotransplantation. Alternatively, liver localised or ectopic transplantation of hepatocyte suspensions or hepatocyte microspheres to support liver function offer alternatives to whole organ transplant.

There is also interest in differentiation of progenitor or stem cells into scaleable functional hepatocyte populations. Implanting cells and observing differentiation in vivo has achieved most success. However, conversion of haematopoietic stem cells to hepatocytes without fusion has also been achieved in vitro in separated injured liver co-culture (Jang *et al,* 2004). Differentiation of mesenchymal stem cells to hepatocyte like phenotype has been stimulated in vitro using fibronectin, SCF, HGF, EGF, and FGF-4 (Lange *et al*, 2005). A combination model of mesenchymal stem cells pre-cultured with damaged liver tissue prior to transplantation appears to have hepatic potential (Luk *et al*, 2005).

1.4 Project background, hypothesis and aims

This thesis describes studies that were designed to evaluate some of the functional and morphological characteristics of an in vitro hepatocyte and hepatic stellate cell co-culture system.

The system entails co-culture of pre-cultured, activated, hepatic stellate cells with freshly isolated hepatocytes on a $P_{DL}LA$ surface. Hepatic stellate cells were used due to their involvement in hepatocyte regulation in vivo (see section 1.2.2.4). In vitro the cells interact to form multicellular 3D spheroids. These co-culture spheroids have previously been shown to maintain low-level albumin production and P450 1A2 function over several months (Riccalton-Banks *et al*, 2003). Also, hepatocytes in co-culture with stellate cells enter into S phase of the cell cycle demonstrating potential for a proliferative hepatocyte culture system (Lewis, 2003); cells in standard mono-culture are blocked at the mid to late G1 phase restriction point (Loyer *et al*, 1996).

It is hypothesised that the hepatocyte stellate cell co-culture system will support a high level of differentiated function and structure. To test this hypothesis, the studies described in this thesis aim to:

- Develop and validate an HPLC method and apply it to quantifying metabolite production by in vitro cell systems.

- Design and validate reverse transcriptase PCR probes to investigate expression of various liver enriched mRNAs and cytokines.

- Use time-lapse photography to study the manner and time frame of cell interactions and spheroid formation.

- Use immunohistology and transmission electron microscopy to investigate the structure of the spheroids.

- Evaluate the potential of the co-culture system as a toxicological model.

CHAPTER 2

Formation and Structure of Hepatocyte Stellate Cell Co-culture Spheroids

2.1 Introduction

2.1.1 The advantages of 3D cell culture

The liver has a complex and highly organised 3D architecture. A 3D culture system is therefore necessary to allow elements of hepatic structure to develop in vitro. Such a system, correctly engineered, is likely to provide a more faithful model of liver function and response than a cell monolayer through allowing cells to create an in vivo like microenvironment and heterotypic cell connections. For example, potential exists for gradients of environmental factors such as nutrients, toxins, waste products, ECM, paracrine mediators and physical stress. In early development of 3D systems these are unlikely to be in the correct balance to provide an in vivo like range, but such a system offers the opportunity to introduce in vivo like complexity.

2.1.2 Methods and mechanisms of formation of 3D aggregates

There is strong evidence that the method and mechanism of hepatocyte spheroid formation is critical to the functional performance of the resultant aggregate. Manipulation of aggregation is therefore important, and has been achieved through numerous methods. Growth factor supplementation and matrix compliance alter the kinetics of aggregation (Semler *et al,* 2000), and different culture surfaces can result in different mechanisms of spheroid formation (Hasebe *et al,* 2005). In the latter case, attachment of cells to the culture surface resulted in relatively slow formation of more functional spheroids and involved the plasminogen activator/plasmin system whereas

formation of spheroids without attachment was faster, did not involve the PA/plasmin system, but produced less functional aggregates. The speed of aggregation may be important in establishing rapid cell-cell contact and function, but the effect of this on function is unclear. An alternative study on the mechanics of formation of 3D hepatocyte structure has suggested that the actin network is essential for hepatocytes to self assemble into aggregates (Emmanouhl *et al,* 2001), but experiments disrupting such an integral cell structure will interfere with multiple other mechanisms. The complexity and multifactorial nature of the events that contribute to aggregation make it difficult to observe and difficult to create appropriately controlled experiments.

2.1.3 Aggregate shape, ultrastructure and ECM organisation

If ECM is presented in a three-dimensional architecture, such as a sandwich culture or encapsulation, both cell shape and some aspects of functionality are preserved (Dunn *et al,* 1989; Engl *et al,* 2004). In contrast, although hepatocytes adhere well to an ECM coated surface to form a 2D monolayer, this is associated with flattening of the cells and loss of function (Michalopoulos *et al,* 1975; Rojkind *et al,* 1980). The composition of any ECM support is also important, demonstrated by enhanced function, morphology, and viability on matrix derived from solubilised acellular liver compared with simple collagen or plastic (Burra *et al,* 2004). Therefore the evidence combines to suggest that the correct ECM mixture presented in the correct orientation could have benefits for hepatocytes in culture.

A 3D structure is thought to be advantageous due to making in vivo like 3D connectivity possible. 3D aggregate structures have more cadherin expression and less integrin expression than monolayers suggesting more cell to cell communication (Hou *et al,* 2001). Increased connectivity between cells, and, in co-cultures, the arrangement of cells to maximise heterotypic interfaces, have both been linked to improved cell function (Bhatia *et al,* 1998; Khetani *et al,* 2004). Aspects of liver ultrastucture such as cellular junctions, membrane polarity and bile canaliculi are commonly reported in 3D culture systems such as aggregate cultures in bioreactors (Zeilinger *et al,* 2004; Powers *et al,* 2002), collagen sandwich cultures (Engl *et al,* 2004), spheroid cultures (Koide *et al,* 1990; Abu-Absi *et al,* 2002; Dvir-Ginzberg *et al,* 2004), and cultures on polymer scaffolds (Hanada *et al,* 2003). Such systems usually also involve some form of ECM support, and, in practice these characteristics are difficult to analyse independently. For example, in a co-culture model of preserved hepatocyte morphology and function, these attributes have been shown to be dependent on the ability of the cells to form aggregates with a 3D ECM network (Kudryavtseva and Engelhardt, 2003).

2.1.4 Cell viability in 3D structures

Functionality and viability of culture systems are not necessarily synonymous. Spheroid structures, repeatedly demonstrated to have improved hepatocyte specific function, suffer central necrosis after one to two weeks presumably as a result of toxin accumulation and/or nutrient deprivation (Dvir-Ginzberg *et al,* 2004). A depth of 80-100 μm has been suggested as the maximum at which

hepatocytes can survive in an aggregate structure (Fukuda *et al,* 2004). However, this range will inevitably depend on the density and composition of the aggregate amongst other factors.

2.1.5 Aims

The studies described in this chapter were designed to characterise the formation, composition and structure of the co-culture spheroids. A range of mono-culture techniques were used to provide 2D and 3D mono-culture comparisons for hepatocyte-stellate cell co-culture and thereby distinguish between the co-culture effect and alternative methods of aggregation. Mono-culture aggregates are formed by agitated culture, or trypsin pre-treatment, the latter being a technique hypothesised to increase cell adhesion through release of sticky glycopeptides, and rearrangement of surface glyco proteins (Deman *et al,* 1974). The chapter specifically aims to:

- Use time lapse photography to study the characteristics of formation of co-culture spheroids.

- Use histology and immunolocalisation techniques to study the structure, heterotypic cell arrangement, and ECM support in the spheroids.

- Use LDH leakage and Live-Dead™ probe in conjunction with confocal microscopy to study the viability of cells in the spheroids.

- Use TEM to investigate the ultrastructure of the spheroids.

2.2.2 Rat stellate cell isolation

Stellate cells were isolated as described previously (Riccalton-Banks *et al.*, 2003). In brief, supernatant from cell washes and the percoll purification spin were combined, and made up to 200 ml with stellate culture media (DMEM (Gibco) supplemented with 5 mM L-glutamine, 100 U Penicillin, 100 μg streptomycin, 250 ng amphotericin B and 10% FCS Gold (PAA laboratories)). This was centrifuged three times at 50g, each time retaining the supernatant and discarding the pellet. The final supernatant was centrifuged at 260g, the resultant pellet suspended in stellate culture medium, and plated into two T75 flasks. Media was changed after 12 to 24 hours and then every three days. Cells were cultured for several weeks until confluent before use.

2.2.3 $P_{DL}LA$ coating of culture plates

$P_{DL}LA$ (Polyscience Inc) was dissolved in 2,2,2-Trifluoroethanol at a concentration of 1.5 mg/ml. 500 μl of $P_{DL}LA$ solution was added to each 962 mm^2 nunclon dish. The dishes were placed in an oven at 60°C for approximately 1 hour to evaporate the solvent and then exposed under a sterilising UV lamp for 30 minutes before being stored at -20°C.

2.2.4 Methods of cell culture

Co-cultures comprised of 300 000 cultured stellate cells and 600 000 freshly isolated hepatocytes combined in a $P_{DL}LA$ coated 962 mm^2 circular plate, and

maintained in 1.5 ml hepatocyte culture medium (William's E media supplemented with 5 mM L-glutamine, 50 μg/ml gentamicin, 5 mM nicotinamide, and 10 mU/ml insulin). Dexamethasone media supplementation is not used as this has a detrimental effect on spheroid formation (also observed and published by Abu-Absi *et al*, 2005). Mono-cultures were conducted using 600 000 hepatocytes in the same types of plates and also in untreated tissue culture plastic (TCP) plates, and were maintained in the same manner. Mono-cultures on TCP were cultured with 10% FCS for the first 2 hours. Agitated cultures were conducted in Primaria™ plates on a rotary plate shaker at 120 rpm. Trypsin treated cultures were incubated for 10 minutes with 2 μM trypsin in 200 μl media, followed by dilution to 1.5 ml with hepatocyte culture media. Incubation for all cultures was at 37°C in a 5% CO_2 atmosphere.

2.2.5 Time Lapse Photography

Cells were kept in a 37°C CO_2 supplemented incubation chamber under a Leica DR IRBE microscope with an attached Leica DC 200 digital camera. Images were acquired every 6 minutes and converted into video using Adobe Premier™ software. Time lapse sequences were constructed such that 1 second of sequence time equates to 2 hours of cell culture.

2.2.6 Histology and immunolocalisation

Five day old spheroids or liver samples were prepared for histology by fixing aggregates in 10% formalin in PBS for approximately 1 hour, suspending the fixed tissue in an agarose gel pellet. The agarose pellet was then dehydrated

through a series of alcohol concentrations (70%, 90% and 100% *v/v*), cleared using xylene and then embedded in paraffin wax. 4 μm paraffin sections were then cut using a Leica RM 2165 microtome and allowed to dry overnight. These were deparaffinised by heating the sections at 60°C for 10 minutes, then passing them through xylene (2 x 5 minutes) and rehydrated through a series of decreasing percentage alcohol solutions. Samples were then subject to the relevant empirical or immunological protocol, dehydrated through increasing alcohol concentrations, and mounted in DPX.

Cell monolayers were prepared for histology by placing a drop of cell suspension on a microscope slide, allowing the cells to attach and develop a typical morphology over several hours, and then fixing for 30 minutes in 10% formalin.

Empirical stains used were Mayer's Haematoxylin and alcoholic eosin 1%, Picrosirius Red; 1mg/ml Sirius red for 1 hour, saturated aqueous picric acid (Junqueira *et al,* 1979), and silver impregnation of reticulin. Immunological studies used various primary antibodies: monoclonal anti-human fibronectin (Sigma IST-3) in a 1:10 dilution, SMA (clone 1A4, 1:250 dilution), GFAP (1:100), desmin (1:10), αBcrystallin (1:250), followed by Dako™ liquid DAB + substrate-chromagen kit used as per manufacturers instructions. All slides were visualised using a Leica DM IRB microscope.

2.2.7 LDH assay of cell viability

The LDH assay was conducted using a commercially available kit (Roche applied science – Cytotoxicity detection kit (LDH)). Supernatant was removed from the cells and centrifuged to remove any cells/spheroids and then refrigerated. Any pelleted cells/spheroids were lysed using 0.5 ml Triton-X 100 (1%) in assay medium that was then transferred to lyse the remaining cells in the culture well. The assay was conducted in a 96 well plate using 100 µl of assay supernatant or cell lysate and 100 µl of freshly prepared Diaphorase, NAD^+, Iodotetrazolium chloride and sodium lactate mixture (from kit). Incubation was 30 minutes. Formazan formation (directly related to LDH content) was measured by detecting absorbance in a plate reader at 490 nm. Percentage cell death was calculated by dividing the LDH activity value from the supernatant by the LDH activity value of the supernatant and remaining cell lysate combined and multiplying by 100.

2.2.8 Live-Dead™ kit assay of cell viability

Live-Dead™ kit (Molecular Probes) was used to further assess cell viability. Five day old spheroids were incubated for 40 minutes at room temperature in hepatocyte culture media containing 4 µM ethidium-homodimer, and 2 µM calcein AM. Spheroids were then washed, re-suspended in fresh media, and visualised using a water immersion lens on a confocal microscope fitted with a 488/568 double dichroic filter, detection at 515 nm and 617 nm.

2.2.9 Transmission Electron Microscopy

Spheroids were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, embedded in 5% (*w/v*) agarose and then fixed in 2% (*v/v*) osmium tetroxide for 2 hours. The agarose-aggregate samples were then dehydrated through a series of alcohols: 50%, 70%, 90% (*v/v*) ethanol (all 2 x 15 minutes), and chemically dried with 100% (*v/v*) ethanol (3 x 30 minutes) and 100% (*v/v*) acetone (2 x 15 minutes). Samples were then set in epon epoxyresin, sliced in to 80 nm sections using a glass knife on a Reichert-Jung Ultracut E microtome and mounted on 100 mesh hexagonal copper grids (TAAB, UK). These were stained with lead citrate and uranyl acetate (saturated solution diluted 1:1 with 100% methanol) and visualised using a Jeol 1010 TEM (80 KV accelerating voltage).

2.3 Results

2.3.1 Light microscopy comparison of cell culture methods

Hepatocytes co-cultured with stellate cells formed spheroids of 100-150 μm diameter over approximately two days that included most cellular material in the culture well (figure 2.1a). The hepatocyte mono-culture control involving trypsin pre-treatment formed similar size aggregates over a similar time period (figure 2.1b), but the initial aggregation was slower than the stellate facilitated aggregation in co-culture. The agitated hepatocyte mono-culture control formed spheroids within 12 hours that were inclusive of most cellular material and were approximately 200 μm or more in diameter (figure 2.1c). Hepatocytes in static mono-culture on a $P_{DL}LA$ surface formed aggregates slowly and inconsistently that were rarely larger than 50-70 μm, and were less inclusive of material in the culture dish (figure 2.1d). Hepatocytes in standard culture on tissue culture plastic attached to the culture surface and flattened to form a typical monolayer (figure 2.1e). The formation and morphology of the co-culture and agitated culture systems are discussed in detail in chapter 5.

2.3.2 Hepatocyte - stellate cell interactions and spheroid formation

Hepatocytes co-cultured with stellate cells on a $P_{DL}LA$ surface formed spheroids of 100-150 μm in diameter over approximately two days. If a gradient of stellate cell density was present spheroids formed only in areas of

high stellate cell concentration with hepatocyte monolayer formation in areas of lower stellate cell density (figure 2.2).

The lowest magnification hepatocyte-hepatic stellate cell co-culture time lapse movie gives a temporal overview of the course of aggregation and spheroid formation (Figure 2.3, Time lapse 1). The process occurred in several distinct phases, each of which is annotated on the time-lapse movies. The first 12 hour period of culture was characterised by low cell motility and some hepatocyte spreading. This was followed by 12 hours of relatively high cell motility in which rapid stellate cell contraction led to aggregate formation. A further 12 hours of consolidation occurred in which the outlines of the spheroids became smooth and distinct, and the remaining cells were amalgamated. After this the spheroids maintained a stable morphology.

The highest magnification hepatocyte-hepatic stellate cell co-culture time lapse movie provides a detailed view of the heterotypic cell interactions (Figure 2.4, Time lapse 2). Stellate cells rapidly developed the normal culture activated multiple process morphology (Sato *et al,* 2003) in the first few hours of culture. Throughout the first 8 hours of culture, despite hepatocyte contact, contraction to aggregates did not occur. After the first 8 hours of culture the stellate processes rapidly retracted creating co-culture aggregates. After 20-24 hours the aggregates underwent some consolidation. The tension developed in the stellate cell contractile response can be seen at the beginning of this period (in the top left of the movie) as a spheroid is literally catapulted off screen by a stellate cell process. This movie clearly shows that the stellate cells were highly motile and

the hepatocytes immobile except under the active contractile control of the stellate cells.

The stellate cells did not display the same level of motility or retract their processes in the same manner when mono-cultured on a $P_{DL}LA$ surface (Figure 2.5, Time lapse 3). However they did retract their processes rapidly in response to hepatocyte conditioned media (Figure 2.6, Time lapse 4). Also, stellate cells exhibited the same contractile response to hepatocyte fragments as whole cells (Figure 2.7, Time lapse 5). This aggregation occurred as soon as the cell fragments were added. Stellate cells partially aggregated epithelial liver cell line Hep G2 cells but slower and with formation of less well defined aggregates than primary hepatocytes (Figure 2.8, Time lapse 6). Stellate cells failed to aggregate epithelial pulmonary cell line A549 cells (not shown).

Hepatocytes were static and did not adhere to the $P_{DL}LA$ culture surface or form aggregates when mono-cultured on $P_{DL}LA$ (Figure 2.9, Time lapse 7).

2.3.3 Stellate cell characterisitics in vitro and their distribution in co-culture spheroids

A study of the stellate cell population in mono-culture conducted after a period of approximately 3 weeks in vitro, immediately prior to incorporation into co-culture, defined some of the immunological characteristics of the stellate cells involved in spheroid formation. The presence of GFAP (figure 2.10) and desmin (figure 2.11) confirmed the identity of the stellate cells (Cassiman *et al,*

2002). Heat shock protein αB-Crystallin (figure 2.12) and fibronectin (figure 2.13) were also detected and are reportedly expressed by activated stellate cells in vitro (Cassiman *et al,* 2001). Detection of SMA filaments (figure 2.14) and SEM (figure 2.15) demonstrated the cells typical contractile multi-process activated morphology (Sato *et al,* 2003). After 5 days in co-culture the stellate cells were identified in spheroid sections by immunolocalisation of SMA. They were located mainly around the periphery and centre of the spheroids (figure 2.16). In some smaller spheroids a single central stellate cell was seen.

2.3.4 Morphology and ultra structure of hepatocyte – stellate cell co-culture spheroids and shaken hepatocyte mono-culture spheroids

Histology techniques and TEM were used to investigate the structure of spheroids formed by hepatocyte – stellate cell co-culture compared with hepatocyte only spheroids formed by agitated culture. Haematoxylin and eosin staining showed healthy looking nuclei evenly distributed throughout the co-culture spheroid, a distinct barrier outline, and a compact structure (figure 2.17). Staining with haematoxylin alone highlighted the high density of cell nuclei in co-culture and elongation of nuclei on the edge of the spheroids (figure 2.17 inset). The co-culture spheroids had a complex ECM support: Picro Sirius red staining demonstrated a widespread fibrous collagen matrix (figure 2.18) and silver impregnation of reticulin detected thick fibre deposition, although this was harder to discern due to nuclear staining (figure 2.19). In addition, immunolocalisation of fibronectin revealed a capsule on the periphery of the co-culture up to 10 μm thick (figure 2.20). All of these ECM types are widely distributed in normal rat liver sections (*inset* figures 2.18-2.20).

In contrast to the co-culture spheroids, the agitated mono-culture spheroids had a lower density of nuclei and occasionally non-viable centres. They had a total absence of fibrous collagen matrix (figure 2.21a) and reticulin fibres (figure 2.21b), and lacked a peripheral fibronectin capsule (figure 2.21c).

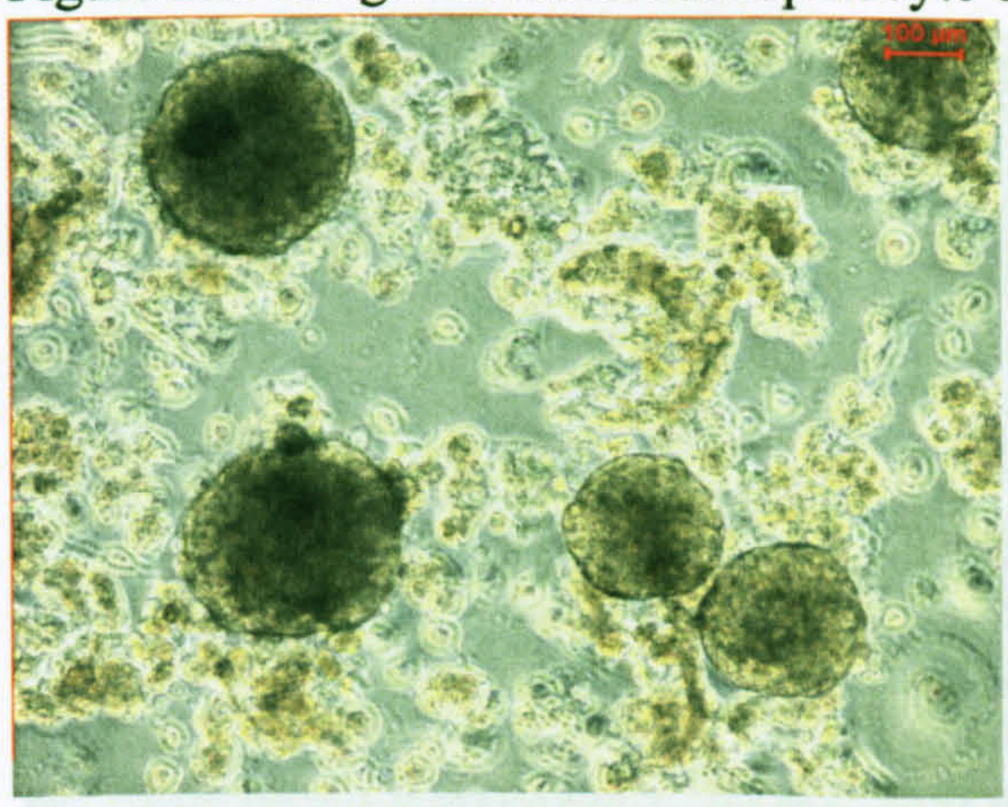
Further support that co-culture spheroids had the capacity to form and maintain hepatic architecture was provided by TEM, which showed a well-developed hepatic ultra structure, including bile canaliculi, tight junctions, desmosomes, fat storage and glycogen storage (figure 2.22a). None of these features were observed in monoculture spheroids. A myosin containing stellate cell was identified near the periphery of one spheroid section, forming multiple junctions with the adjacent hepatocyte (figure 2.22b).

2.3.5 Viability of the hepatocyte – stellate cell co-culture spheroid

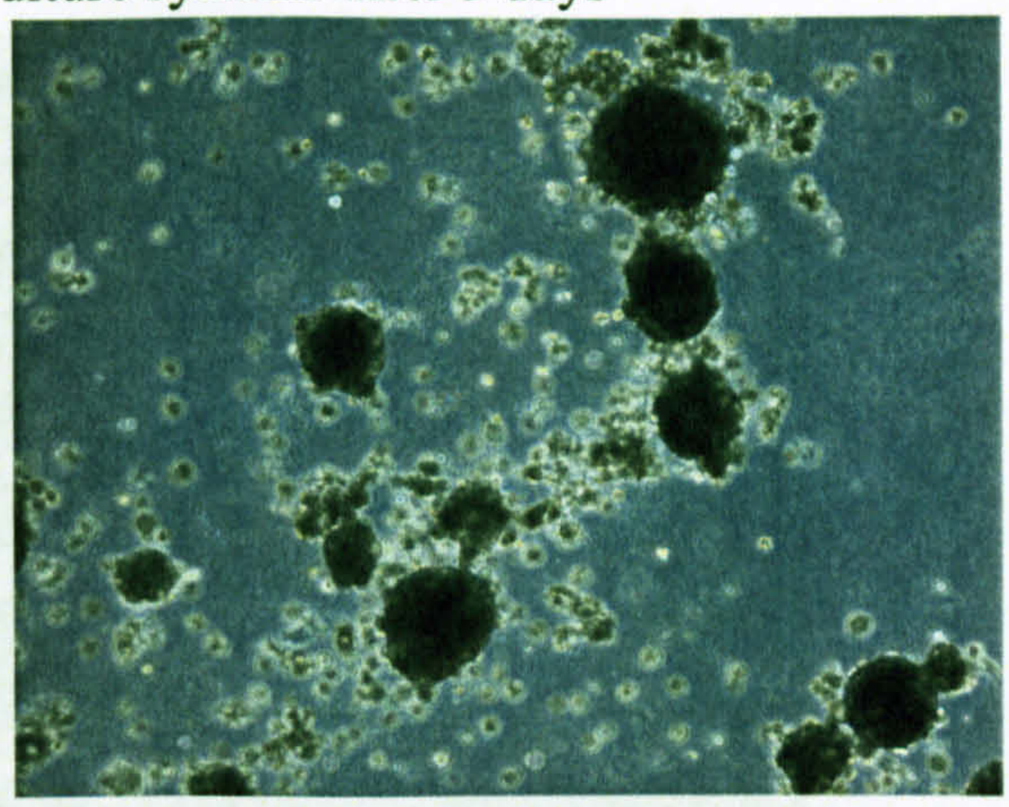
A study of the viability of the co-culture spheroids relative to cells in mono-culture demonstrated a stabilisation of cell viability after spheroid formation in co-culture (figure 2.23a). Between 24 and 48 hours of culture, cell death assessed by LDH leakage was approximately 10% in both mono-culture and co-culture. However, over the subsequent 3 days cell death was 30% of remaining cells in co-culture compared to 58% in mono-culture. A similar benefit of 24% cell death in co-culture to 49% in mono-culture was maintained over the following two days. Absolute levels of LDH leakage were also higher in mono-culture relative to co-culture showing this result was not a distortion of the

leaked to lysed LDH ratio due to an increasing stable stellate cell population in co-culture (figure 2.23b). This viability data was supported by confocal microscopy of Live-Dead™ treated co-culture spheroids after 5 days in culture that showed spheroids were viable throughout (figure 2.24).

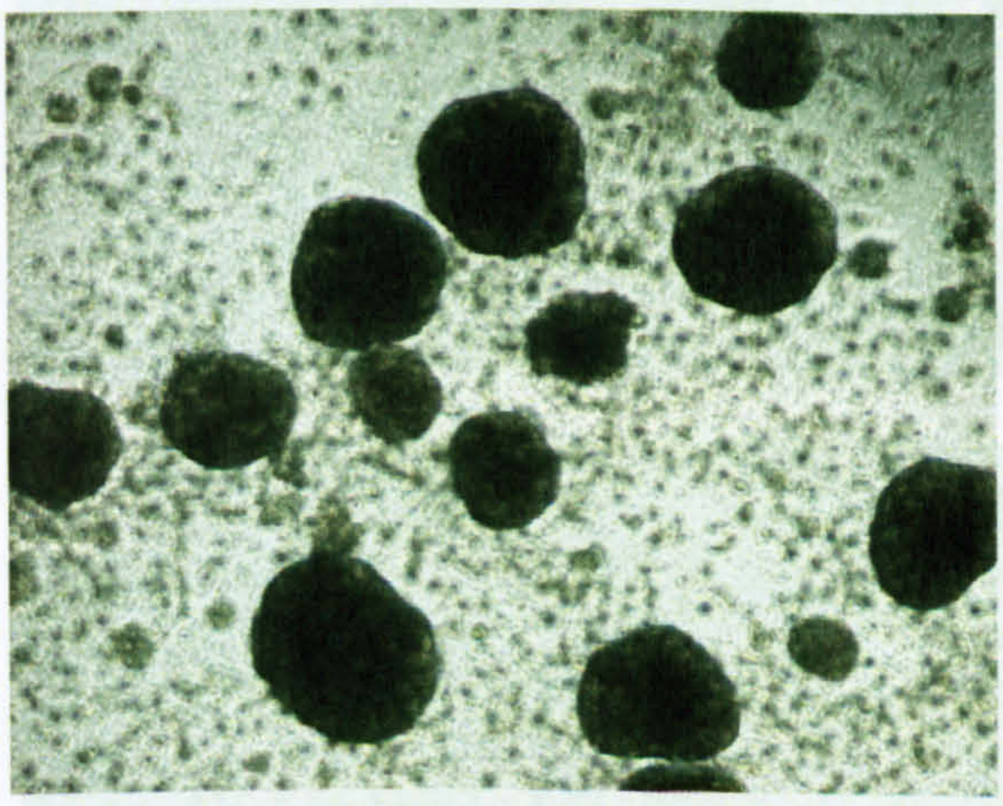
Figure 2.1: Images of different hepatocyte culture systems after 5 days



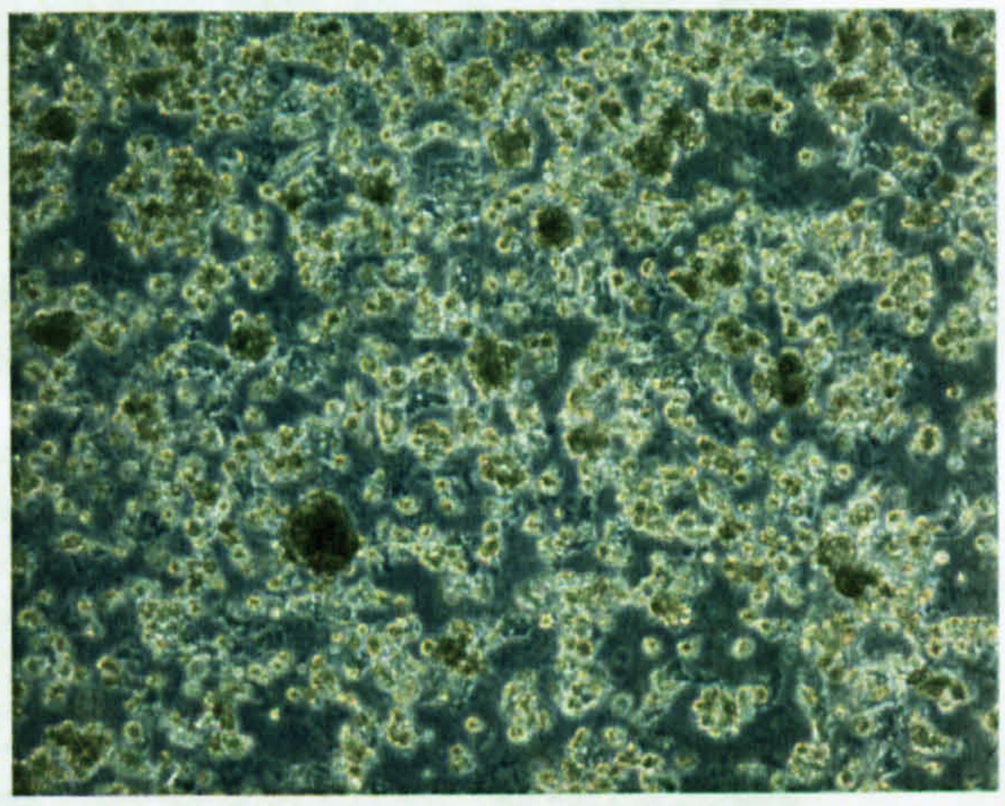
(a) Co-culture of hepatocytes and stellate cells on a $P_{DL}LA$ surface



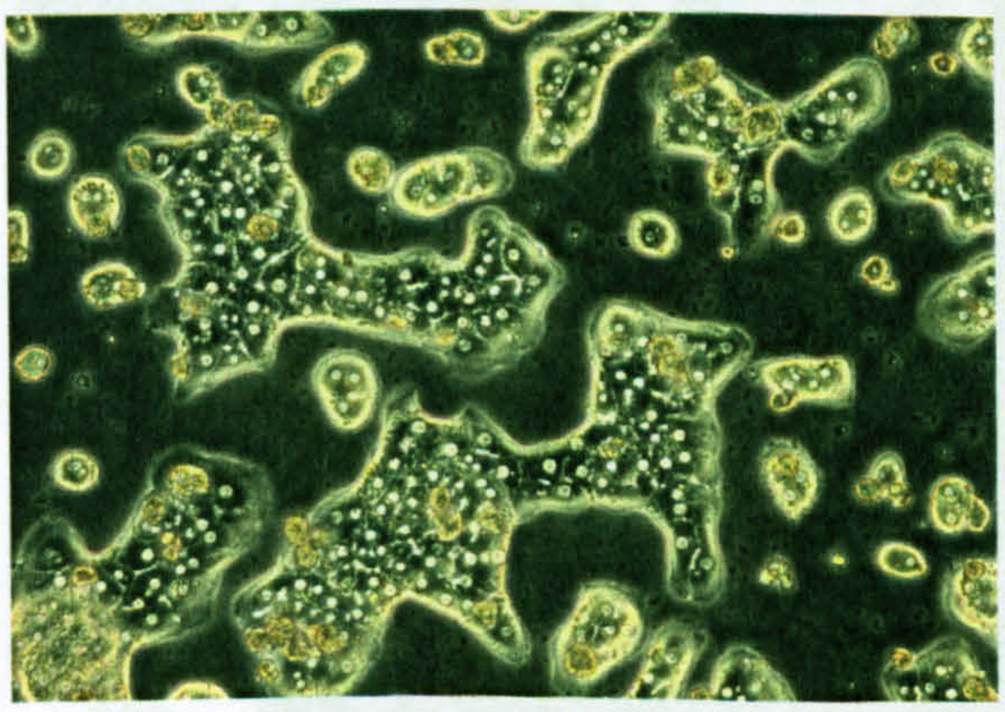
(b) Mono-culture of trypsin pre-treated hepatocytes on TCP



(c) Mono-culture of agitated hepatocytes on TCP



(d) Mono-culture of hepatocytes on a $P_{DL}LA$ coated surface



(e) Mono-culture of hepatocytes mono-culture on TCP

Figure 2.2

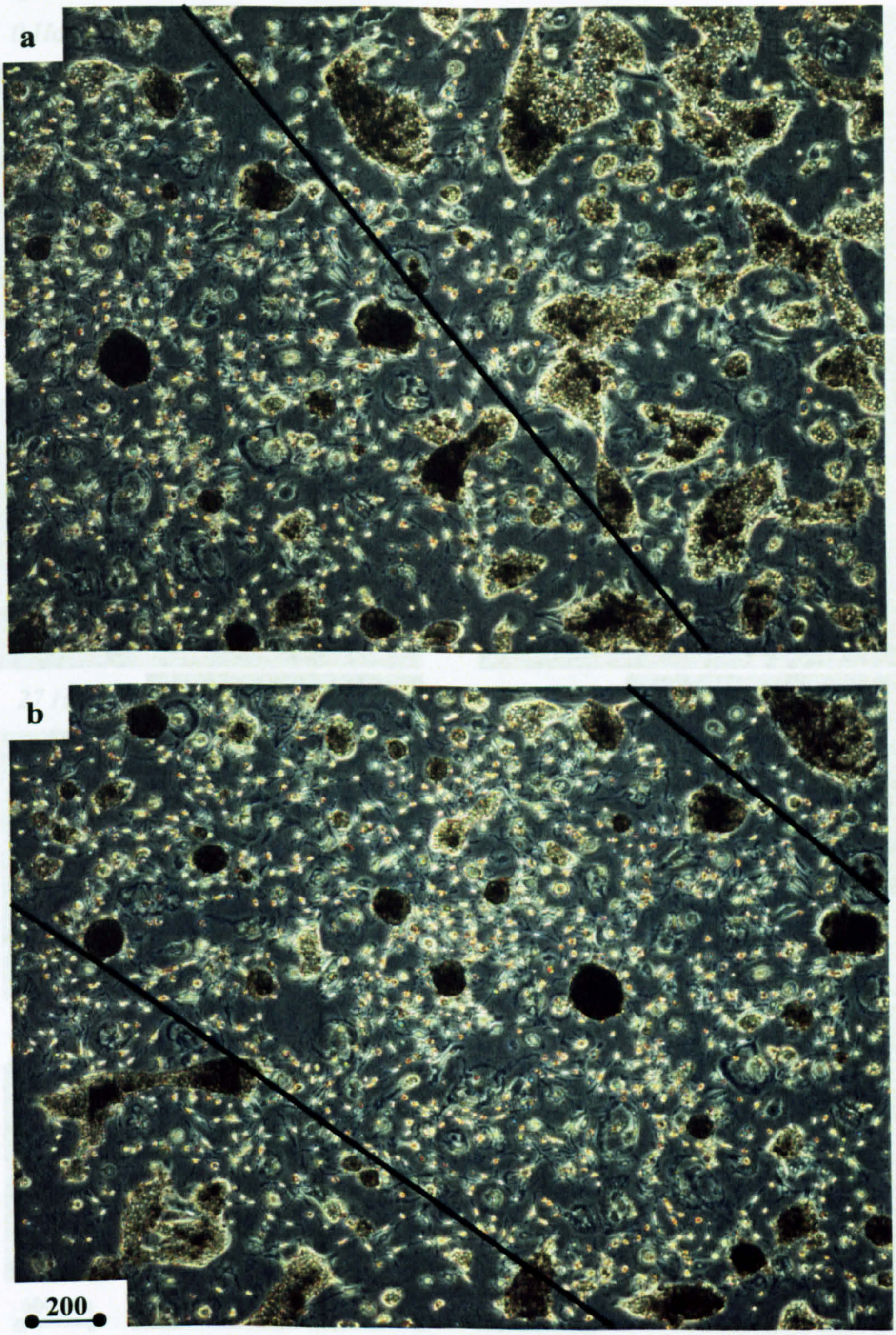


Figure 2.2: Images of hepatocyte and stellate cell co-culture on a $P_{DL}LA$ surface. Spheroid formation occurred only in areas of high stellate cell density (marked to the left of the line in image (a) and in between the lines in image (b)). Outside these areas, where stellate cell density was lower, hepatocytes adhered to the culture surface.

Figure 2.3

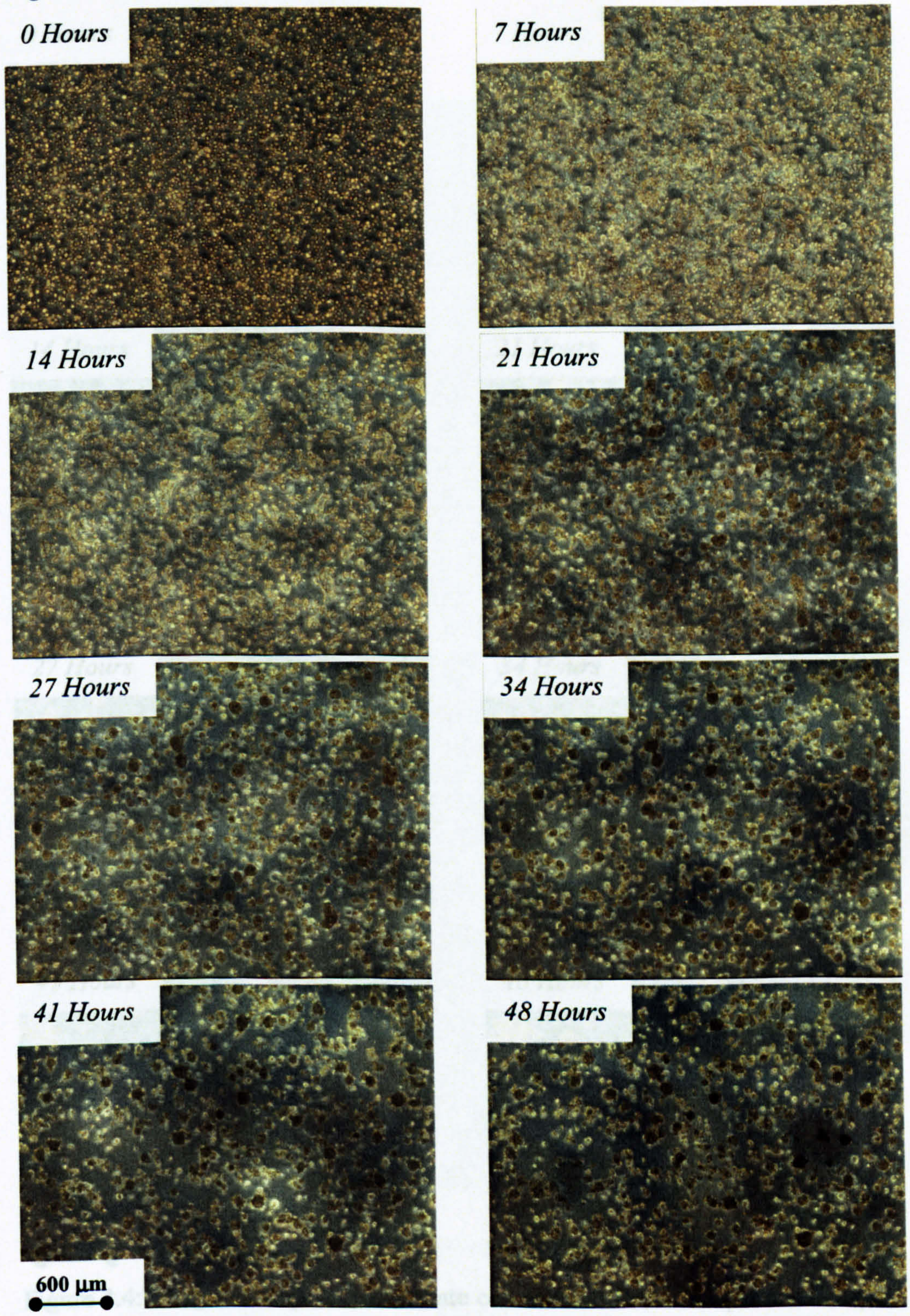


Figure 2.3: Images of hepatocyte-stellate cell co-culture (time lapse movie 1) on a $P_{DL}LA$ surface showing the time course of formation of spheroids. Stellate cell motility, initially low, increased with time in culture with hepatocytes. Spheroid formation occurred rapidly in an approximately 6 hour window in the region of 15 hours into co-culture. After this time spheroids were stable.

Figure 2.4

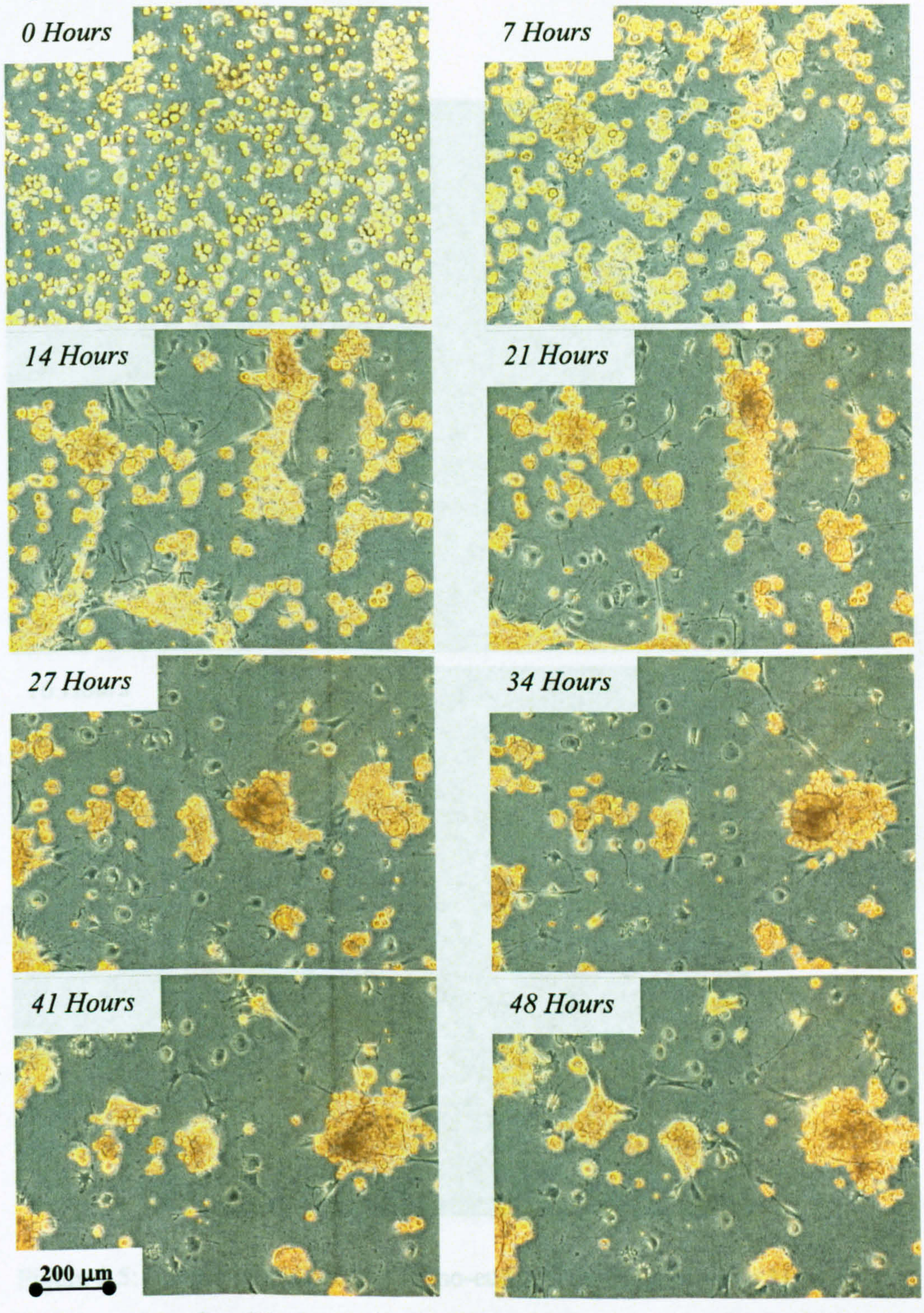


Figure 2.4: Images of hepatocyte-stellate cell co-culture (time lapse movie 2) on a $P_{DL}LA$ surface showing that stellate cell processes contracted when contacted by hepatocytes. Motility and contractility increased with time in culture. This is demonstrated in the top left of the movie after 21 hours when a newly formed spheroid under tension from a contracting stellate cell process is pulled off screen.

Figure 2.5

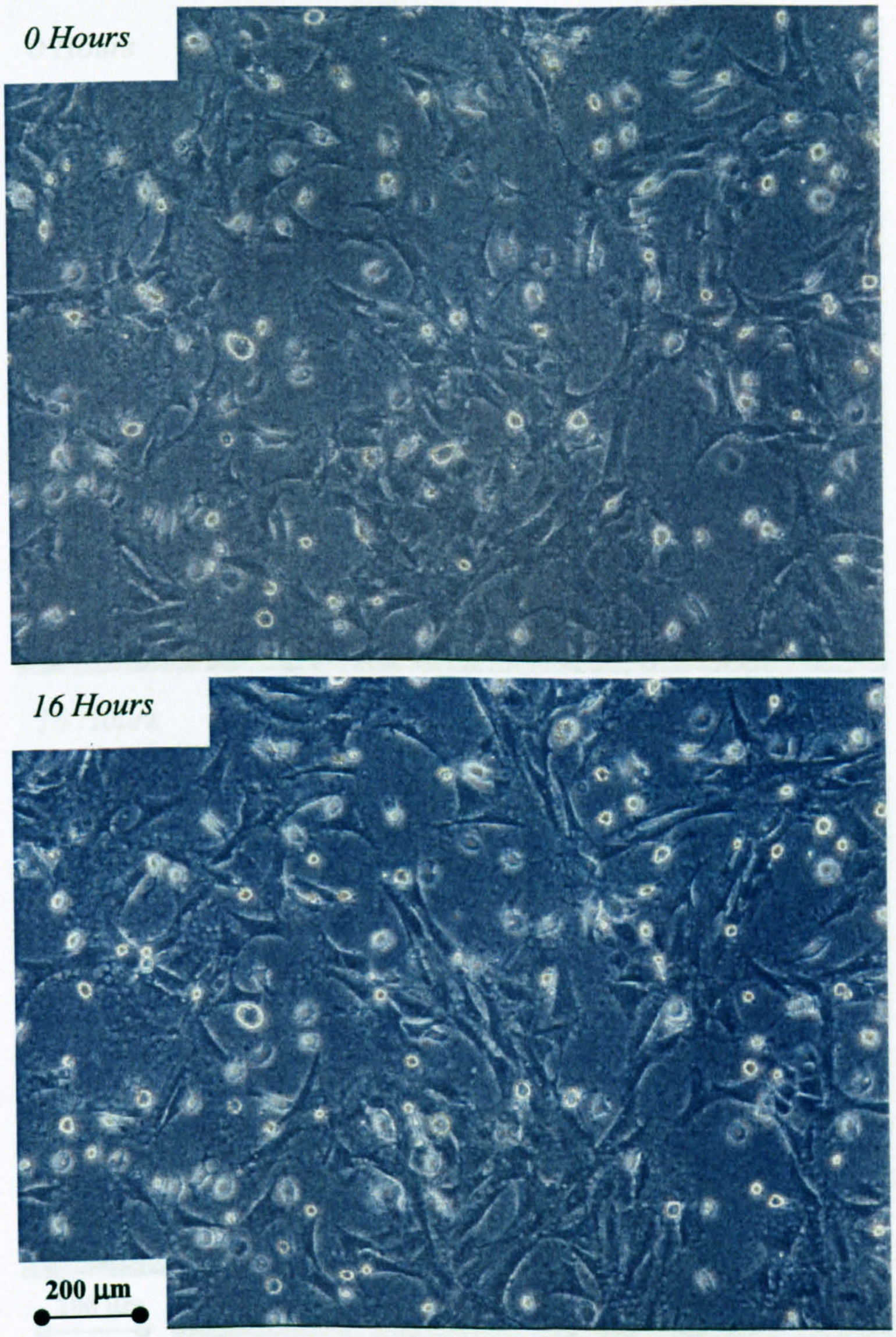


Figure 2.5: Images of stellate cell mono-culture (time lapse movie 3) on a $P_{DL}LA$ surface showing that activated stellate cell processes did not contract, and stellate cell motility was relatively low, in mono-culture after a similar period in culture to that at which maximum motility was observed in co-culture.

Figure 2.6

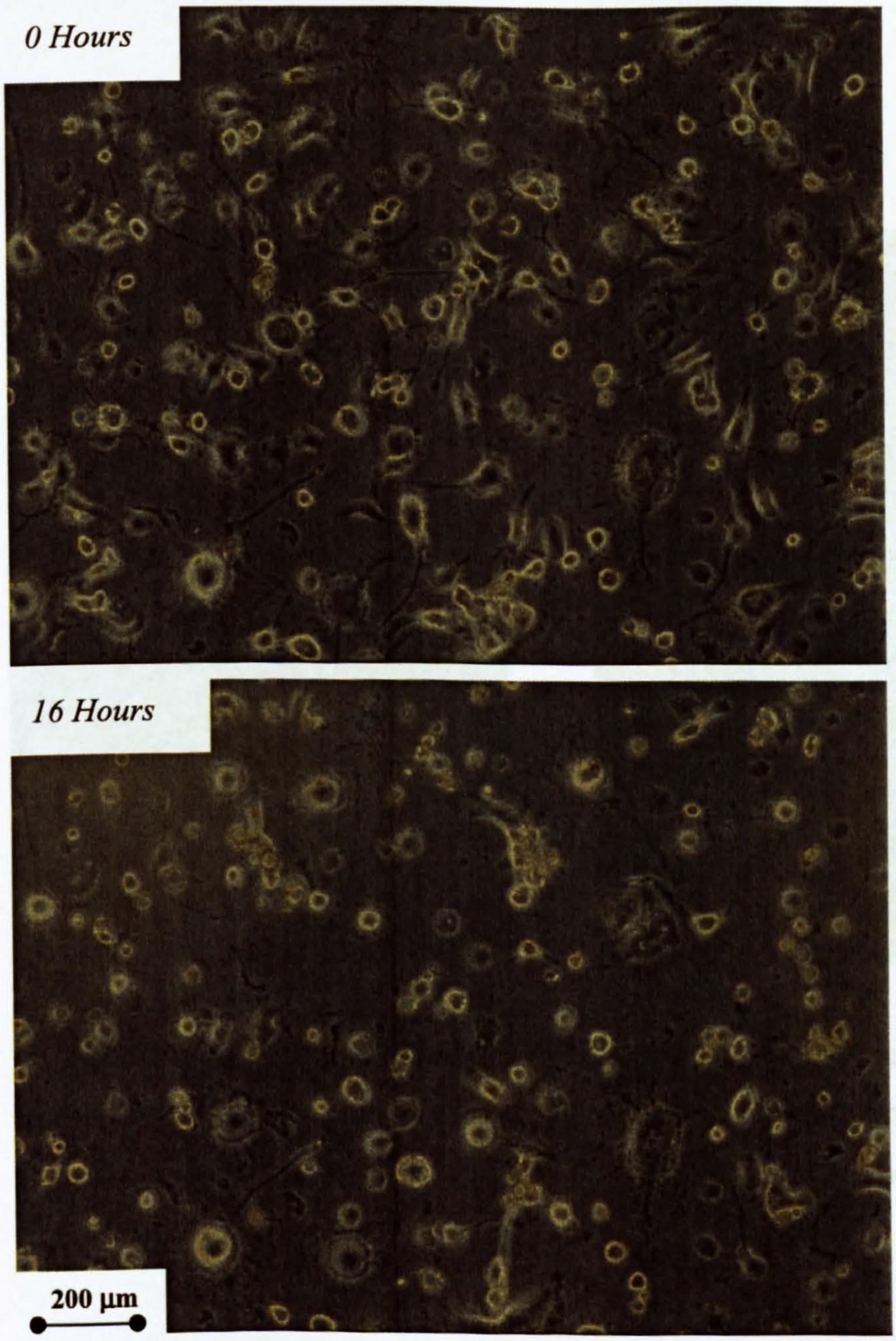


Figure 2.6: Images of stellate cell mono-culture (time lapse movie 4) on a $P_{DL}LA$ surface showing that activated stellate cells in mono-culture rapidly retracted processes in response to hepatocyte conditioned media (added immediately prior to 0 hours image).

Figure 2.7

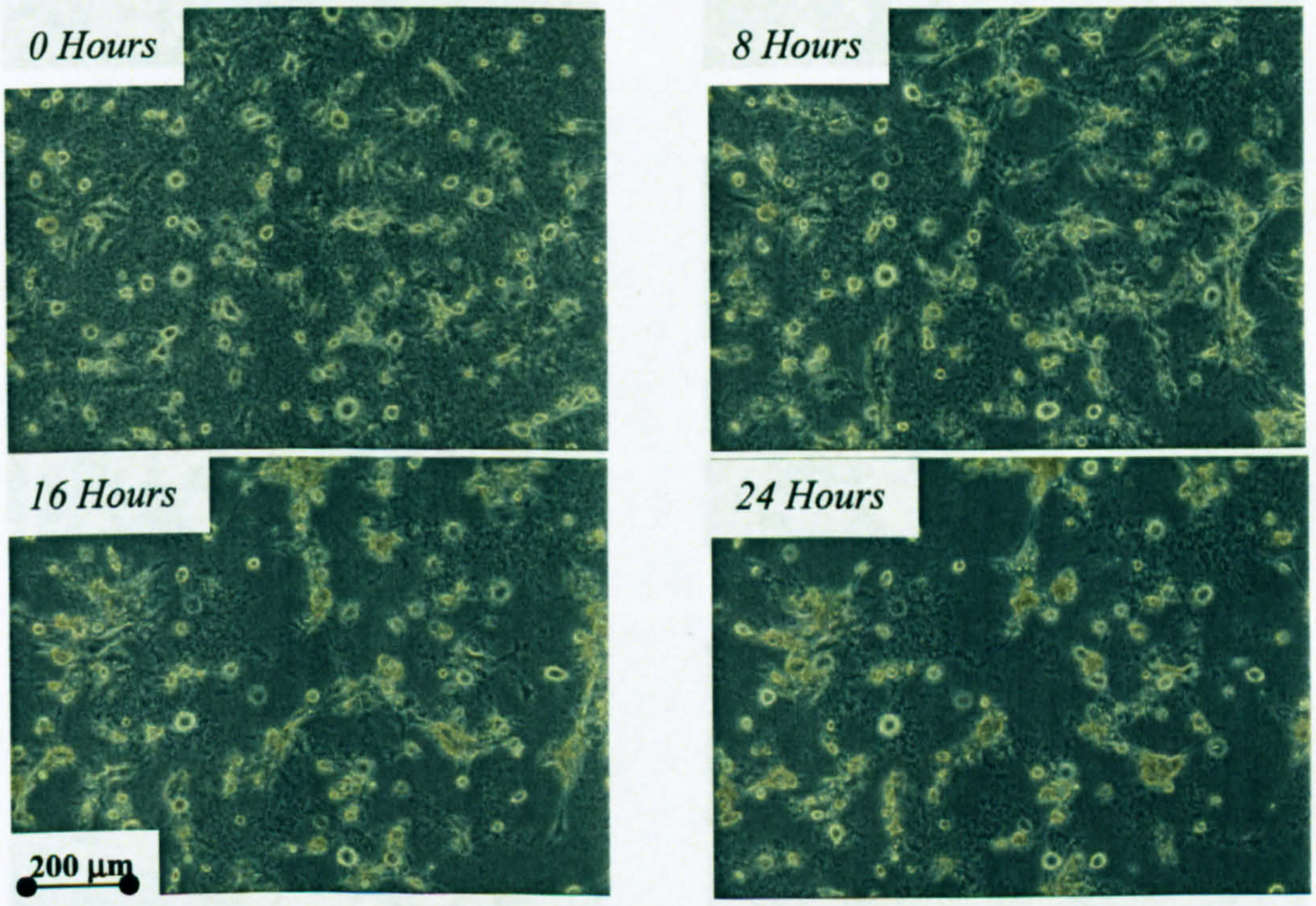


Figure 2.7: Images of stellate cell mono-culture (time lapse movie 5) on a $P_{DL}LA$ surface showing that activated stellate cell processes contracted in response to hepatocyte fragments (added immediately prior to 0 hours image).

Figure 2.8

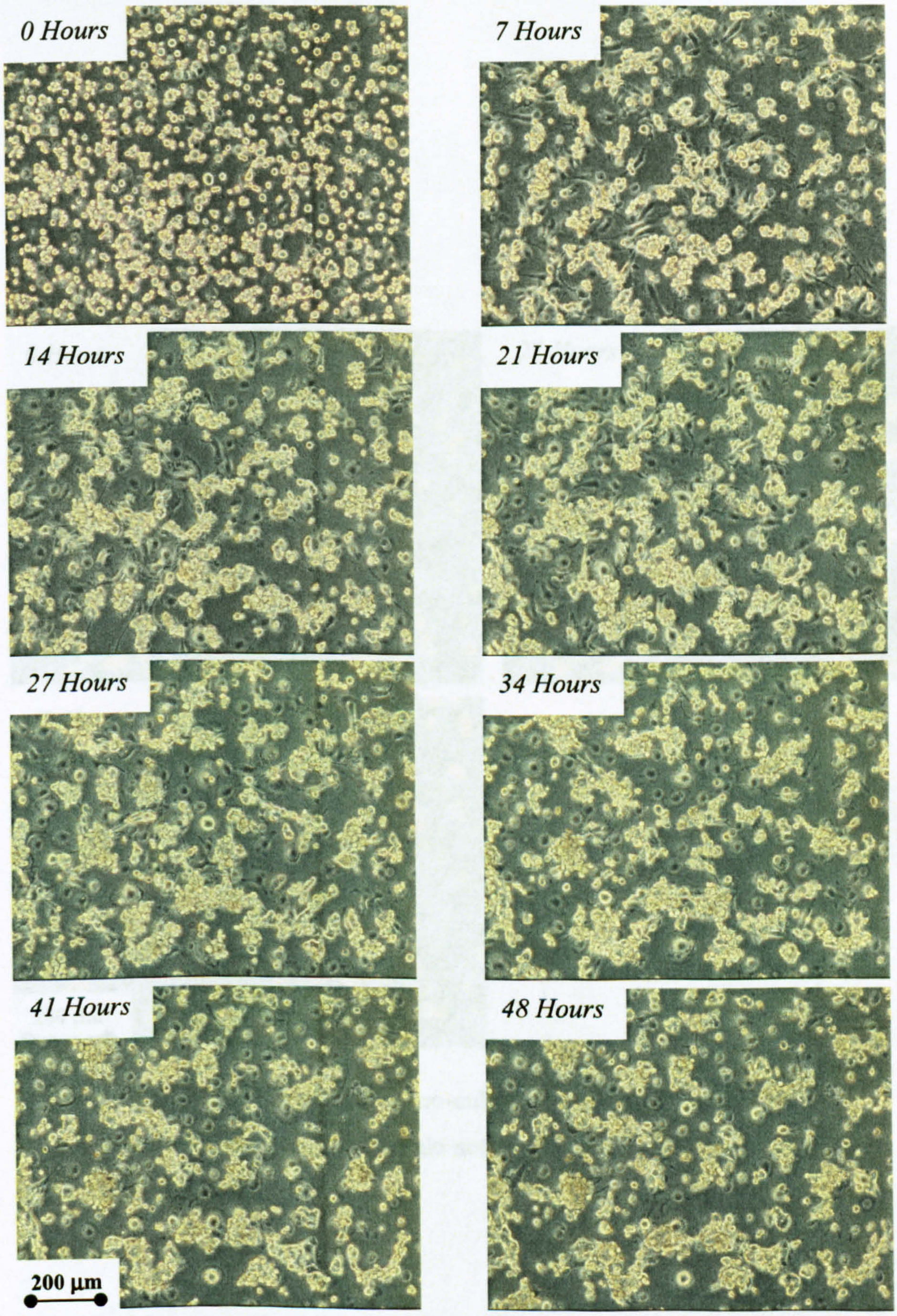


Figure 2.8: Images of primary stellate cell and Hep G2 cell line co-culture (time lapse movie 6) on a $P_{DL}LA$ surface showing activated stellate cells do not aggregate Hep G2 cells in the same manner as freshly isolated primary hepatocytes.

Figure 2.9

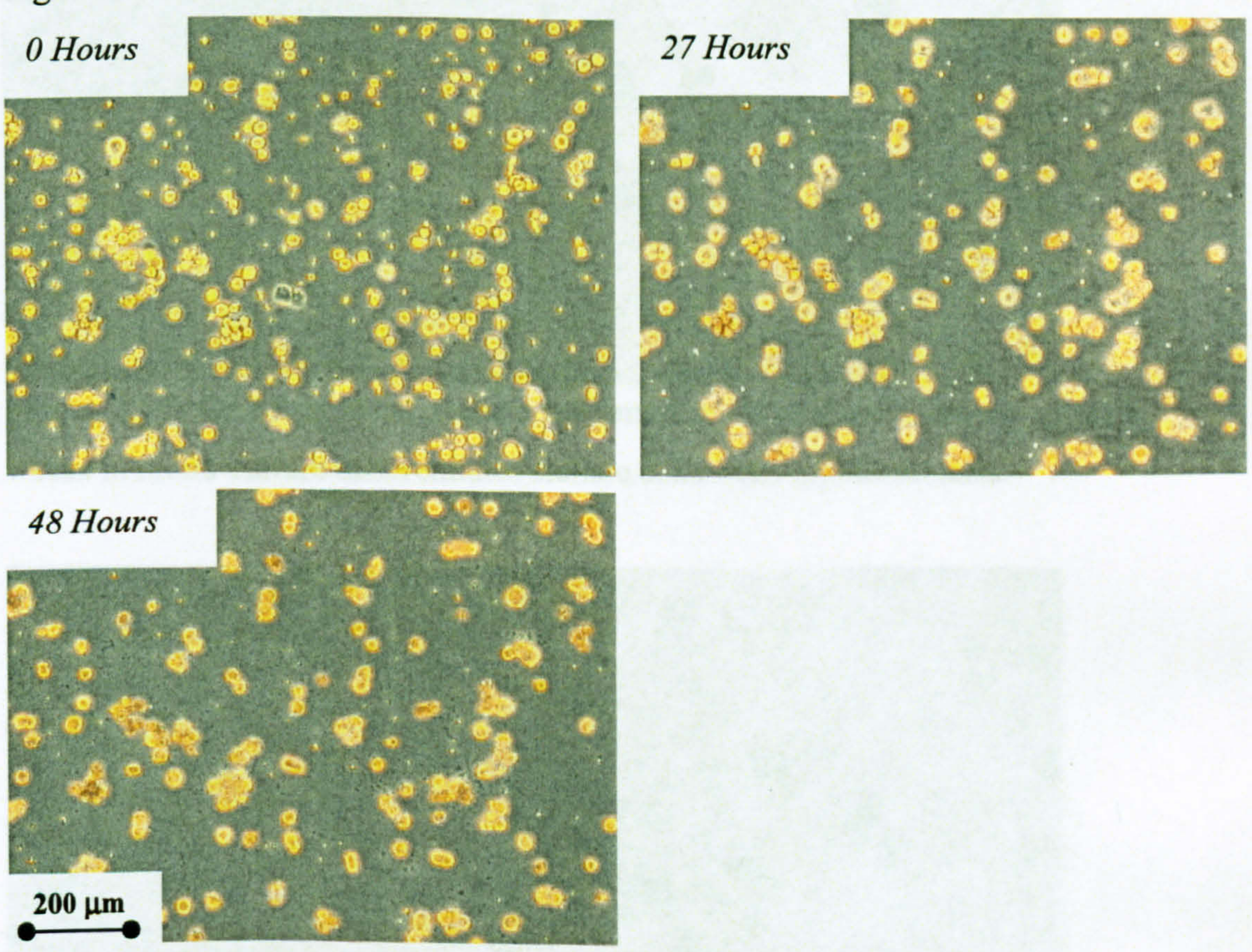


Figure 2.9: Images of hepatocyte mono-culture (time lapse movie 5) on a $P_{DL}LA$ surface showing hepatocytes do not aggregate or attach to the surface under these conditions.

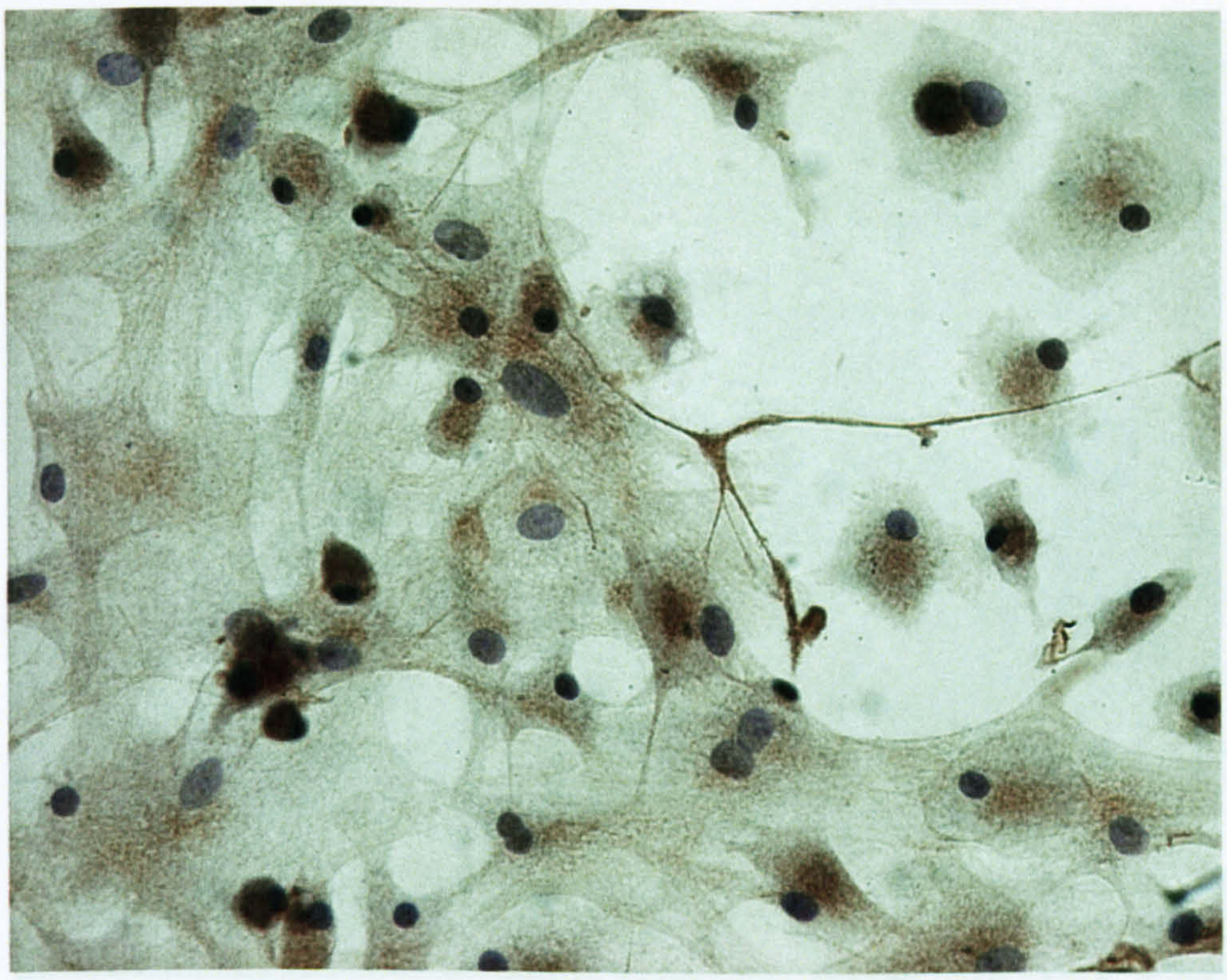


Figure 2.10: GFAP is a rat stellate cell marker. Immunolocalisation after 3 weeks in mono-culture differentiates stellate cells from myofibroblasts.

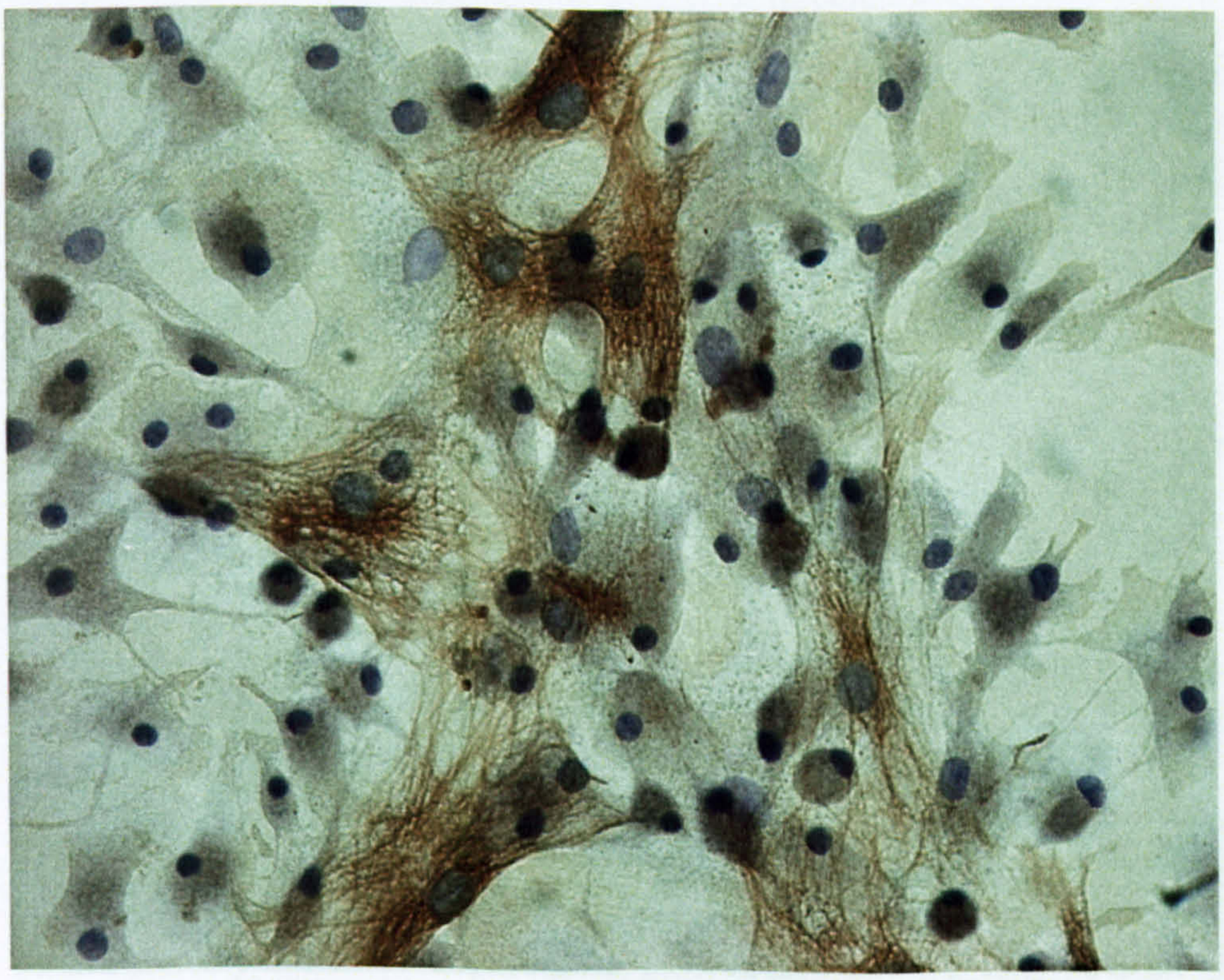


Figure 2.11: Desmin is a rat stellate cell marker. Immunolocalisation after 3 weeks in mono-culture differentiates stellate cells from myofibroblasts.

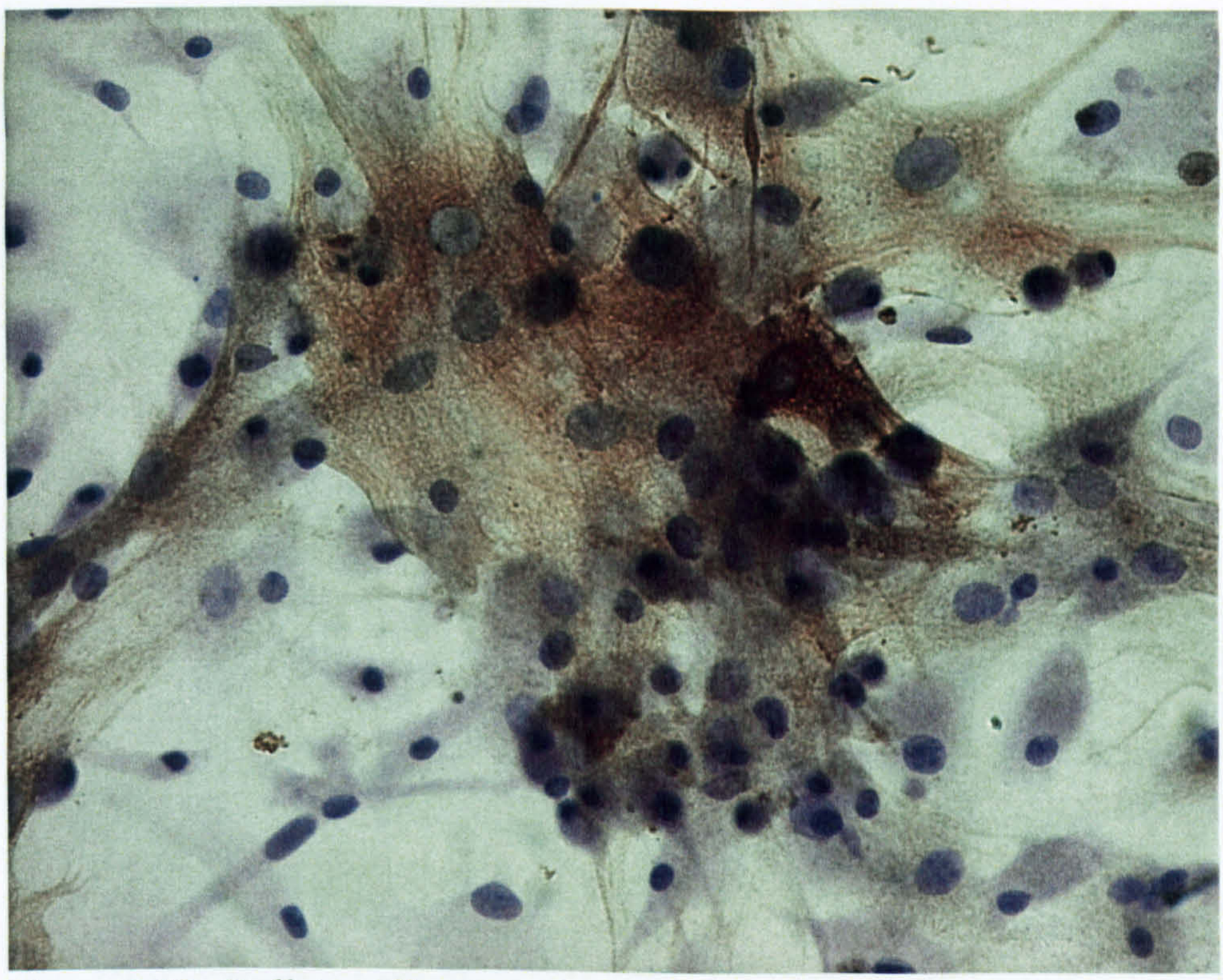


Figure 2.12: Stellate cells are an important source of ECM. This image shows fibronectin immunolocalisation in rat stellate cells after 3 weeks in vitro.

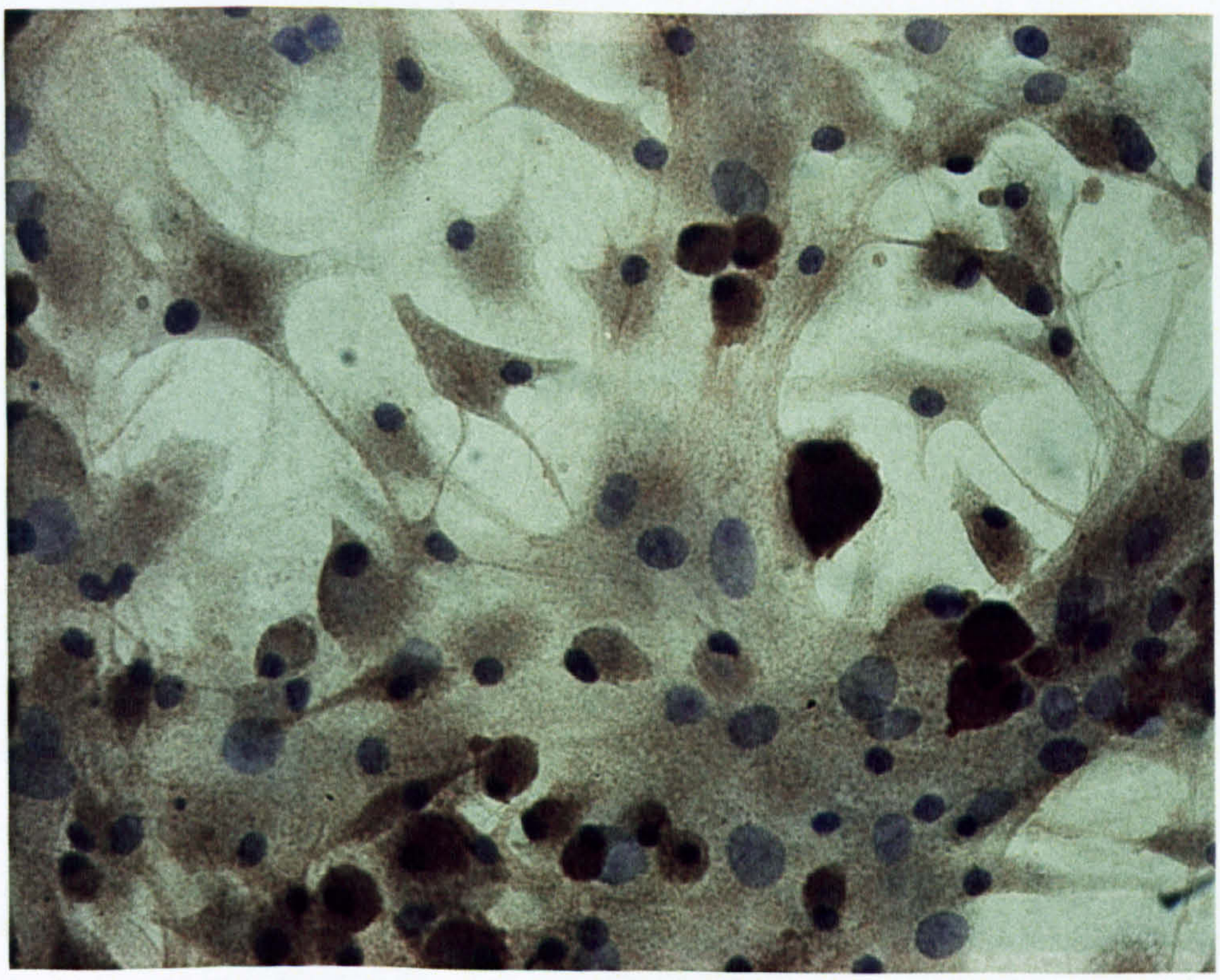


Figure 2.13: Stellate cells are known to express αB-Crystallin. Immunolocalisation in rat stellate cells after 3 weeks in mono-culture is an indicator of activated phenotype.

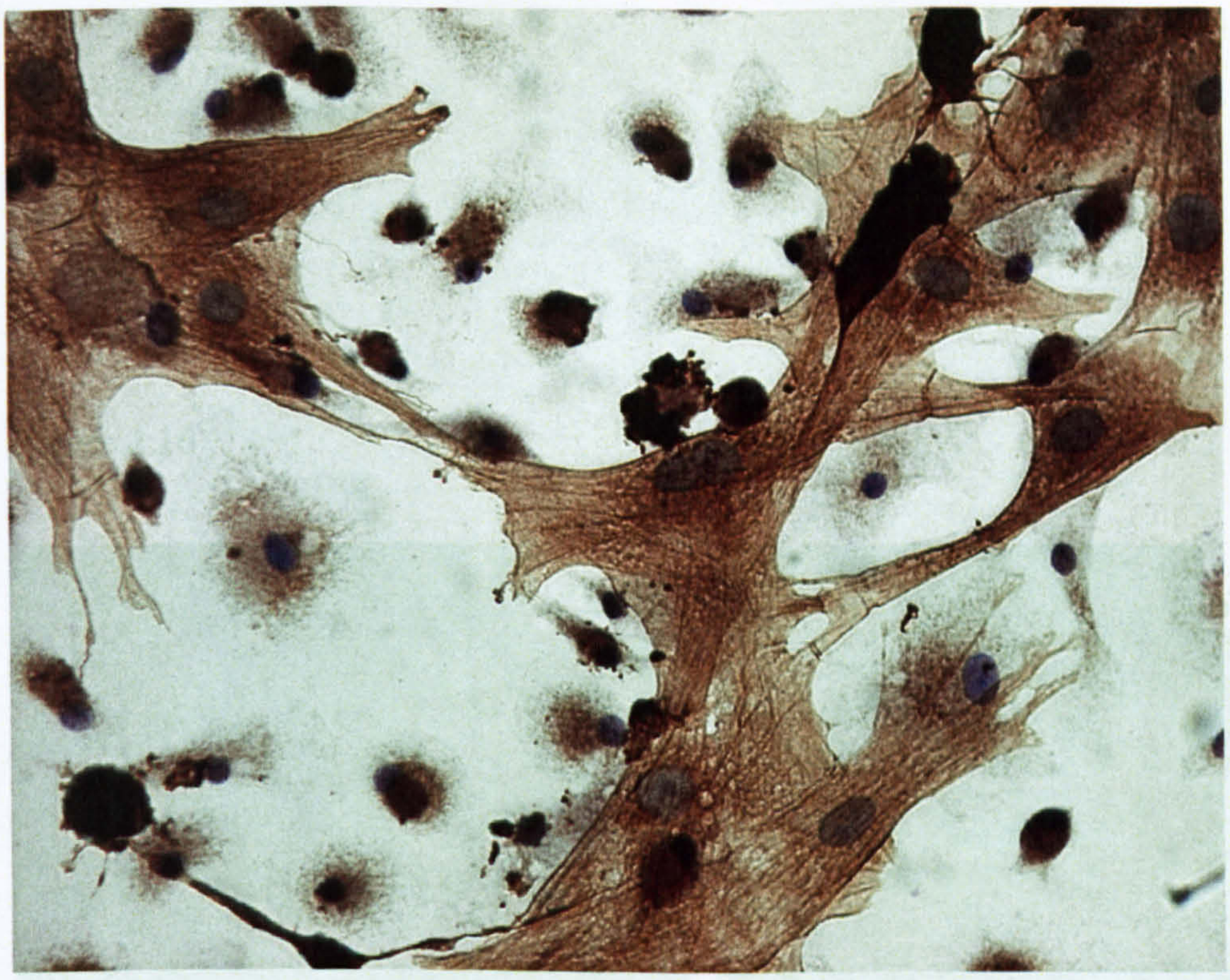


Figure 2.14: Activated stellate cells express SMA. Immunolocalisation in rat stellate cells after 3 weeks in culture is evidence of the contractile morphology of the cells at this time.

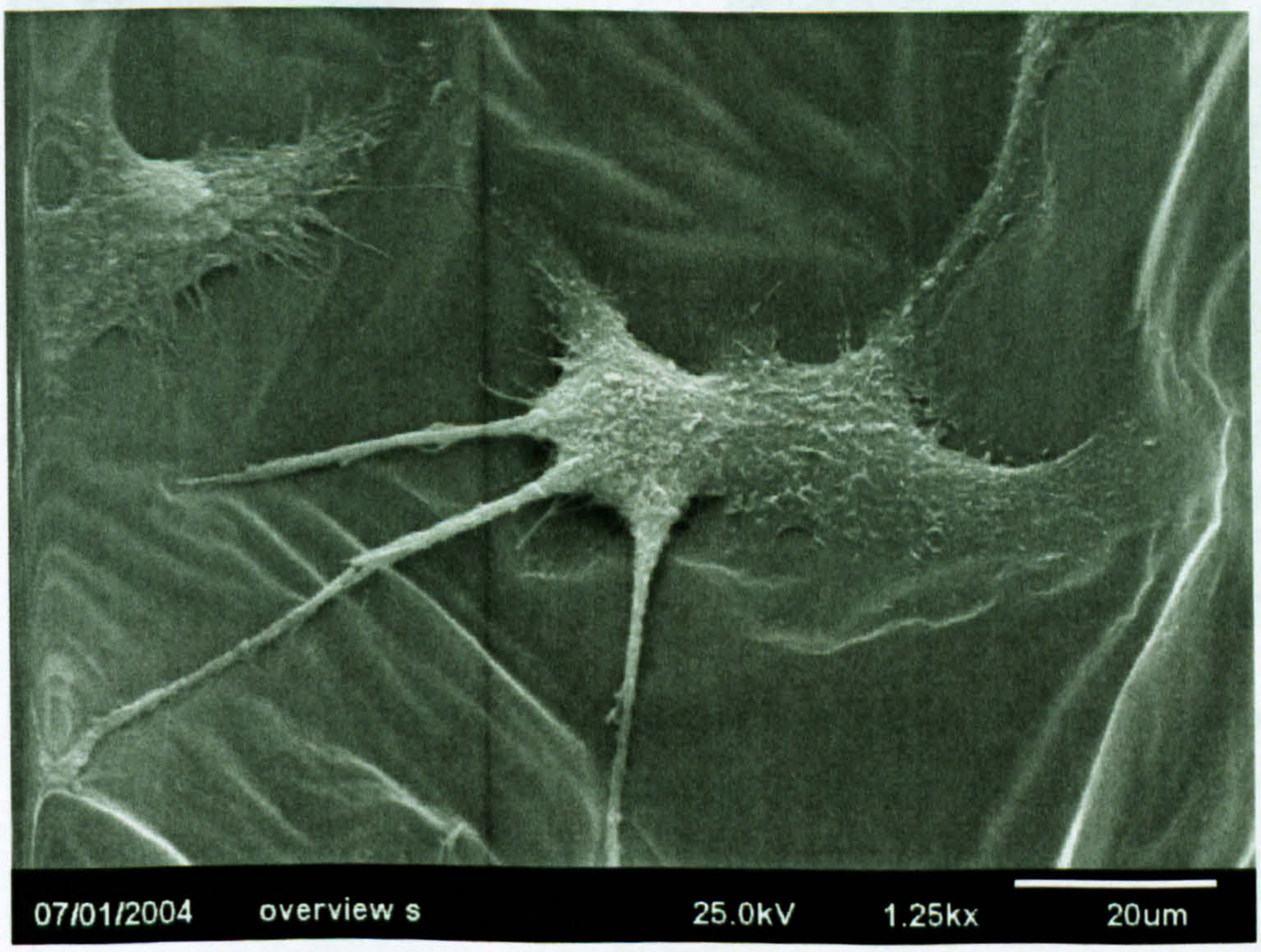


Figure 2.15: An SEM image of a rat stellate cell showing activated morphology after 3 weeks in mono-culture. This is typical of the stellate cell morphology prior to co-culture with hepatocytes.

Figure 2.16

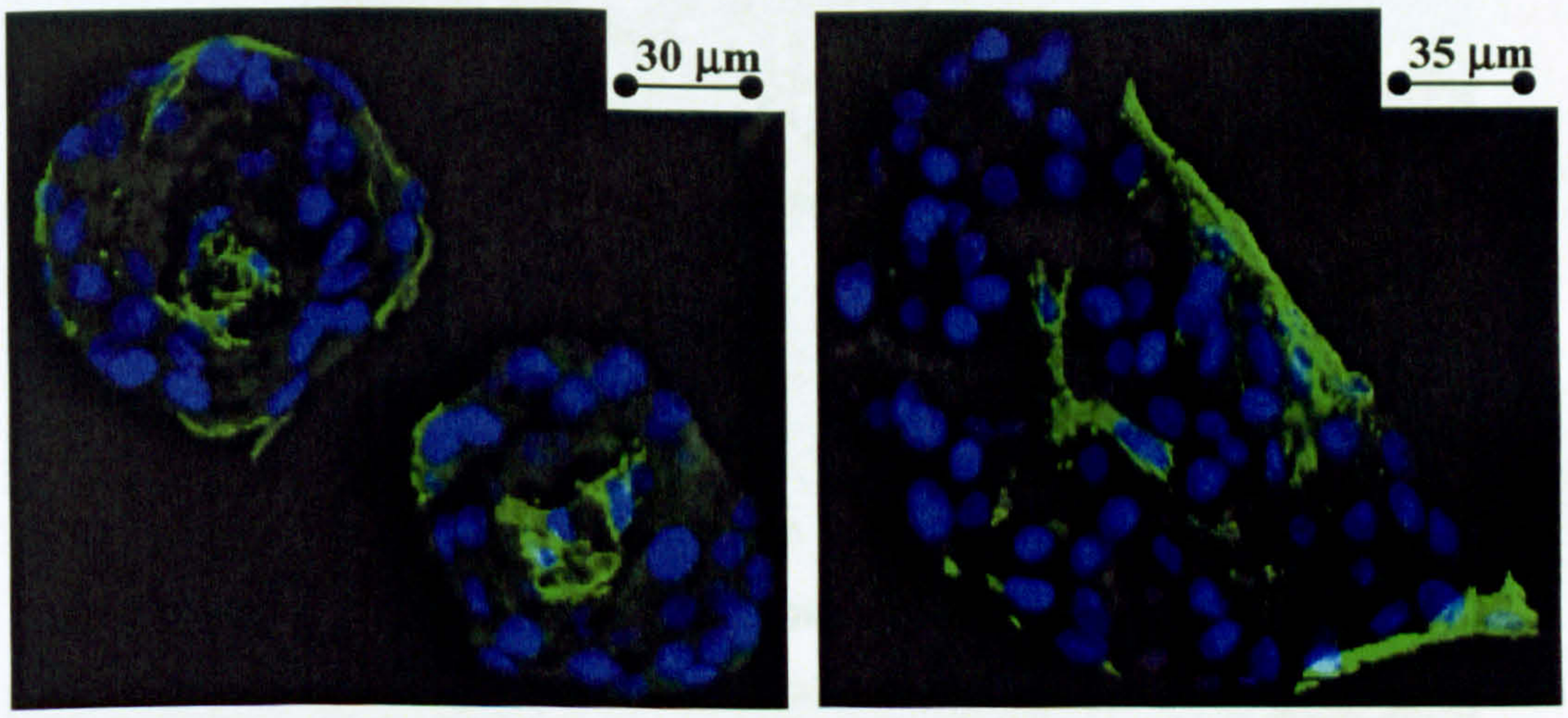


Figure 2.16: Images of SMA (green) immunolocalisation in spheroid sections prepared after 5 days of co-culture showing the arrangement of stellate cells around the periphery and in the centre of hepatocyte-stellate cell co-culture spheroids. Nucleus counterstained with DAPI (blue).

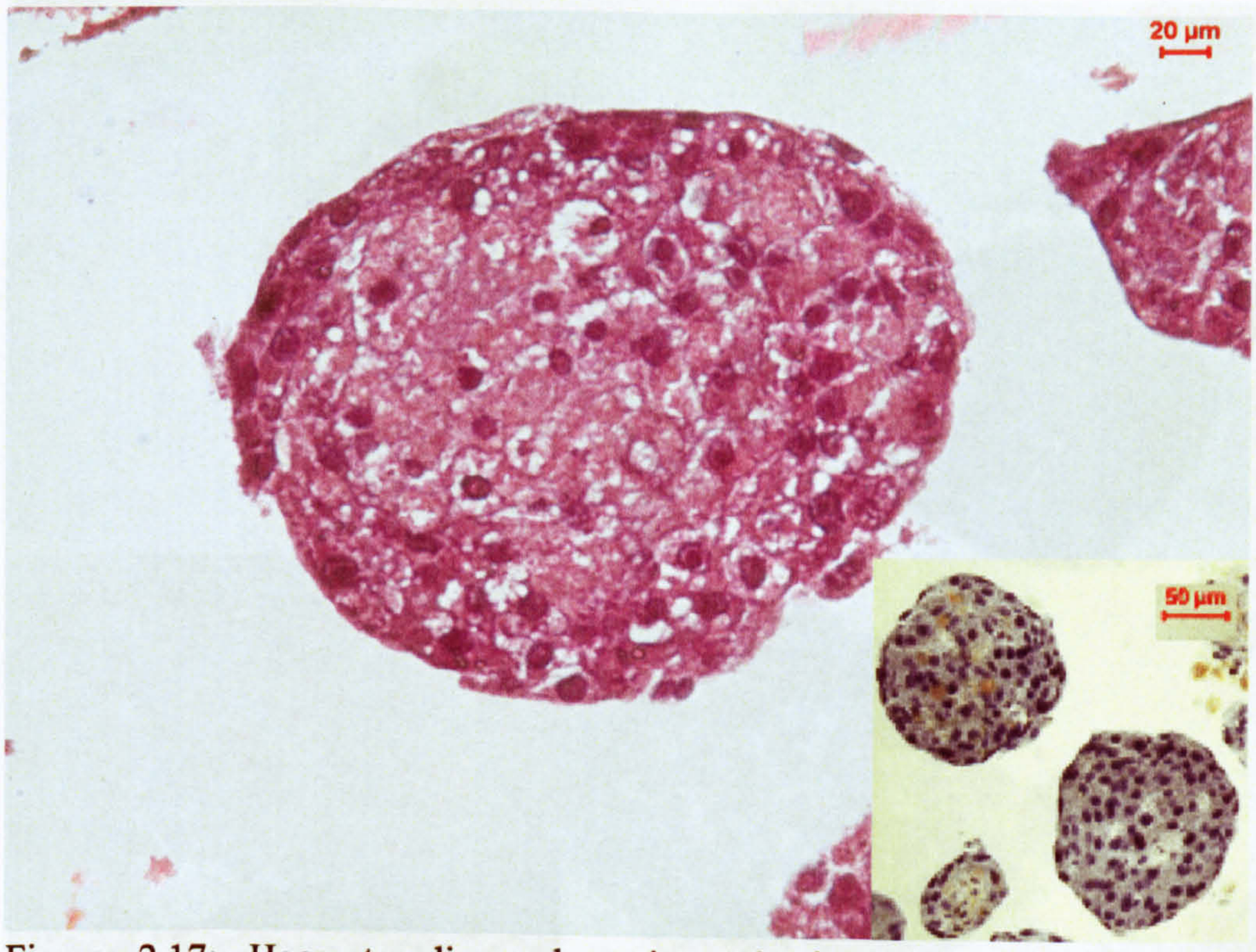


Figure 2.17: Haemotoxylin and eosin stained sections from 5-day-old hepatocyte-stellate cell spheroids shows viability and compact morphology.

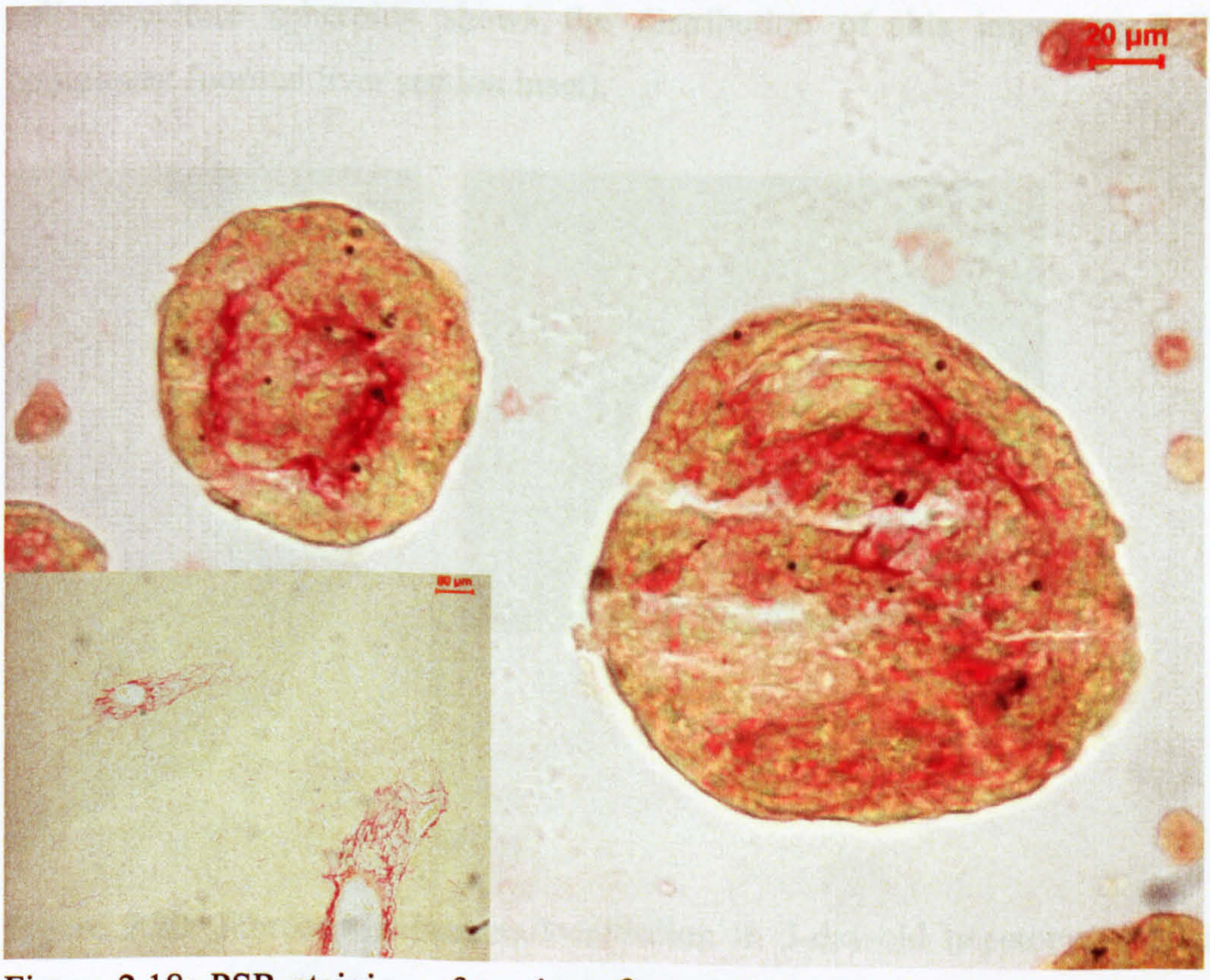


Figure 2.18: PSR staining of sections from 5-day-old hepatocyte-stellate cell co-culture spheroids shows the distribution of this important ECM component (normal liver section inset).

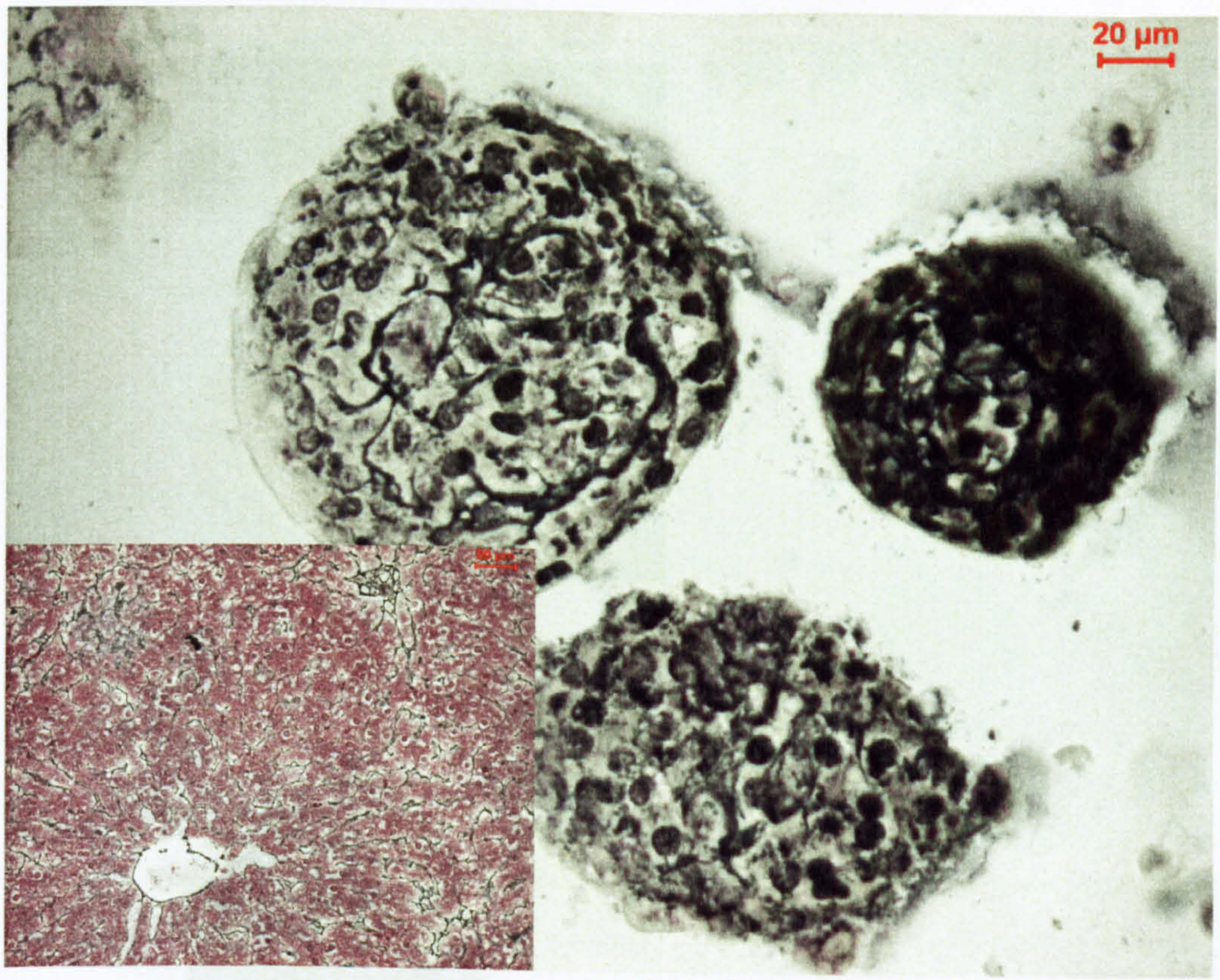


Figure 2.19: Reticulin staining in sections from 5-day-old hepatocyte-stellate cell co-culture spheroids shows the distribution of this important ECM component (normal liver section inset).

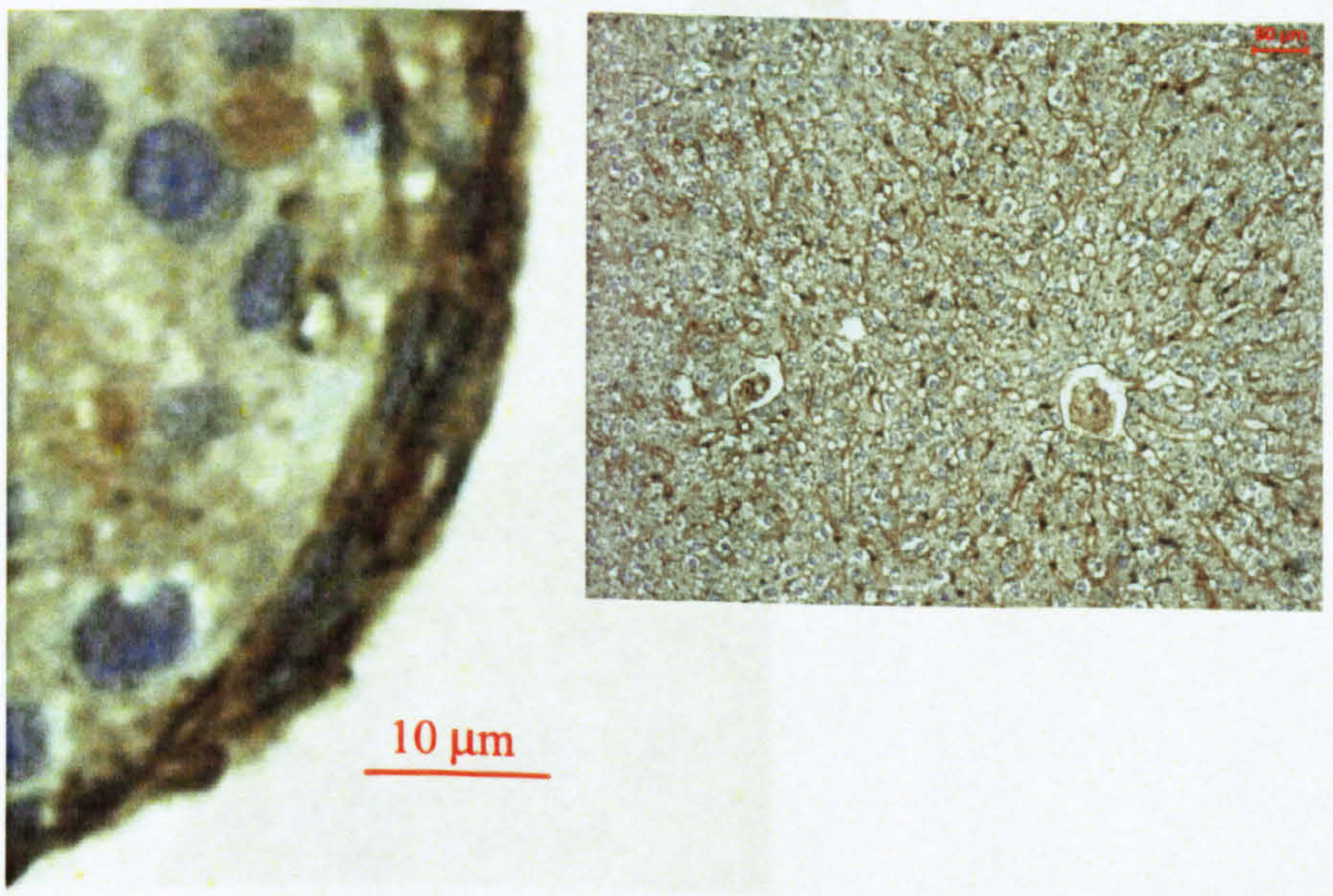


Figure 2.20: Fibronectin immunolocalisation in 5-day-old hepatocyte-stellate cell co-culture spheroid sections shows the peripheral deposition of this important ECM component (normal liver section inset right).

Figure 2.21a

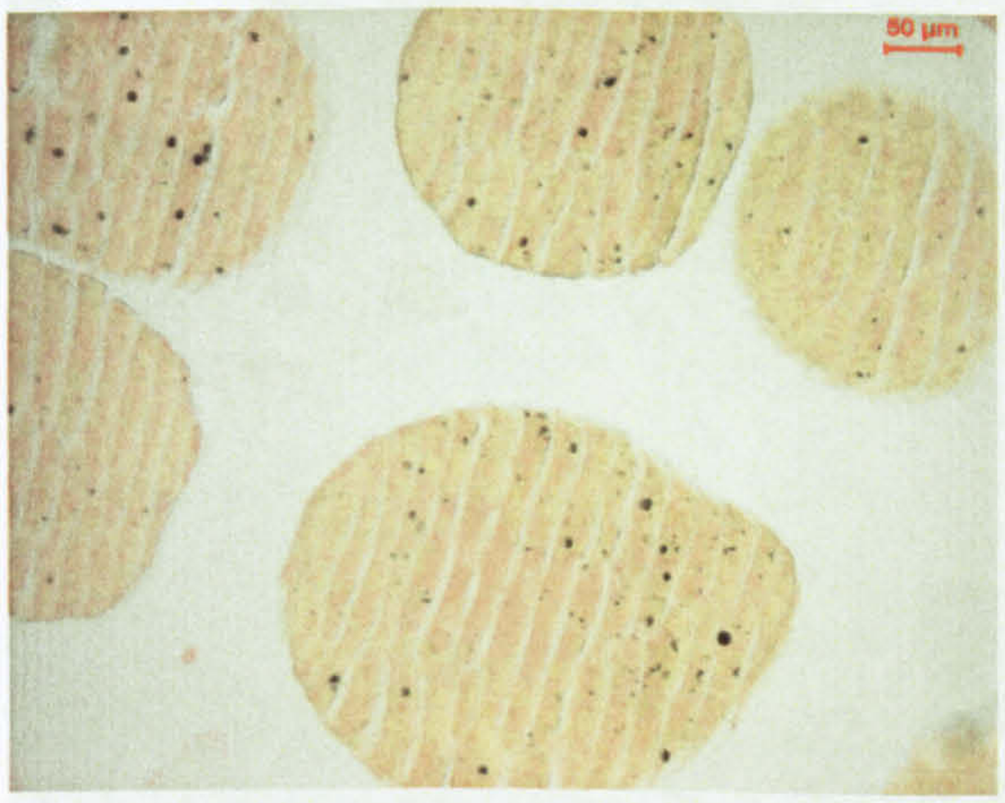


Figure 2.21b

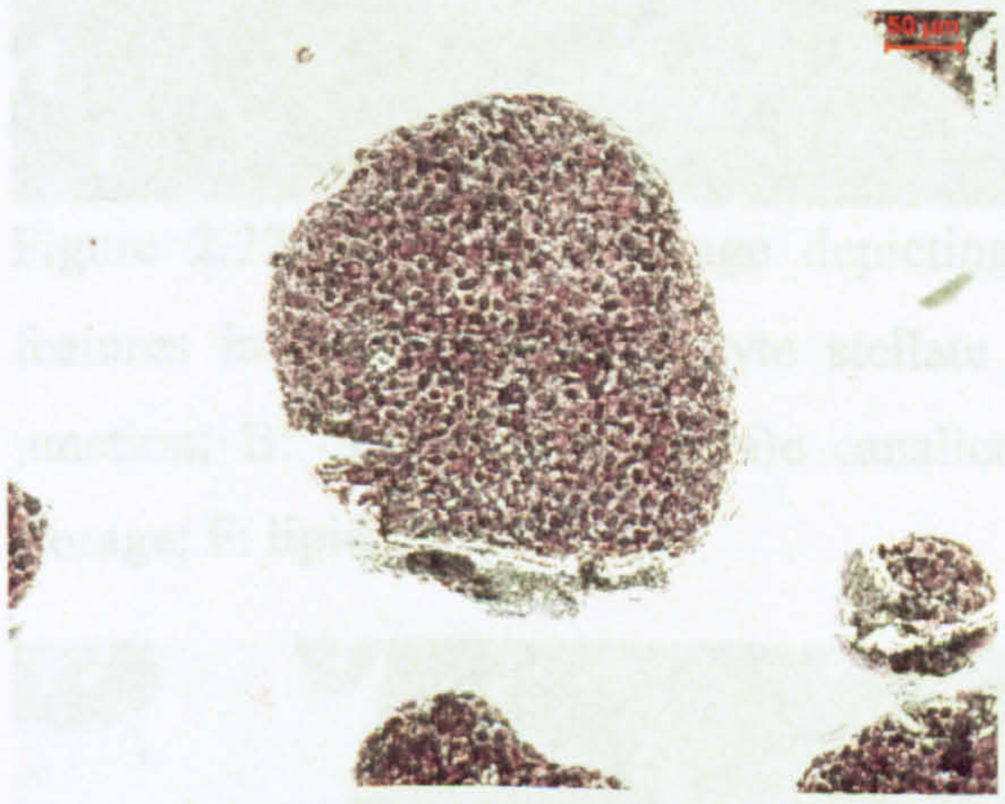


Figure 2.21c

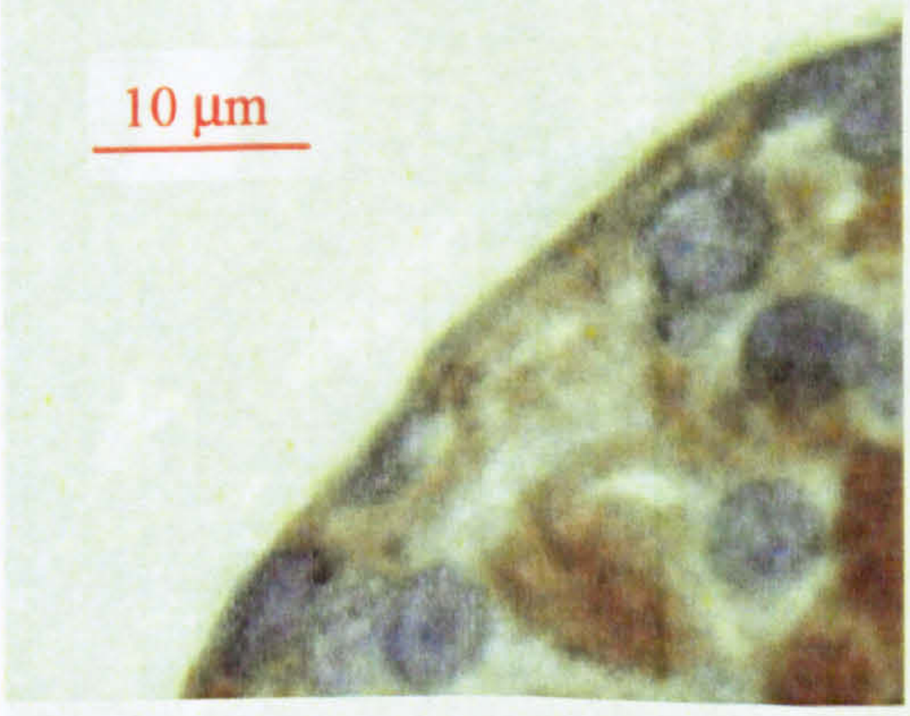


Figure 2.21: Collagen staining (a), reticulin staining (b) and fibronectin immunolocalisation (c) in sections from 5 day old hepatocyte mono-culture spheroids formed by culture agitation demonstrated an absence of all these ECM components.

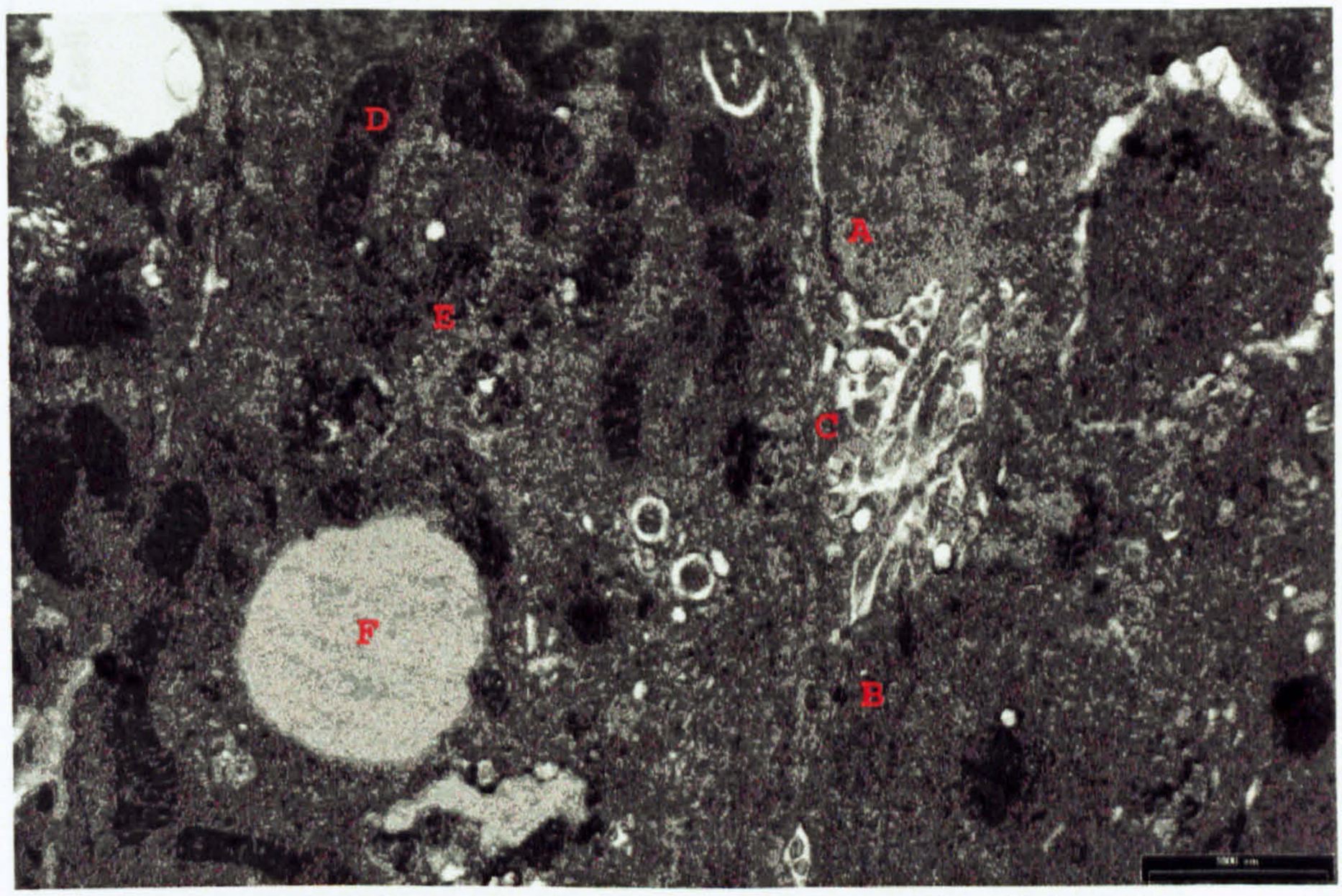


Figure 2.22(a): A TEM image depicting a range of cellular ultrastructure features in 5-day-old hepatocyte stellate cell co-culture spheroids; A: tight junction; B: desmosome; C: bile canaliculus; D: mitochondria; E: glycogen storage; F: lipid deposition.

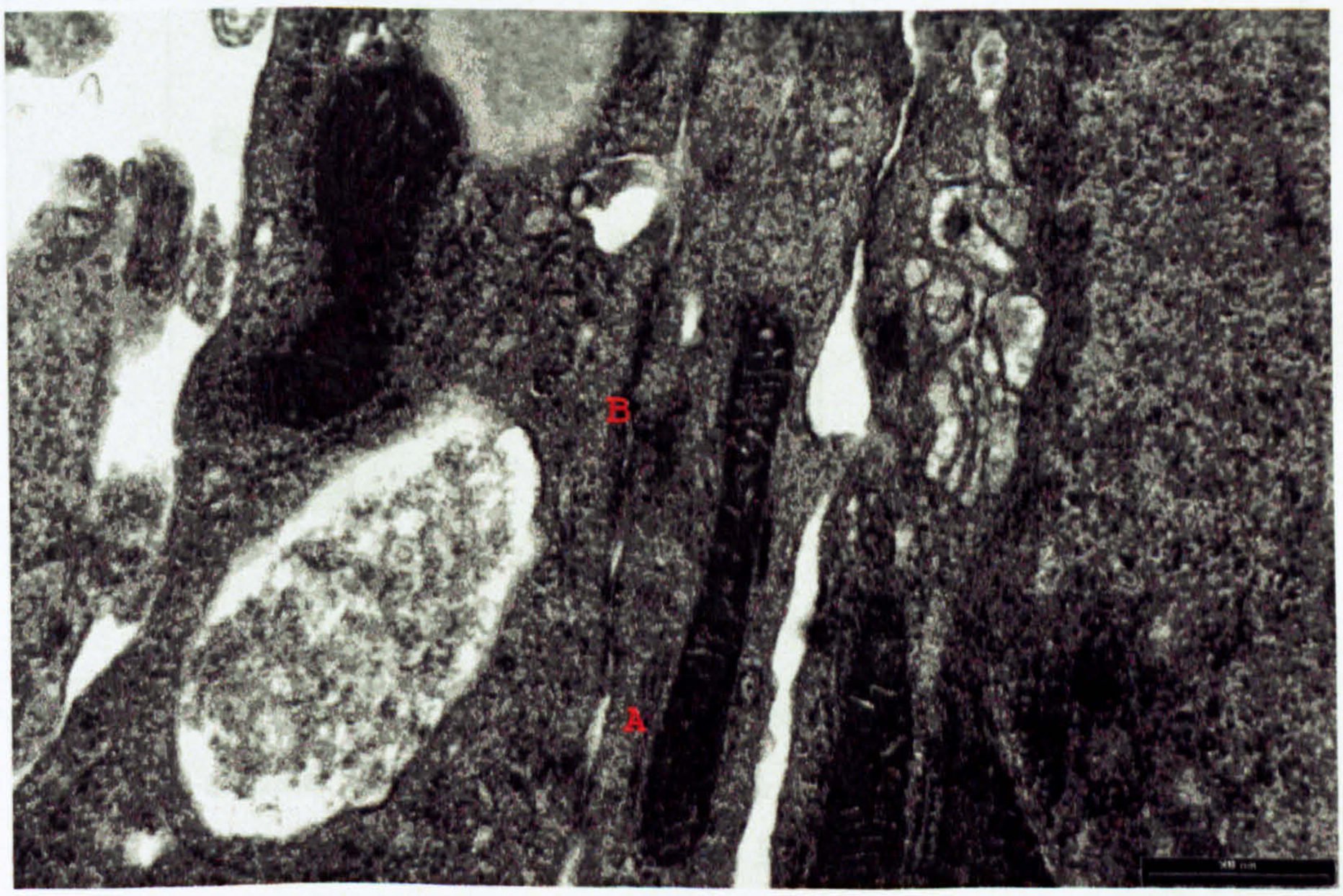


Figure 2.2(b): A TEM image of a 5-day-old hepatocyte-stellate cell co-culture spheroid showing a thin stellate cell or cellular process near the spheroid border, identified by A: myosin fibres and B: multiple junctions with the adjacent cell.

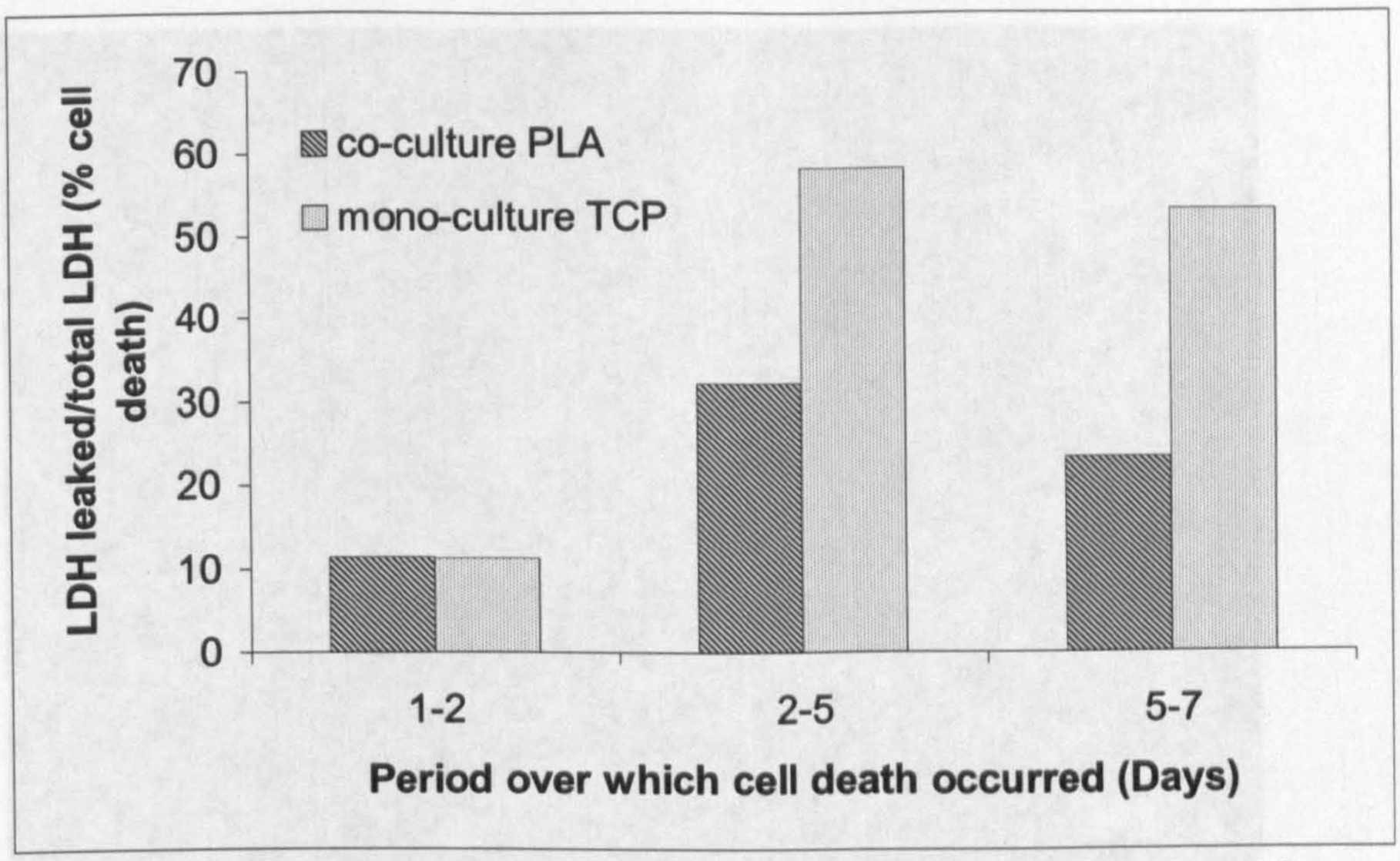


Figure 2.23(a): A graph showing cell death over time in hepatocyte-stellate cell co-culture spheroids compared to hepatocyte mono-culture. Data shows the percentage of LDH leaked during a culture period as a proportion of the total LDH in the well after total cell lysis. This gives an indication of the percentage cell death *since the previous measurement*.

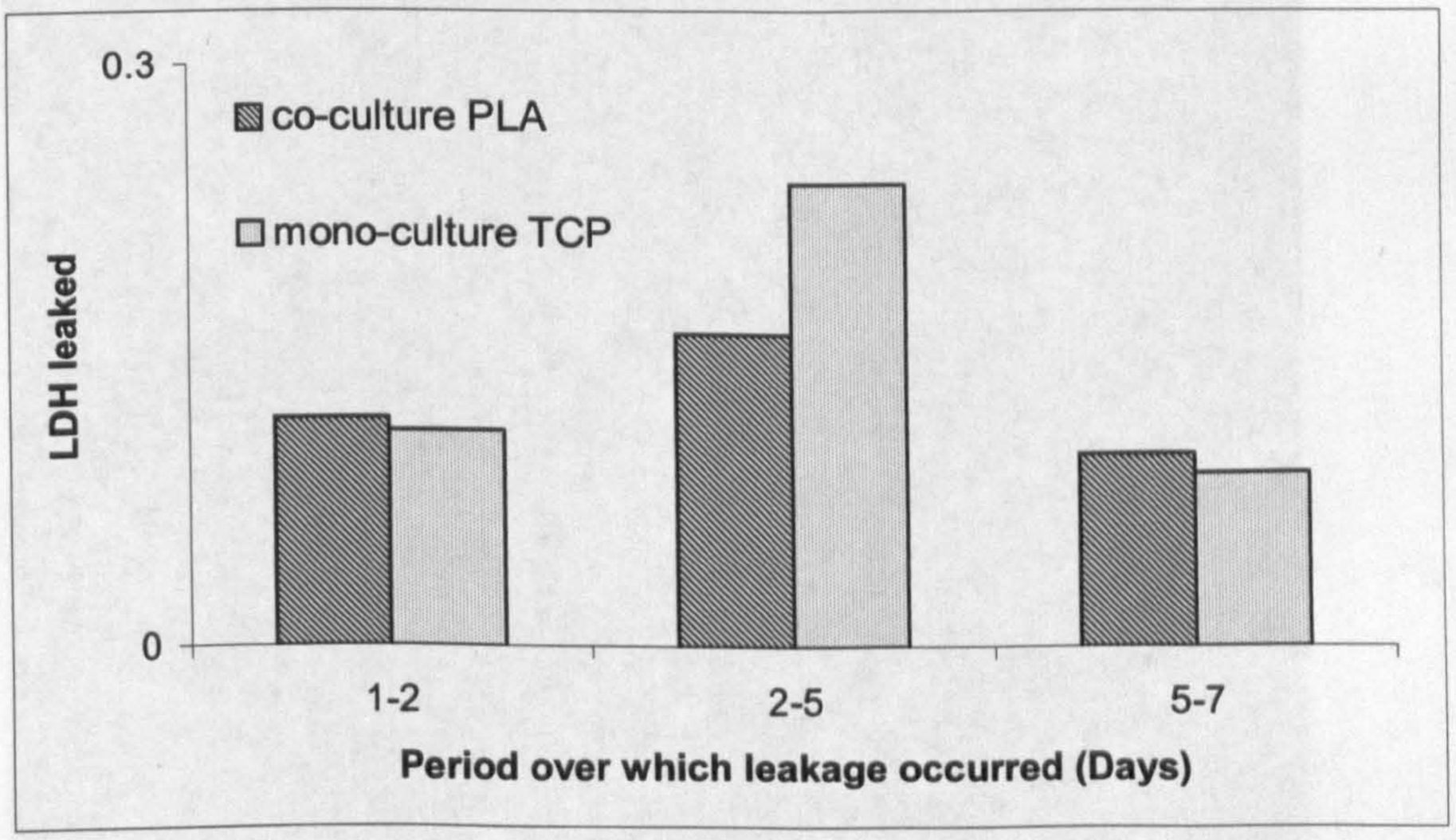
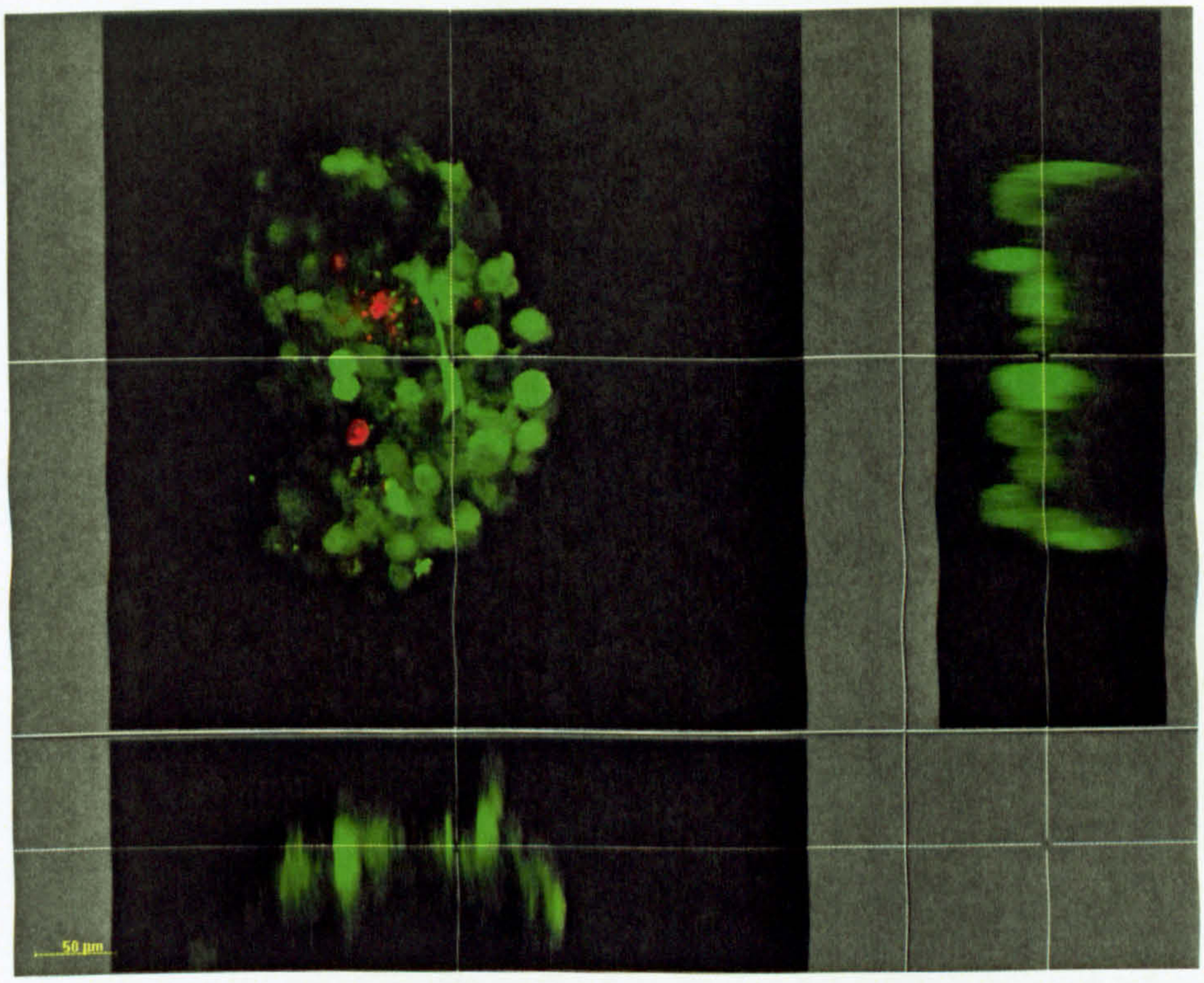


Figure 2.23(b): A graph showing absolute LDH leakage over time in hepatocyte-stellate cell co-culture spheroids compared to hepatocyte mono-culture. The higher absolute LDH leakage in mono-culture between 2 and 5 days indicates the viability result in figure (a) is not due to a stable viable stellate cell population.



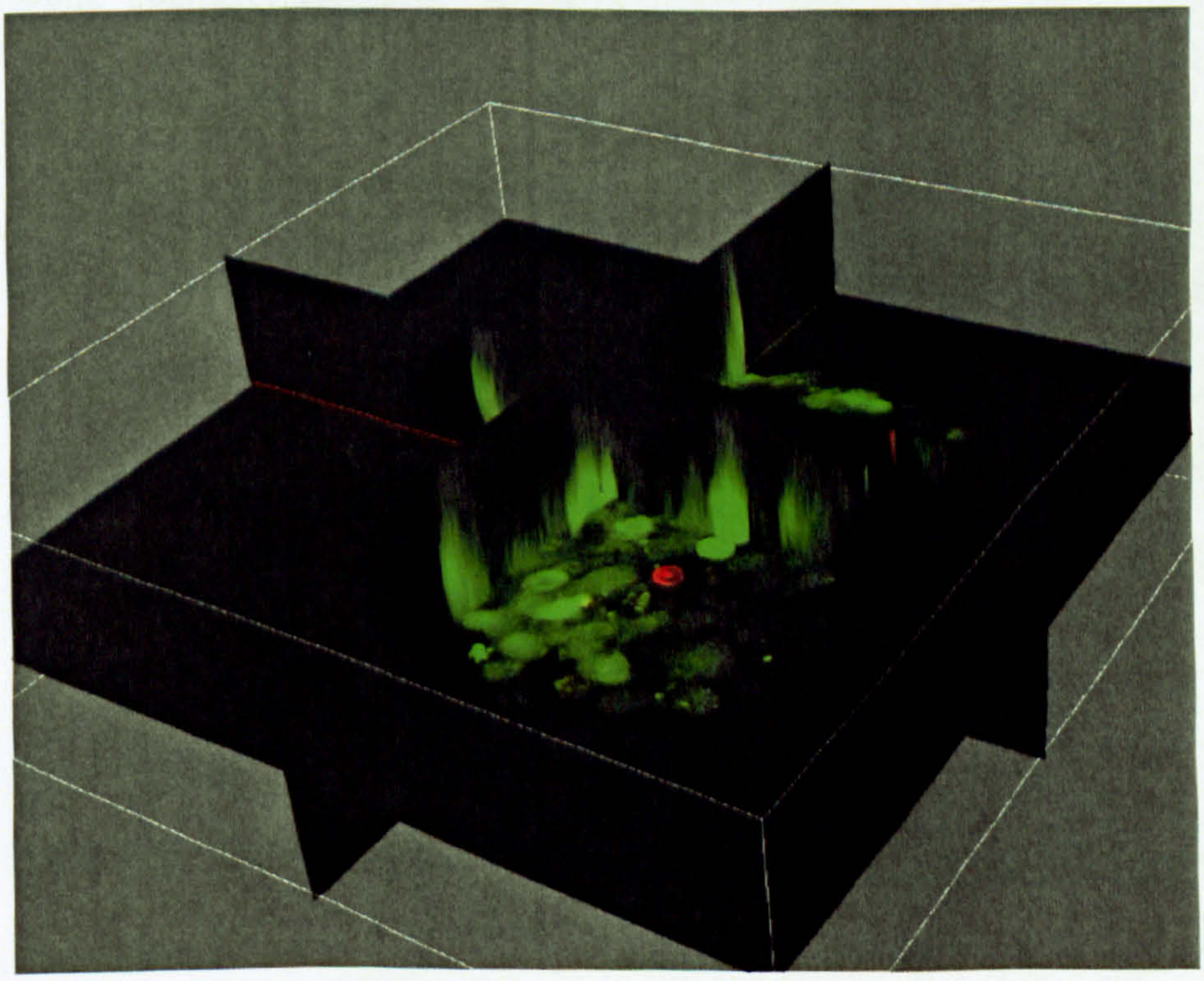


Figure 2.24: Live dead™ treated 5-day-old hepatocyte-stellate cell co-culture spheroids after 5 days in culture visualised by confocal microscopy are viable throughout. The images are presented with sections to show the viability at different levels of penetration.

2.4 Discussion

A range of techniques was used to look at the cellular arrangement, ECM support and ultra structure of co-culture and mono-culture spheroids. Co-culture with stellate cells had a major effect on the organisation of hepatocytes into spheroidal aggregates both in the dynamics of the aggregation process and in the structure and composition of the resulting aggregates.

2.4.1 Characterisation of the stellate cell population in vitro prior to co-culture

Some characterisation of the stellate cell population involved in spheroid formation was necessary due to the potential changes, such as transdifferentiation to myofibroblasts, that can occur over an extended time in culture (Cassiman *et al,* 2002). Identification of the key stellate cell markers GFAP and desmin discount the possibility of a simple myofibroblast population. However, the strong expression of SMA, fibronectin and αB-crystallin indicate an activated contractile myofibroblast like morphology at the time of incorporation into the co-culture.

2.4.2 The characteristics of hepatocyte-stellate cell co-culture spheroid formation

In hepatocyte-stellate cell co-culture, aggregation is not a result of passive cell collision and adhesion. It is an active process dependent on stellate cell motility. The stellate cell process contraction and morphology is influenced by

hepatocyte co-culture and this process is informative about the factors influencing cell aggregation. The delay before the start of stellate cell process contraction, despite cell contact, and the increase in stellate cell process contractility over time, has a number of potential explanations including adaptation of gene expression leading to build up of cell surface protein or soluble mediator expression. Formation of co-culture spheroids only in areas of high stellate cell density, combined with the failure of stellate cells to facilitate alternative epithelial cell line spheroid formation as effectively as primary hepatocyte spheroid formation, suggests factors from both stellate cells and differentiated hepatocytes are required for stellate facilitated spheroid formation. The contraction of stellate cells in response to hepatocyte fragments and hepatocyte conditioned media supports the involvement of a hepatocyte soluble mediator(s) and eliminates the involvement of complex cross talk between the two cell types (i.e. a signal from one cell type triggering a signal from the second cell type that then acts on the first) in eliciting this response.

2.4.3 The structure and cellular arrangement of the hepatocyte-stellate cell co-culture spheroids

The structure of the co-culture produced spheroids is related to the mechanism of formation. Stellate cells on the periphery of the spheroids are hypothesised to be those that were beneath the hepatocytes during the initial plating phase and therefore end up on the exterior of the spheroid; these are often thicker on a flatter side of a spheroid, presumably the side that was on the culture surface. When a stellate cell is located in the centre of a spheroid this is thought to be

the result of the stellate cell attaching on the top of hepatocytes, or pulling two smaller aggregates together. The benefit of this type of aggregation in which one cell type actively pulls a second passive cell type around it is seen in the high degree of heterotypic contact.

Important structural characteristics of liver tissue developed only when stellate cells were present. The organised ECM support with a specifically capsular peripheral fibronectin deposition, relative to the pervasive collagen, is particularly interesting due to the importance of the former as a component of the basal lamina. Both collagen and fibronectin are highly expressed components of the liver ECM in vivo, and recreation of this support in vitro will therefore probably be an important element for any long term culture environment. A hepatocyte stellate cell co-culture system will potentially improve on previous ECM supports as it incorporates the major ECM producing cell type of the liver and has previously been shown to produce a liver like collagen pattern (Takai *et al,* 2001). Furthermore, high mRNA expression of various ECM components is found in the regenerating liver (Jakowlew *et al,* 1991). Paradoxically, the ECM could have the disadvantage of a diffusion barrier for nutrients or waste products, although cell viability was not detrimentally affected over the period of this study, and spheroids are not wider than the limit suggested by previous studies (Fukuda *et al,* 2004).

The functional effectiveness of the cell contacts and intercommunication in the stellate-hepatocyte co-culture was further demonstrated by development of a well defined hepatic ultra structure including bile canaliculi, desmosomes and

tight junctions. Hepatocytes also showed evidence of fat deposition and glycogen storage. These features are all likely to be indicative of preserved cell function and communication. Their absence in mono-cultures demonstrates that signalling to the hepatocytes from the stellate cells or from the ECM that develops in the stellate cells presence is required for hepatocytes to form these structures.

2.4.4 Viability of the hepatocyte-stellate cell co-culture spheroids

The improved maintenance of cell viability in co-culture relative to mono-layer only occurs after the cells have adopted a multicellular spheroid morphology. This suggests fast aggregation could be important in preventing early cell death. Also, hepatocytes in contact with a heterotypic cell type reportedly have greater function than their isolated counterparts (Bhatia *et al,* 1998). In combination this suggests that methods such as this that rapidly establish heterotypic cell contact and maximise heterotypic cell interfaces through 3D morphology are an important tool in producing viable and functional tissue aggregates.

2.4.5 Conclusion

Co-culture on a $P_{DL}LA$ surface demonstrates an optimised environment for cell interaction and aggregation with associated liver like ultra structure. This study provides a generic model of aggregation and cellular arrangement for multicellular structures where aggregation is imposed on one cell type by the contractility of another. Such methods have a potentially important role in

functional and structural support of tissue engineered cell culture aggregates, and provide a valuable method of aggregating non-motile cells.

CHAPTER 3

CYP 450 Maintenance in Hepatocyte – Stellate Cell Co-culture

3.1 Introduction

3.1.1 Defining liver function

The liver is a multifunctional organ. Therefore a large number of functions can potentially be assayed to indicate the state of differentiation of a hepatocyte population in culture. Some of the more popular assays include detection of blood protein production, ammonia metabolism, membrane transport, and phase I oxidation or phase II conjugation reaction products. The rank of importance of the functions maintained in an in vitro culture system is defined by the potential applications of the system being investigated. Ammonia metabolism will be particularly vital for an effective bio-artificial liver, whilst drug metabolising enzymes are of more importance for in vitro toxicology and metabolism assays. Furthermore, it is not yet clear whether mechanisms that enhance certain functions, such as xenobiotic metabolism, may be detrimental to others such as transport. It is therefore important that an attempt to define the functionality of a culture system strictly defines the remit.

3.1.2 Why measure cytochrome P450 enzyme function?

The studies in this chapter investigate P450 oxidation reactions. These reactions are required in in vivo proportions in models of metabolism to give accurate metabolic profiles. They are also important in toxicology models if P450 metabolically activated toxins are to be detected, and in bio-artificial liver devices for xenobiotic clearance and oxidation of endogenous compounds.

Specifically, the function of P450 3A was investigated. P450 3A function was chosen due to its importance in xenobiotic metabolism; 50% of metabolically oxidised pharmaceuticals are substrates. It is also a highly labile liver function and therefore provides a stringent test for a long-term culture system. Furthermore, a well established method is available for accurate determination of P450 3A activity that can simultaneously provide information on other P450 activities.

3.1.3 Long term liver culture with maintenance of cytochrome P450 enzyme function

The consensus of published information suggests that P450 3A activity can be maintained over two to three weeks in human hepatocytes cultured in modified systems (e.g. collagen gel sandwich or epithelial cell co-culture) whilst the duration of activity in rat cells is far shorter. In collagen gel sandwich culture P450 3A mediated testosterone metabolism to 6β-hydroxytestosterone by human hepatocytes has been maintained without deterioration for 9 days (Kern *et al,* 1997). In contrast rat function deteriorated after only 3 days. In rat liver slices testosterone metabolism significantly decreases over the first 48 hours (Muller *et al,* 1998). In human hepatocytes in sandwich culture rifampicin is an effective inducer of P450 3A function after 3 days, however rifampicin is not effective after this culture period in rat hepatocytes (Kern *et al,* 1997). In rat liver slices induction of P450 3A, 1A1, 1A2 and 2B have all been achieved 3 days after isolation, although this involved supplementation with dexamethasone from the start of culture (Gokhale *et al,* 1997).

Co-culture of hepatocytes with other liver epithelial cells has been studied over the last 20 years. Maintenance of non-specific total P450 content over 10 days in rat hepatocytes has been shown compared with a rapid drop in conventional culture (Begue *et al,* 1984). This effect was also observed in human hepatocytes, although over a longer time period (Ratanasavanh *et al,* 1986). Evidence of sustained phase II function has also been presented (Vandenberghe *et al,* 1992). More recently P450 mediated lidocaine metabolism to MEGX, to which P450 3A contributes a part, has been maintained up to three weeks and improved relative to monoculture in human hepatocytes co-cultured with biliary epithelial cells (Auth MK *et al,* 2005).

3.1.4 Methods of measuring cytochrome P450 enzyme activity

A range of assays is available to measure P450 function. They vary in specificity and accuracy. Generally the test cells are exposed to a P450 substrate and then the levels of metabolite produced are recorded. Substrates with fluorescent metabolites (ie metabolism of ethoxyresorufin to resorufin via the ethoxyresorufin dealkylase activity of P450 1A1) can be used to give a fluorescence endpoint, easily read in a plate reader. Alternatively, metabolite levels can be assayed more accurately using analytical methods such as HPLC. However, substrates and metabolites vary in their usefulness. Detecting MEGX, a product of lignocaine metabolism, is a method of assaying P450 activity, but the metabolism is contributed to by several different P450 isozymes, making definite conclusions about specific activity difficult. Gold standards have been

developed for a number of the P450 isozymes i.e. a substrate that has a metabolite produced only or predominantly by a single isozyme. Testosterone is the gold standard substrate for analysis of P450 3A activity and is therefore the substrate used in this work.

The regiospecific hydroxylation of testosterone by various P450 enzymes enables the production of hydroxytestosterone metabolites to be related to P450 activity. The 6β-hydroxylation of testosterone is predominantly mediated by P450 3A, the major isoform being 3A4 in human (Waxman *et al,* 1991) and 3A1 in rat (Sonderfan *et al,* 1987). Oxidation of testosterone to 4-androstene-3, 17-dione in rats is predominantly catalysed by P450 2B1 (greater than 80%) with minor contribution by 2C11 and 2B2 (Sonderfan *et al,* 1989). In human hepatocytes P450 2C19, and to a lesser extent 2C9, are responsible for production of 4-androstene-3, 17-dione, with potential contribution from P450 2B6 (Yamazaki *et al,* 1997). 2α-hydroxylation is predominantly P450 2C11 mediated in rat (Sonderfan *et al,* 1989). An orthologous enzyme to P450 2C11 has not yet been identified in humans, but 2α-hydroxylation is presumably mediated by an enzyme in the 2C subfamily (Raucy *et al,* 2002). 11β-hydroxylation is catalysed by P450 11B in both rat and humans. An HPLC method is used to detect the quantity of these testosterone metabolites in the incubation medium. This method can therefore provide detailed information about the activity of P450 3A, but can also provide an indication of other P450 activities via those metabolites produced from a range of isozymes.

3.1.5 Aims

The studies described in this chapter were designed to investigate the characteristics of P450 enzyme function in the hepatocyte-stellate cell co-culture system. Specifically, the work aimed to:

- Validate an HPLC based testosterone metabolism assay of P450 activity.

- Apply the HPLC method to investigate the pattern and rate of reduction in function of various P450 enzymes over time in the hepatocyte – hepatic stellate cell co-culture system relative to the range of control culture systems described in chapter 2.

- Investigate the response of the cells to inducing agents.

- Conduct a preliminary investigation into the effect of rat stellate cell co-culture on the P450 function of a single preparation of human hepatocytes.

3.2 Materials and Methods

3.2.1 Cell isolation and culture

Rat cells were isolated as described in chapter 2 and co-cultured in 6 well plates.

Human hepatocytes were obtained from the UK Human Tissue Bank. Cells were from a hepatic resection necessitated by colorectal metastasis in a non-smoking Caucasian female, aged 63. Cells were transported at 4°C and plated down at approximately 8 hours after surgery, at which point viability was still 75%. 125000 human hepatocytes were combined with 62500 cultured rat stellate cells per well of a PLA coated 24 well plate and maintained in 320 µl of hepatocyte culture medium. The control was a human hepatocyte mono-culture.

3.2.2 Testosterone metabolism assay

Rat or human cell cultures were incubated for 1 hour at 37°C in 1.5 ml or 320 µl respectively of EBSS supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} and containing 100 µM testosterone. Supernatant was centrifuged to remove cell debris, and frozen for later analysis. For the induction study, cell cultures were maintained for 4 days after cell isolation before a 3 day incubation with hepatocyte culture media supplemented with 10 µM dexamethasone and 100 µM Phenobarbital.

3.2.3 HPLC

Samples were analysed using a Beckman HPLC 1090 fitted with a Zorbax 300 SB-C18 4.6 mm x 15 cm column maintained at 50°C. Mobile phase A consisted of 450 ml H_20:50 ml Acetonitrile:250 µl Formic acid, and mobile phase B consisted of 50 ml H_20:450 ml Acetonitrile:75 µl Formic acid. Mobile phase was run at 1 ml/minute, starting at 15% B, increasing linearly over 10 minutes to 50% B. UV absorbance was detected at 245 nm using an integral diode array detector. Sample injection volume was 40 µl. Each run was controlled by intermittent injection of an external standard of testosterone, as well as a mix of all metabolite standards. The run was valid if the standard varied no more than 2% from injection to injection, or from that of previous runs.

3.2.4 Experimental design and data analysis

Rat hepatocytes were subject to the five different culture systems described in chapter 2. The purpose of comparing the co-culture to so many different types of culture was to attempt to isolate the effect of co-culture from aggregation. Each culture was carried out at least five times in duplicate except for the induction experiment that was carried out three times in duplicate. Duplicates were averaged to create independent data points for statistical analysis. Data was analysed using one way ANOVA for differences in metabolite production between the various culture conditions. Post tests with Tukeys error control were conducted to identify these differences. All data analysis was done in excel using Analyze-it™ excel add in for the post tests. Graphs show mean values +/-

the standard error of the mean. A single sample of human cells in duplicate was also assayed in co-culture and TCP mono-culture. In these graphs the error bars represent the standard error of the duplicate.

P450 function is expressed as a percentage of initial activity, not per mg protein. This is due to the complication of cell populations changing in co-culture where only one cell type, the hepatocyte, is responsible for function. The change in total protein in the culture may not reflect the change in hepatocyte population and therefore is invalid to normalise function. The value for initial function used to calculate the percentage function is a single average value from all experiments. This is necessary because experimental variation means that the initial assay of function cannot be done at exactly the same time after each cell isolation. Figure 3.6 shows the dynamic changes in function in the few hours following isolation that would lead to a large source of error if a separate initial assay was used for each experiment.

3.3 Results

3.3.1 Development and validation of an HPLC method for detection of testosterone metabolites

3.3.1.1 HPLC testosterone metabolite elution times

The standards of testosterone and its metabolites were run individually to determine their elution times. A mix of all standards was then run intermittently throughout each experiment and samples and compounds were identified by order and time of elution. Figure 3.1 shows an HPLC trace from a sample containing a mix of all standards with labelled elution times. Figure 3.2 shows a sample HPLC trace from a biological sample with identifiable peaks labelled.

3.3.1.2 Validation of testosterone metabolite elution times and quantitation of UV absorbance

Validation of the linearity of UV absorbance of testosterone metabolites was carried out over 0-10 μM to cover the concentrations of metabolites preliminary studies indicated were likely to be present in experimental samples. Six repeat injections of 0, 2.5, 5, 7.5 and 10 μM concentration standards of each compound were carried out. Acceptable analytical validation requires a plot of concentration against UV absorbance to have a linear correlation coefficient greater than 0.99 (figure 3.3). The coefficient of variation over the range of analytical values should be less than 2%. A worked example of the calculation

of coefficient of variation for 6α-hydroxytestosterone is shown in table 3.1. Table 3.2 shows values for the other metabolites without the repeat injection values. Validation was also carried out up to 200 μM in the same manner, and all R squared values were also greater than 0.99. Samples could be frozen for several weeks with a 2% reduction in detection. Recovery was 98% i.e. if incubation media was not exposed to cells, 98% of starting material was detected.

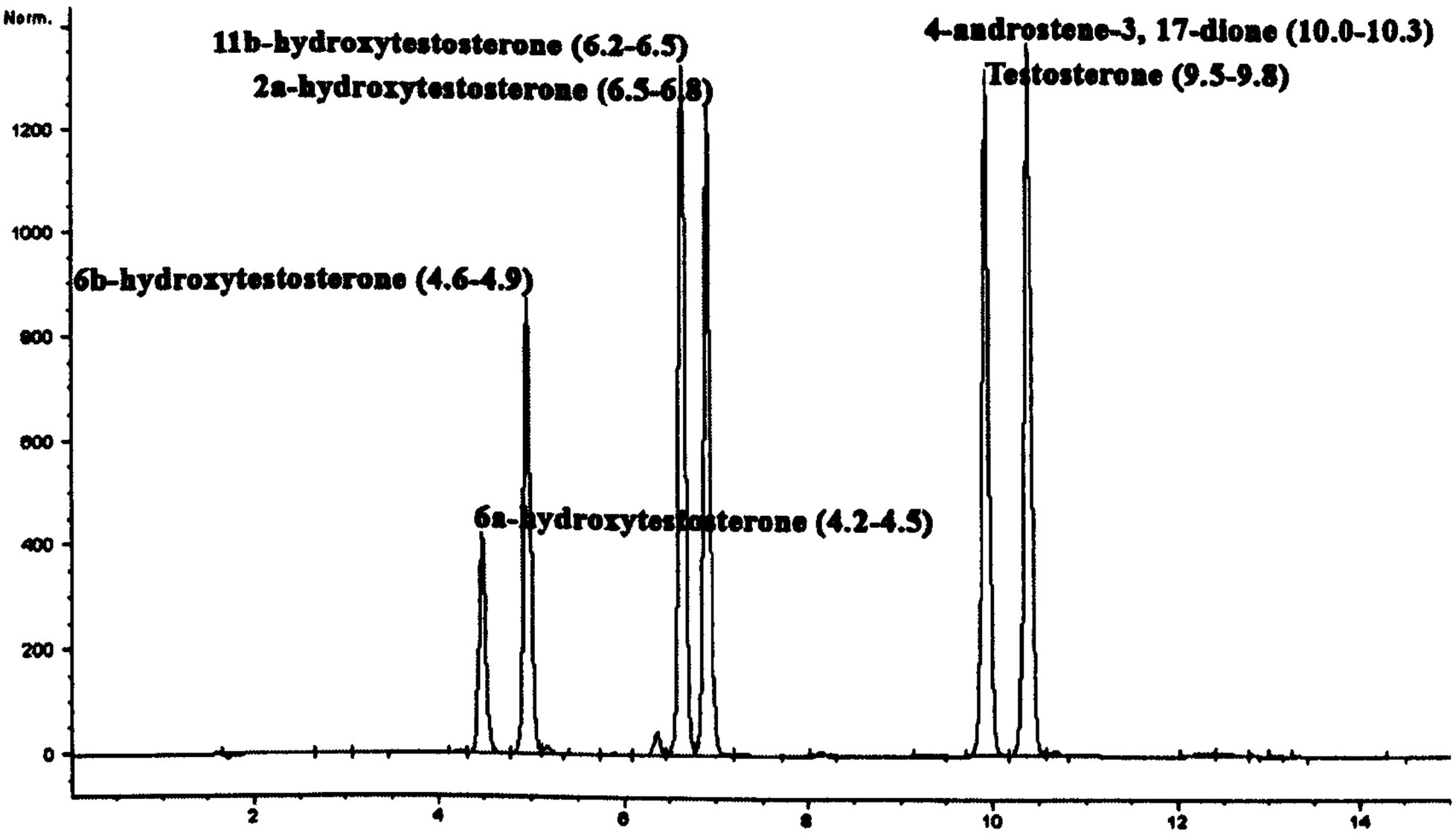


Figure 3.1: HPLC elution times for standard solutions of testosterone and a selection of important testosterone P450 phase I metabolites: 6α-hydroxytestosterone, 6β-hydroxytestosterone, 2α-hydroxytestosterone, 11β-hydroxytestosterone and 4-androstene-3, 17-dione.

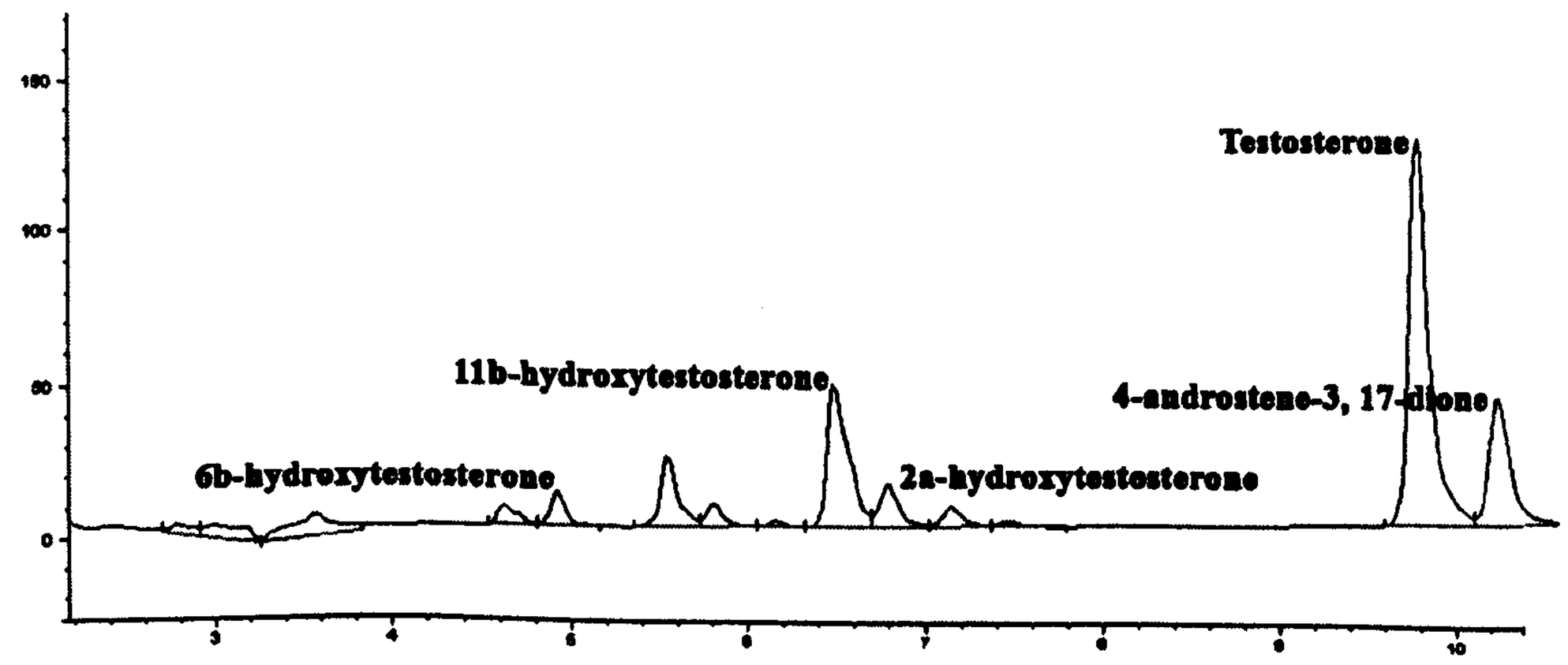


Figure 3.2: An HPLC trace of the testosterone metabolite profile from freshly isolated hepatocytes incubated for 1 hour with 100μM testosterone with identifiable peaks labelled.

	Repeat injection values						Mean	SD	Variance
10 μM	303	304	308	298	301	306	303	3.56	12.67
7.5 μM	220	224	224	223	226	225	224	2.07	4.27
5 μM	150	155	153	153	151	151	152	1.83	3.37
2.5 μM	72	74	73	74	75	71	73	1.47	2.17
0 μM	0	0	0	0	0	0	0	0	0

Average mean absorbance	Average variance	Average standard dev	Co-efficient of variation %
188	5.62	2.37	1.26

Table 3.1: A worked example of the calculation of the coefficient of variation for repeat injections of 6α-hydroxytestosterone to establish the variability of the HPLC method.

Metabolite	Average mean absorbance	Average variance	Average standard dev	Co-efficient of variation %
6-alpha	188	5.62	2.37	1.26
6-beta	128	5.62	2.37	1.85
11-beta	204	7.44	2.73	1.33
2-alpha	164	9.38	3.06	1.87
Testosterone	228	8.05	2.84	1.24
4-androstene 3,17 dione	251	10.46	3.23	1.29

Table 3.2: Coefficients of variation similarly calculated for other hydroxyl testosterone metabolites.

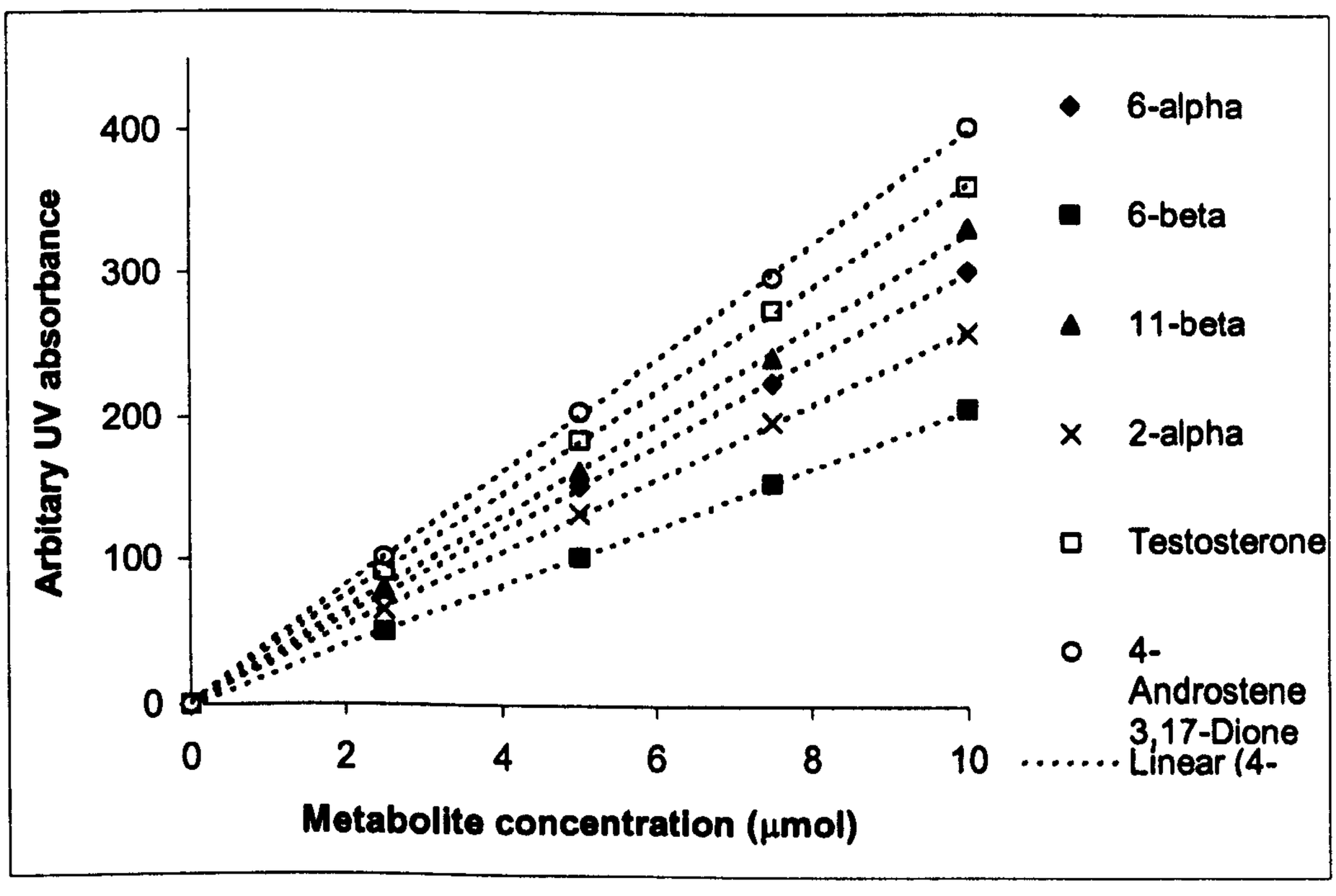


Figure 3.3: A graph showing the linearity of UV absorbance at 245 nm relative to concentration of the different isomeric hydroxytestosterone metabolites over the experimental range. All R^2 values are greater than 0.99.

3.3.2 Application of the HPLC testosterone metabolite assay to detect cytochrome P450 enzyme activity in hepatocyte culture systems

3.3.2.1 Absolute quantities of testosterone metabolite production by freshly isolated hepatocytes

Absolute levels of testosterone metabolite production by freshly isolated cells were assessed to inform about baseline function. The quantities of metabolite produced by the cells are very small. The average area under the curve representing UV absorbance of 6β-hydroxytestosterone is 47 (standard deviation 9.6, standard error 3.4). Using the trend line from figure 3.3, this equates to 2.2 μM 6β-hydroxytestosterone in the media after the initial assay. In 1.5 ml this is a total of 3.3 nmol in a well which would roughly equate to 0.0055 pmol of metabolite being produced by each cell over the 1 hour incubation. Table 3.3 shows these figures for the other metabolites. This is an estimate with a number of potential sources of error. Subsequent long term culture experimental data is expressed as a percentage of this function because other forms of expression become increasingly unreliable with time in culture and as cell populations change.

Table 3.3

Species, metabolite	Arbitrary UV absorbance	Media concentration	Media content	Metabolite per cell
Rat, 6β-hydroxy testosterone	47	2.3 μM	3.3 nmol	0.0055 pmol
Rat, 4-androstene-3, 17-dione	189	4.7 μM	7.1 nmol	0.0118 pmol
Human, 6β-hydroxy testosterone	452	21.8 μM	7.0 nmol	0.0349 pmol
Human, 4-androstene-3, 17-dione	114	2.8 μM	0.9 nmol	0.0045 pmol
Human, 11β-hydroxy testosterone	54	1.7 μM	0.5 nmol	0.0027 pmol
Human, 2α-hydroxy testosterone	74	2.8 μM	0.9 nmol	0.0045 pmol

Table 3.3: The area under the curve (arbitrary units) representing UV absorbance of measured metabolites at the start of the culture period and calculated production of metabolite per cell.

3.3.2.2 Relative maintenance of cytochrome P450 3A function in hepatocyte culture systems

Hepatocyte production of 6β-hydroxytestosterone is assayed as an indicator of P450 3A function (figure 3.4). This metabolite is detected after two and five days of culture in all culture systems but not thereafter. Analysis of variance identifies a difference between culture systems at both these time points ($P \leq 0.0001$). Table 3.4 shows the confidence level for differences identified between each culture system using Tukeys post test to control for type I error. The most striking difference after two days of culture is the inferior function of shaken cells compared to all other cultures. Trypsin and co-culture aggregated cells have an advantage over other cultures at this point, but statistical confidence is relatively weak. After five days in culture the co-culture spheroids had far superior function to all other culture models. This is significant in magnitude with metabolism of testosterone to 6β-hydroxytestosterone by P450 3A 7.2 fold greater than the closest comparator.

3.3.2.3 Relative maintenance of cytochrome P450 2B1, 2B2 and 2C11 function in hepatocyte culture systems

Hepatocyte production of 4-androstene-3, 17-dione was assayed as an indicator of P450 2B1 function (with minor contributions from 2B2 and 2C11)(figure 3.5). This metabolite was detected after two, five, ten and fourteen days in culture. Analysis of variance identified a difference between culture systems at all these time points ($P \leq 0.0001$), and table 3.5 displays the significance of

differences identified using Tukeys post test. After two days of culture the only difference observed with confidence was the inferior function of the shaken cells. After five days of culture the co-culture system had maintained superior function to all other culture systems, similarly after ten days and 14 days, although at the later two time points comparators were reduced to mono-culture on TCP and PLA due to practical constraints. The magnitude of the differences are less than those observed with P450 3A function. Metabolite levels in co-culture are 1.3 fold greater than the most active mono-culture after 5 days, 4.3 fold greater after 10 days, and was the only culture producing this metabolite after 14 days.

Figure 3.4

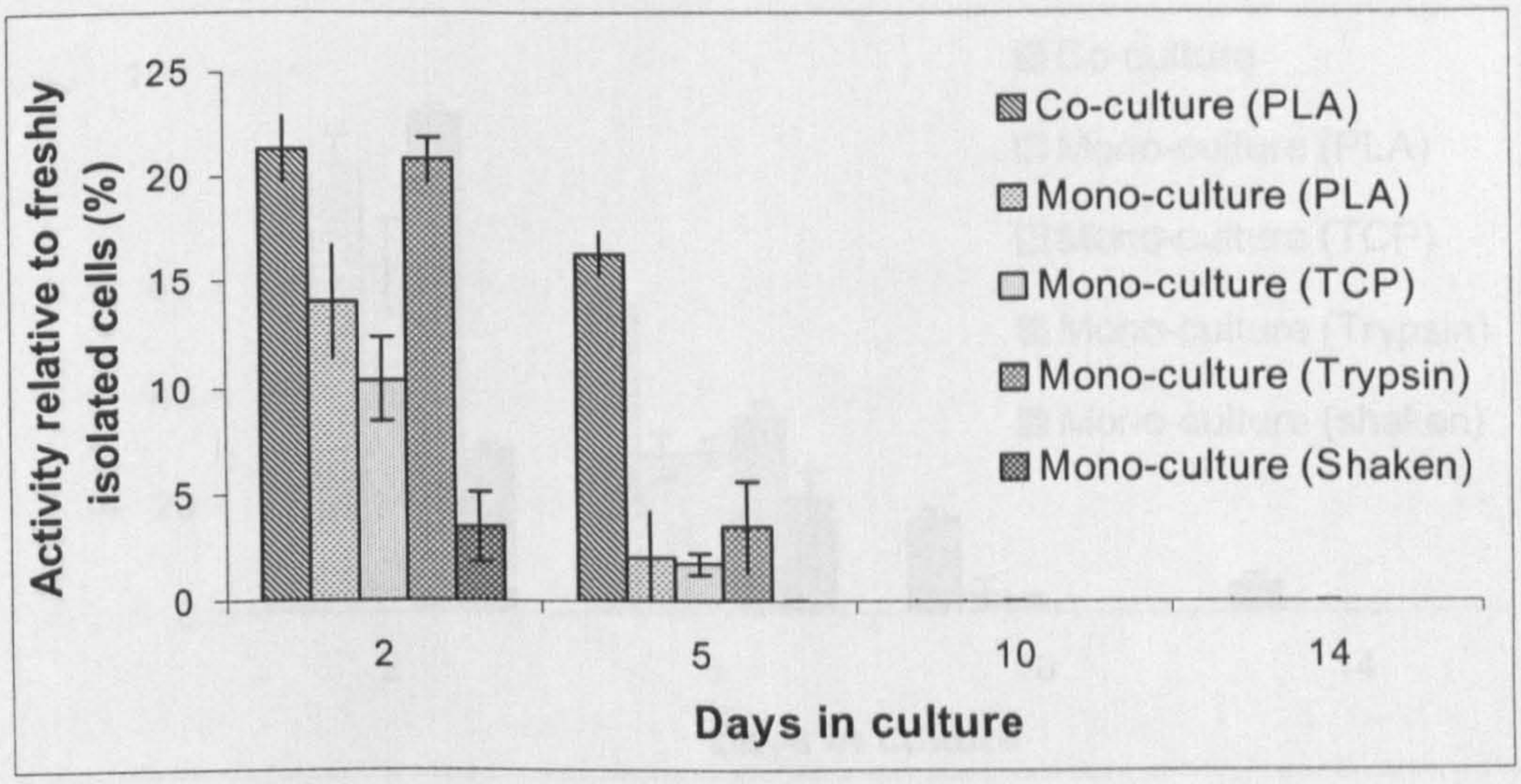


Table 3.4

	Day 2	Day 5
Co vs TCP	P≤0.01	P≤0.01
Co vs mono PLA	-	P≤0.01
Co vs Trypsin	-	P≤0.01
Co vs Shaken	P≤0.01	P≤0.01
TCP vs mono PLA	-	-
TCP vs Trypsin	P≤0.05	-
TCP vs Shaken	P≤0.05	-
PLA mono vs Trypsin	-	-
PLA mono vs Shaken	P≤0.01	-
Trypsin vs Shaken	P≤0.01	-

Figure 3.4: The production of 6β-hydroxytestosterone (indicating P450 3A functionality) from the substrate testosterone over time in various culture systems, namely hepatocyte monocultures on TCP or PLA, hepatocyte mono-culture aggregates formed by agitation or trypsin pre-treatment, and hepatocyte-stellate cell co-culture aggregates. Function is expressed as a percentage of the function of freshly isolated hepatocytes.

Table 3.4: Statistical significance of the differences shown in figure 3.4 calculated using ANOVA and Tukey's post test.

Figure 3.5

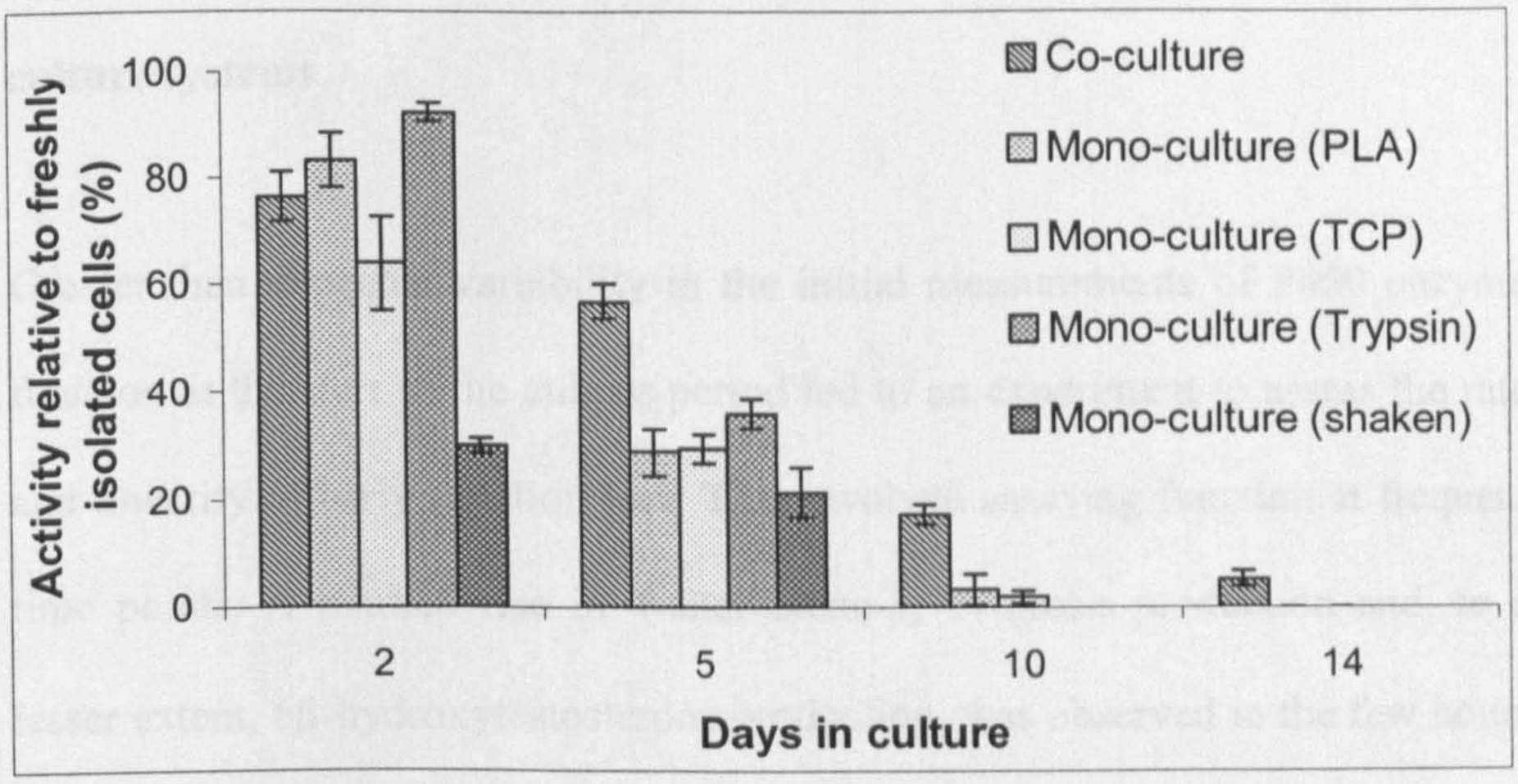


Table 3.5

	Day 2	Day 5	Day 10
Co vs TCP	-	P≤0.01	P≤0.01
Co vs mono PLA	-	P≤0.01	P≤0.01
Co vs Trypsin	-	P≤0.05	
Co vs Shaken	P≤0.01	P≤0.01	
TCP vs mono PLA	-	-	-
TCP vs Trypsin	-	-	
TCP vs Shaken	P≤0.01	-	
PLA mono vs Trypsin	-	-	
PLA mono vs Shaken	P≤0.01	-	
Trypsin vs Shaken	P≤0.01	-	

Figure 3.5: The production of 4-androstene-3, 17-dione (indicating P450 2B functionality) from the substrate testosterone over time in various culture systems, namely hepatocyte monocultures on TCP or PLA, hepatocyte mono-culture aggregates formed by agitation or trypsin pre-treatment, and hepatocyte-stellate cell co-culture aggregates. Function is expressed as a percentage of the function of freshly isolated hepatocytes.

Table 3.5: Statistical significance of the differences shown in figure 3.5 calculated using ANOVA and Tukey's post test.

3.3.2.4 Characteristics of cytochrome P450 function loss in hepatocyte culture systems

Greater than expected variability in the initial measurements of P450 enzyme function at the start of the culture period led to an experiment to assess the rate and linearity of early function loss. This involved assaying function at frequent time points. A marked rise in 4-androstene-3, 17-dione production and, to a lesser extent, 6β-hydroxytestosterone production, was observed in the few hours after isolation and commencing culture, followed by an exponential reduction in function (figure 3.6). After this rapid deterioration of P450 activity a low level of relatively stable function was observed (figure 3.4, 3.5, and 3.6).

3.3.2.5 Relative levels of induction of P450 function in hepatocyte culture systems

Rat hepatocytes on TCP, PLA, or in co-culture were all assayed for response to induction with phenobarbital and dexamethasone between days four and seven of culture. The resulting maintenance of combined P450 2B1, 2C11 and 2B2 activity is shown in figure 3.7 as a percentage of initial activity. ANOVA detects a difference between these culture systems ($P \leq 0.0001$). Tukeys post test identifies a difference between induced co-culture and all other cultures, induced or not ($P \leq 0.01$ for all). The difference between non-induced co-culture and mono-culture is also reiterated, though at a lower confidence level to above due to a smaller sample size ($P \leq 0.05$ for all). No difference is detected between the

other mono-culture groups, induced or not. Also, P450 3A function is not detected at this time.

Figure 3.6

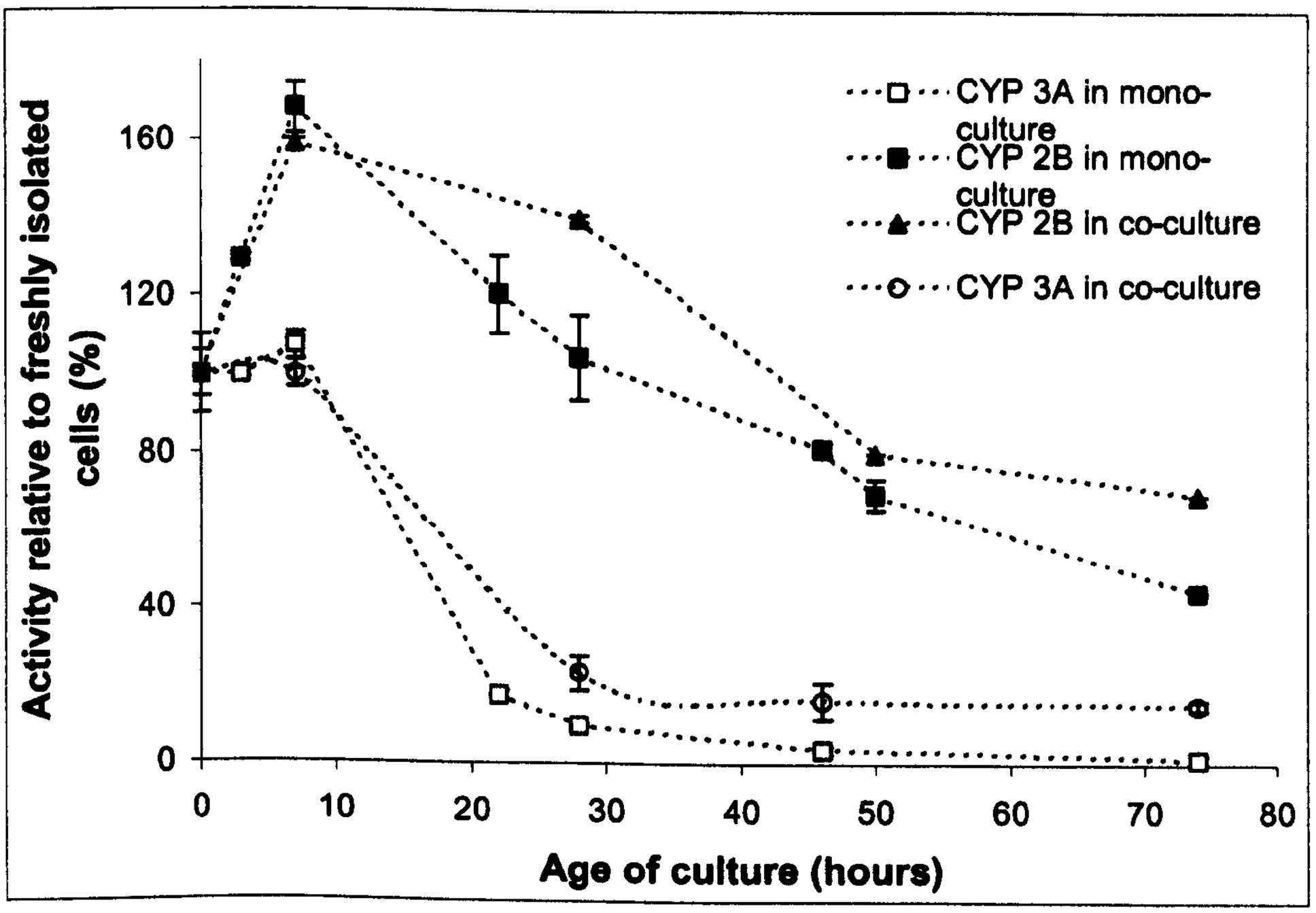


Figure 3.6: The pattern of function loss of CYP 450 3A and 2B over the first 72 hours of rat hepatocyte mono-culture and hepatocyte-stellate cell co-culture.

Figure 3.7

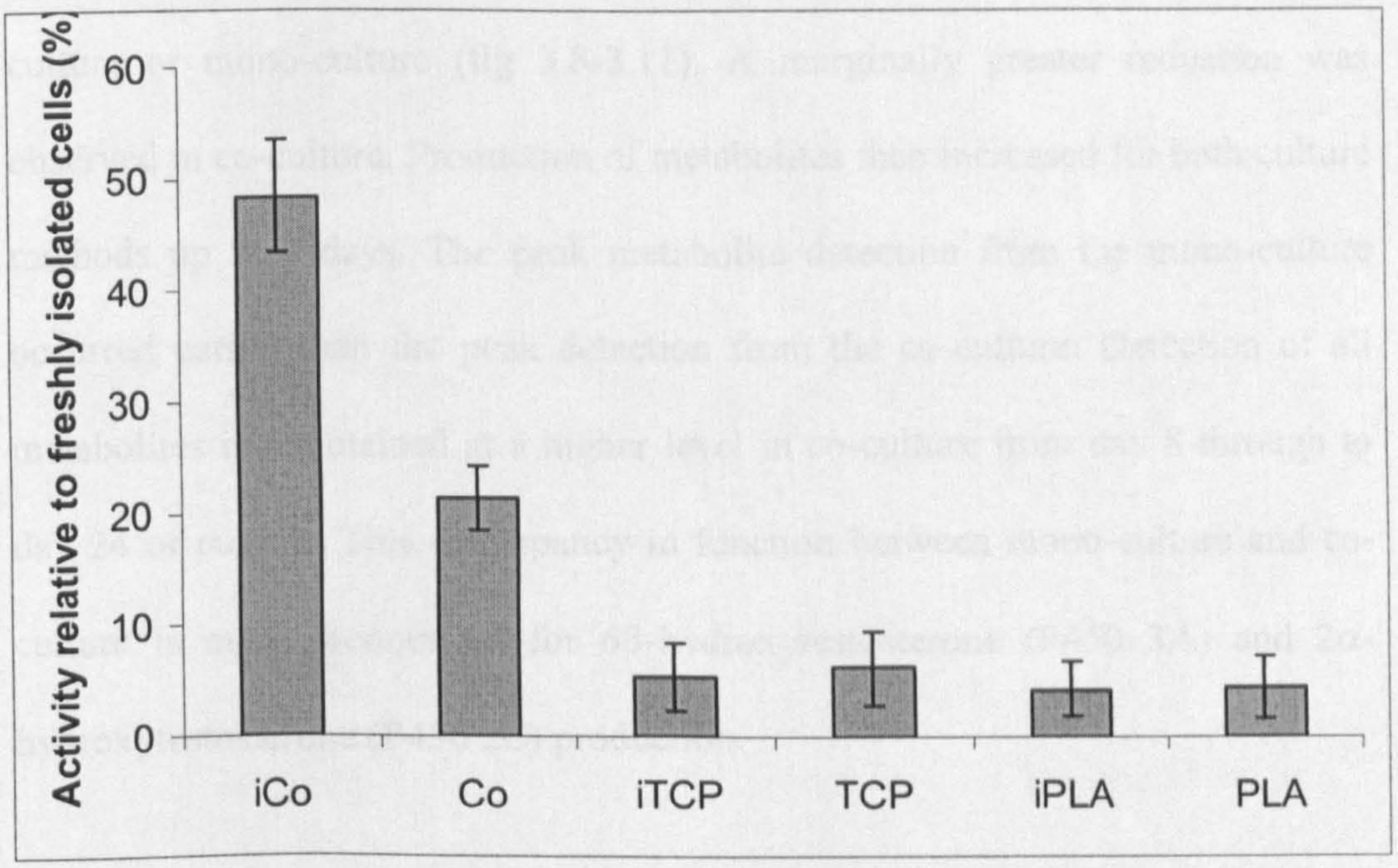


Figure 3.7: The 4-androstene-3, 17-dione production by 7-day-old cultures after culture either in the presence (prefix i) or absence (no prefix) of the enzyme inducers dexamethasone and phenobarbital since day 4 of culture.

3.3.2.6 Relative maintenance of cytochrome P450 enzyme function in human hepatocyte co-culture and mono-culture

P450 function of a single sample of human hepatocytes was assayed in duplicate. In this single sample, metabolism of testosterone to four different hydroxy metabolites was reduced by up to 90% after two days of either co-culture or mono-culture (fig 3.8-3.11). A marginally greater reduction was observed in co-culture. Production of metabolites then increased for both culture methods up to 8 days. The peak metabolite detection from the mono-culture occurred earlier than the peak detection from the co-culture. Detection of all metabolites is maintained at a higher level in co-culture from day 8 through to day 24 of culture. This discrepancy in function between mono-culture and co-culture is most pronounced for 6β-hydroxytestosterone (P450 3A) and 2α-hydroxytestosterone (P450 2C) production.

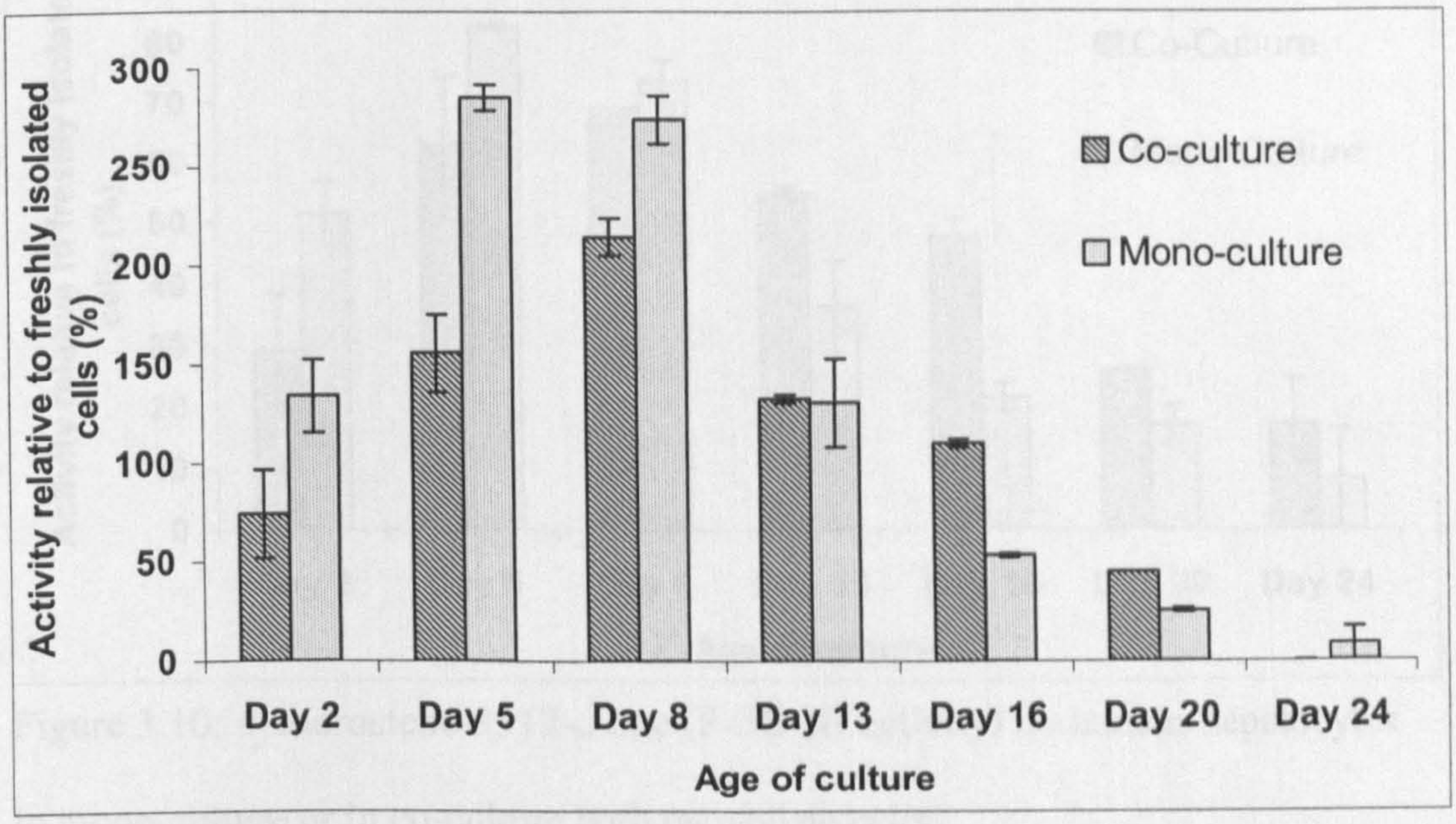


Figure 3.8: 11β-hydroxytestosterone (P450 11B activity) production by human hepatocytes in mono-culture or in co-culture with rat stellate cells.

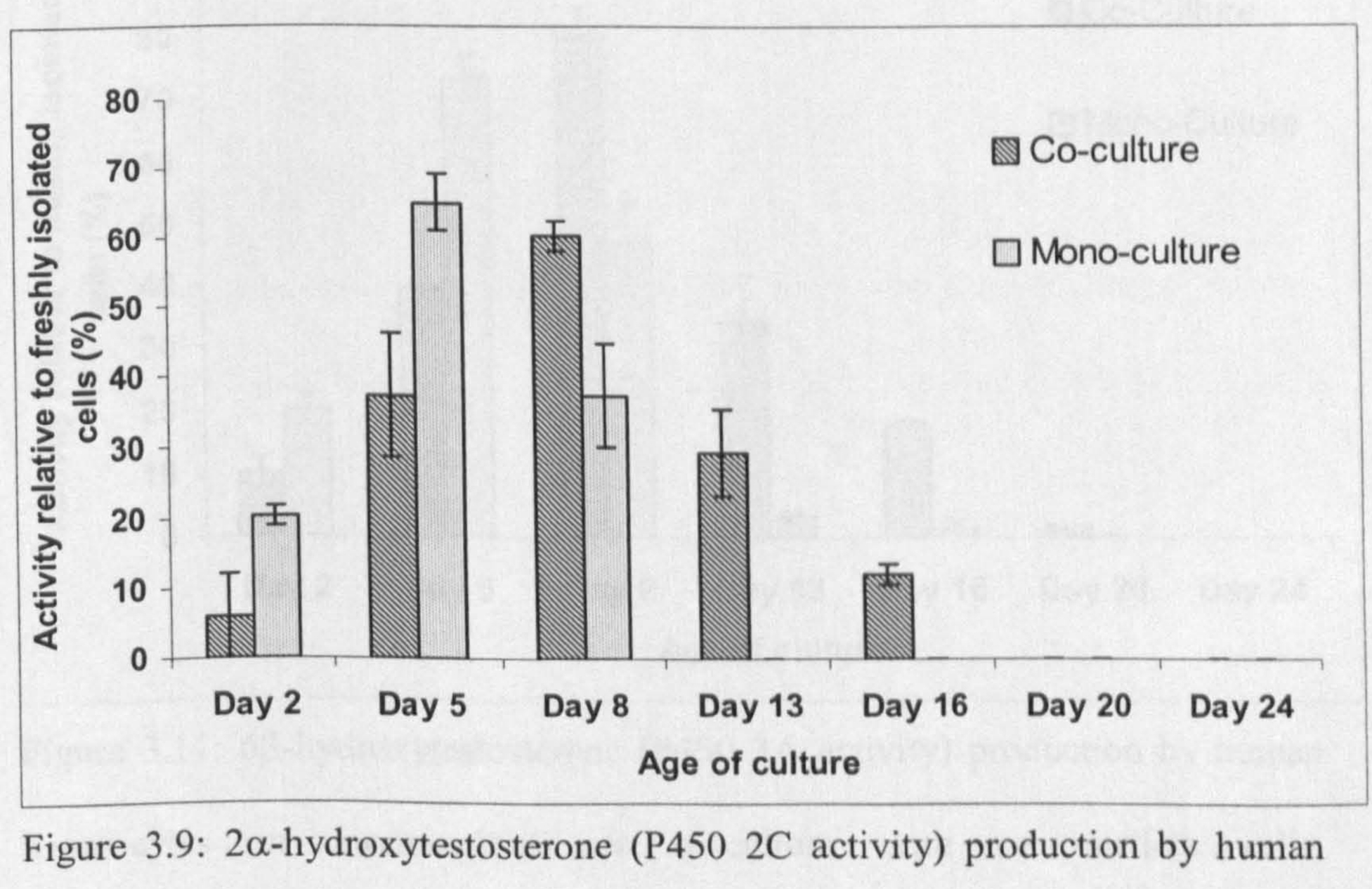


Figure 3.9: 2α-hydroxytestosterone (P450 2C activity) production by human hepatocytes in mono-culture or in co-culture with rat stellate cells.

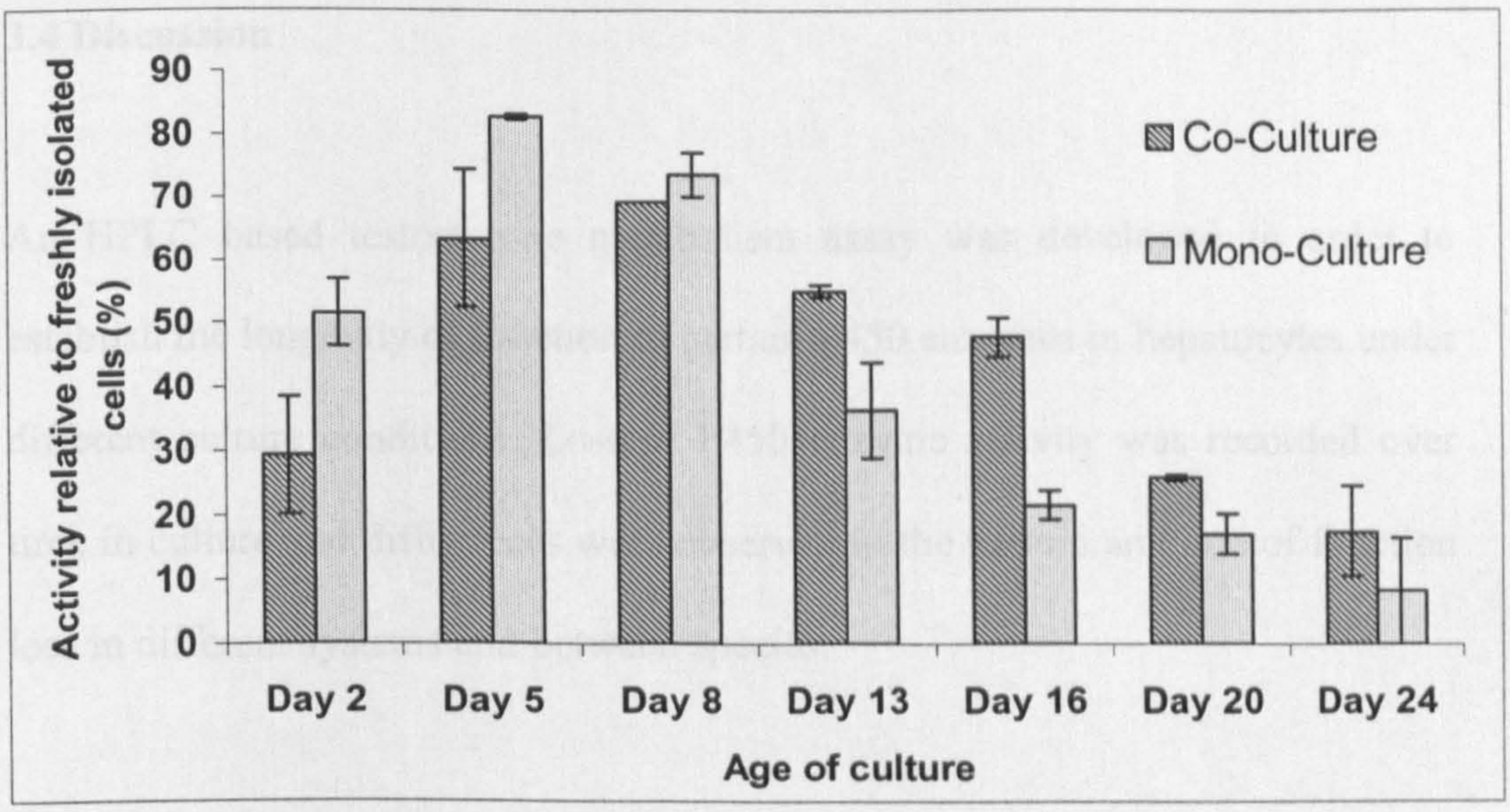


Figure 3.10: 4-androstene-3, 17-dione (P450 2B activity) by human hepatocytes in mono-culture or in co-culture with rat stellate cells.

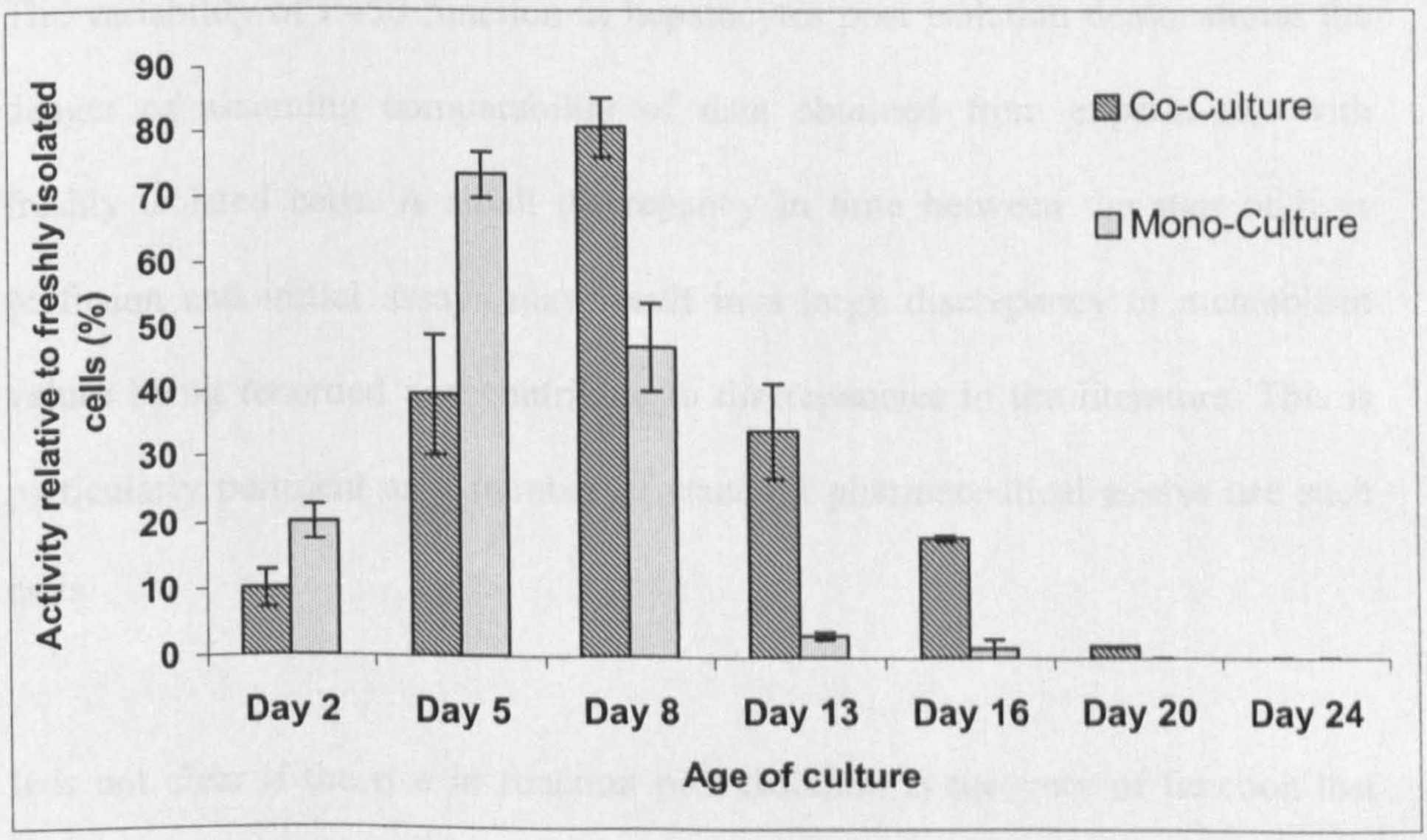


Figure 3.11: 6β-hydroxytestosterone (P450 3A activity) production by human hepatocytes in mono-culture or co-culture with rat stellate cells.

3.4 Discussion

An HPLC based testosterone metabolism assay was developed in order to establish the longevity of function of certain P450 enzymes in hepatocytes under different culture conditions. Loss of P450 enzyme activity was recorded over time in culture and differences were observed in the pattern and rate of function loss in different systems and between species.

3.4.1 The degradation of cytochrome P450 enzyme function in cultured hepatocytes

The variability of P450 function in hepatocytes post isolation demonstrates the danger of assuming comparability of data obtained from experiments with freshly isolated cells. A small discrepancy in time between the start of liver perfusion and initial assays may result in a large discrepancy in metabolism values being recorded and contribute to discrepancies in the literature. This is particularly pertinent as a number of standard pharmaceutical assays use such cells.

It is not clear if the rise in function post isolation is recovery of function lost during isolation or induction of function above in vivo levels due to stress or other factors associated with isolation or culture. The pattern of changing P450 activity is the same but slower in human hepatocytes relative to rat cells showing the pattern is not species specific other than in its time course. These results are consistent with those of a previous study that demonstrated chemical

inhibition of protein synthesis augmented P450 gene expression, suggesting that these genes were under the control of negative transcription factors (Degawa *et al,* 2003). If a similar global reduction in protein synthesis occurs due to the in vitro culture environment, a similar rise in P450 activity would be anticipated. The only inconsistency with this theory is the preliminary human data indicating that P450 activity is initially high. This could be due to the harsh environment of isolation and cooled transport having a temporary major effect on protein synthesis. This would explain why a similar initial high value is not seen in rat cells that have been isolated and transferred to a relatively hospitable culture environment far faster than the human cells. Irrespective of the mechanism, this complexity presents further problems in timing of initial base level function or standardised assays in rat hepatocytes.

Increasing P450 function post isolation in the absence of inducing agents has been reported previously. The most direct comparison is a hepatocyte sandwich culture in which it was reported that rat cells display dynamic changes in function that are not exhibited by human cells. A sharp rise in P450 3A activity is detected in rat cells over the first three days, compared to steady maintenance of human cell function over 9 days (Kern *et al,* 1997). This apparent reversal of species effect compared with the studies reported here is perhaps evidence of the ability of culture conditions to influence the time course and pattern of enzyme function over time. In rat cells a rapid deterioration of P450 3A function similar to that observed in these studies is more commonly reported (Muller *et al,* 1998).

A notable anomaly in this work is the testosterone metabolism detected in mono-culture hepatocytes on a PLA surface. This low level of function is possibly attributable to the aggregation and consequent survival of a small number of cells.

3.4.2 The effect of stellate cell co-culture on hepatocyte cytochrome P450 function

The stellate cell co-culture has a clear moderating effect on the loss of P450 function. If this were simply caused by more cells being lost from mono-cultures during cell maintenance, or a higher death rate in mono-culture, there would be a lower function result in mono-culture from the second assay, with a growing discrepancy at each time point. The higher levels of function in the early stages of human hepatocyte mono-culture give confidence in a true stellate mediated effect. Further, mono-culture spheroid controls eliminate substrate availability as a confounding factor in comparison to monolayer.

As discussed above, rat and human hepatocytes in culture lost P450 3A and P450 2B activity in a distinctive pattern of rising and falling function. The significant prolonging of both these phases observed in rat and human co-culture spheroids relative to mono-culture could be attributable to slower deterioration of global protein synthesis in the co-culture. If the P450 enzymes were under the control of negative transcription factors (Degawa *et al,* 2003), function would rise faster in the mono-culture as the negative influence diminished but would also peak and fall earlier than co-culture where both the negative transcription

factors and P450 expression could be maintained longer, effectively moving the dedifferentiation curve to the right.

3.4.3 Comparison of the hepatocyte – stellate cell co-culture system with alternative culture systems

The hepatocyte – stellate cell co-culture contrasts favourably with the limited comparable published data. Human hepatocytes in co-culture with stellate cells exhibited rising P450 function for an 8 day duration. Hepatocytes cultured in a collagen sandwich are reported to support stable P450 function for the same period (Kern *et al,* 1997). Furthermore, the duration of detection of testosterone metabolism to 6β-hydroxytestosterone by human hepatocytes co-cultured with stellate cells is similar to that reported for metabolism of lidocaine to mono-ethyl-glycine-xylidide (MEGX) in co-cultures of human hepatocytes with BECs (although MEGX production is contributed to by enzymes other than CYP 3A) (Auth *et al,* 2005). This implies that functional deterioration sets in after a similar time in each of these systems, but the response during the viable culture period varies in the different culture environments. Detailed CYP 3A function and expression in comparable rat systems has not been reported. However, rat hepatocytes co-cultured with biliary epithelial cells (BECs) have been shown to maintain acute phase protein production and non specific total P450 content (Guillouzo *et al,* 1984; Begue *et al,* 1984), and co-cultures with the stellate cell line CFSC-2G have been shown to reduce albumin production (Arnaud *et al,* 2003) or elevate albumin mRNA expression (Rojkind *et al,* 1995). This implies that the functional benefits of co-culture are obtained through a range of

different co-culture partners and raises questions regarding the specificity of the support. A further study to compare a range of alternative co-cultures and endpoints in a single lab is required to ascertain if one cell type offers clear benefits over the others.

3.4.5 Conclusions

This work demonstrates the need for a well characterised stable system. The volatility of function in the first few hours of culture, the point at which many assays are currently conducted, and the deterioration of function over the longer term are both barriers to producing a culture system that can be validated to regulatory requirements or deliver the stability of function required for a BAL. The co-culture of hepatocytes and hepatic stellate cells provides a functional improvement on standard mono-culture techniques through a reduction in the initial peak of P450 function and an increased longevity of P450 function in both human and rat cultures. This work in conjunction with that of others indicates the initial rise in function is part of the dedifferentiation process as opposed to a general recovery of cell function, and the whole process is slowed by co-culture with stellate cells. However, the overall benefit of the hepatocyte - stellate cell co-culture technique with respect to other modified culture systems remains equivocal.

CHAPTER 4

Maintenance of mRNA Expression in Hepatocyte – Stellate Cell Co-culture

4.1 Introduction

The production of liver enzymes and liver derived blood proteins is the culmination of a complex regulatory network. Experiments on hepatocyte culture systems in vitro have predominantly considered final function as opposed to the underlying gene transcription. This is probably partially due to the potential scope of gene transcription studies. A transcription factor network containing multiple cross-talking components underlies the transcription and translation of mRNA for a single metabolic enzyme or blood protein (Review: Akiyama and Gonzalez, 2003). Furthermore, such networks are not discrete. For these reasons it is easier to draw simple conclusions, with immediate relevance to an in vitro systems end goals, from a functional assay such as that described in chapter 3. However, understanding the patterns of gene transcription underlying function, and ultimately controlling them, will be essential in achieving prolonged and manipulable hepatocyte function in vitro.

4.1.1 Prolonged cytochrome P450 mRNA transcription by hepatocytes in vitro

There are problems in direct comparability of transcriptional data from different research groups similar to those for comparisons of functional data. The consensus of information available suggests that P450 mRNA can be detected in isolated primary rat hepatocytes for several days in standard mono-culture and up to a week in modified culture systems e.g. sandwich cultures or culture with inducers (Boess *et al*, 2003; Davila and Morris, 1999). The typical pattern

of P450 mRNA loss in isolated rat hepatocytes in the absence of inducers involves a rapid decrease over one or two days of culture, followed by very low sustained expression if a modified culture system is used (Hodgkinson, *et al* 2000; Fujita *et al*, 2005). A comprehensive micro-array study to detect differences in total gene expression profile between various culture techniques found increasing divergence relative to intact liver across liver slices, sandwich culture, standard mono-layer culture and several hepatic cell lines (Boess *et al*, 2003). Unsurprisingly, divergence from intact liver also increased with time in culture. However, rapid down regulation of P450 mRNA was still observed in all systems.

4.1.2 Liver regeneration and the roles of HGF and TGFβ_1

Cytokines are a vital element of hepatocyte regulation. HGF and TGFβ_1 are of particular interest due to their roles in regulating liver regeneration. They are both induced rapidly by chemical or physical liver injury (Asami *et al*, 1991; Mitsue *et al*, 1995) and have a complex relationship with other cytokines. HGF is vital for successful liver regeneration subsequent to partial hepatectomy (Phaneuf *et al*, 2004; Burr *et al*, 1998) acting through modulation of TGFα (Tomiya *et al*, 1998, 2000). TGFβ_1 inhibits proliferation of hepatocytes, up regulates HGF and down regulates TGFα (Nozato *et al*, 2003). It is also thought to play some role in inducing hepatocyte apoptosis (Samson *et al*, 2002), although it is not thought to be responsible for termination of regeneration (Oe *et al*, 2004), and to have a general differentiating effect. The ability of regenerating liver hepatocytes to activate latent TGFβ_1 provides

further evidence of the importance of this cytokine in hepatic regeneration (Jakowlew *et al*, 1991). Further interplay between these cytokines is observed in the capacity of $TGF\beta_1$ to enhance $TGF\alpha$ mediated cell motility, yet drastically reduce HGF mediated chemotaxis (Stolz and Michalopoulos, 1998).

4.1.3 The reverse transcription – polymerase chain reaction technique

In this chapter reverse transcriptase polymerase chain reaction (RT-PCR) is used to investigate levels of significant mRNAs in co-culture and mono-culture. RT-PCR is a powerful technique for assaying mRNA levels. Cells are lysed and processed to isolate total RNA using a simple partition method. Total RNA is then reverse transcribed to cDNA. The cDNA is magnified through PCR using a specialised probe. The probe is modified to have a fluorescent reporter marker at the 5' end and a fluorescence quencher at the 3' end. If the target sequence is present the probe will anneal to the cDNA downstream of one of the primer binding sites (fig 4.1 A). At this point, whilst the probe is intact, fluorescence is effectively quenched by the proximity of the quencher. In a PCR cycle, primers bind and the probe is displaced and broken down by the 5' nuclease activity of the DNA polymerase as the primer is extended (fig 4.1 B). Release of the fluorescent reporter from the proximity of the quencher during polymerisation results in a rise in fluorescence proportional to the product produced (fig 4.1 C).

Figure 4.1

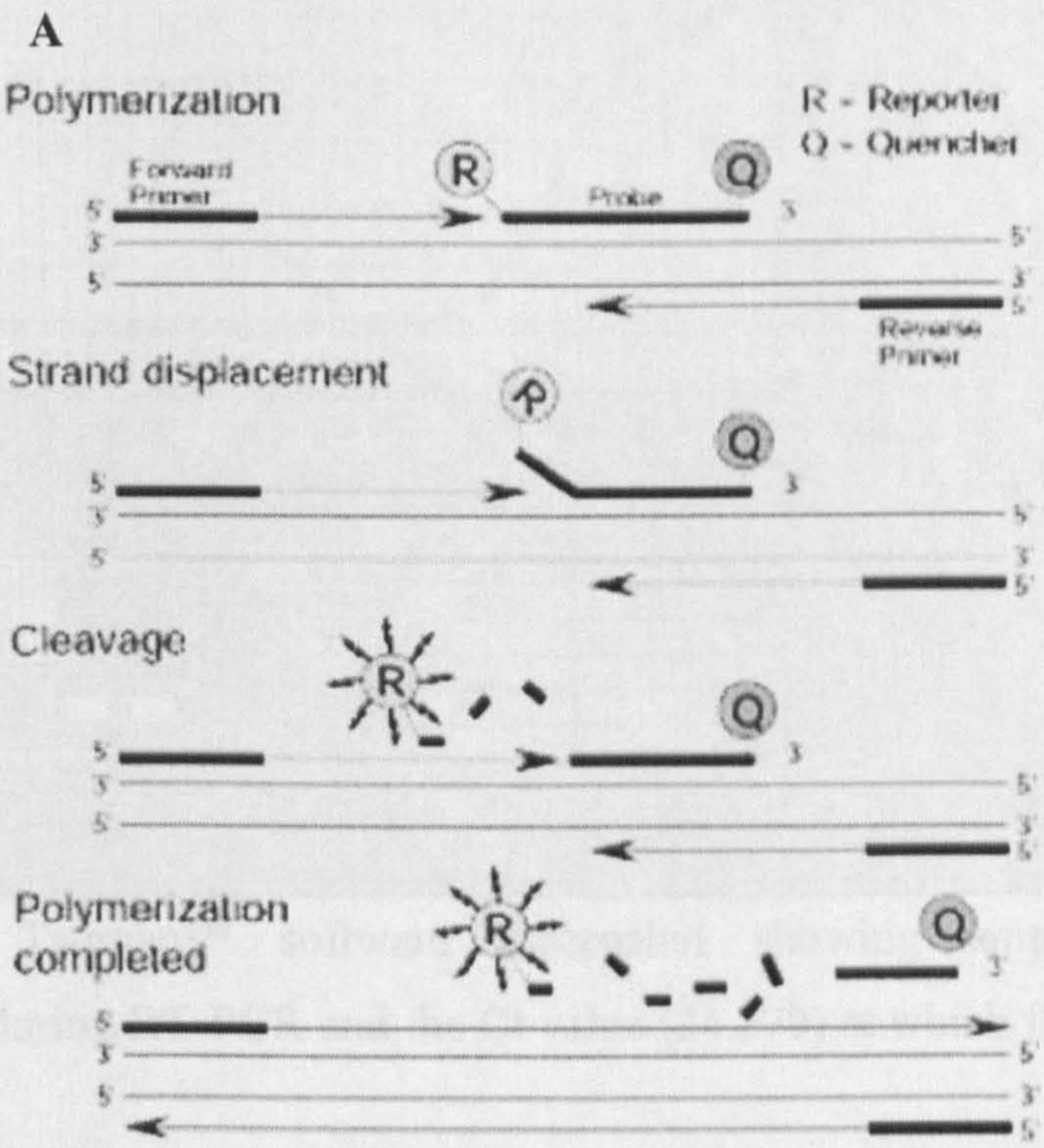


Figure 4.1: The process of PCR and associated fluorescent signal amplification.

In the work described here the Taqman™ real time PCR system is used. The innovation of real time PCR allows monitoring of fluorescence throughout the PCR cycling so the cycle number at which fluorescence is first detected (cycle threshold (Ct)) can be used instead of analysing fluorescence after a set number of cycles. Figure 4.2 shows a chart of fluorescence plotted against number of PCR cycles, with an arrow to show the Ct value. The greater the quantity of starting product, the lower the Ct i.e. the less PCR cycles required to reach a detectable quantity of product. Figure 4.3 shows a chart of changes in detection of the quencher and reference dye.

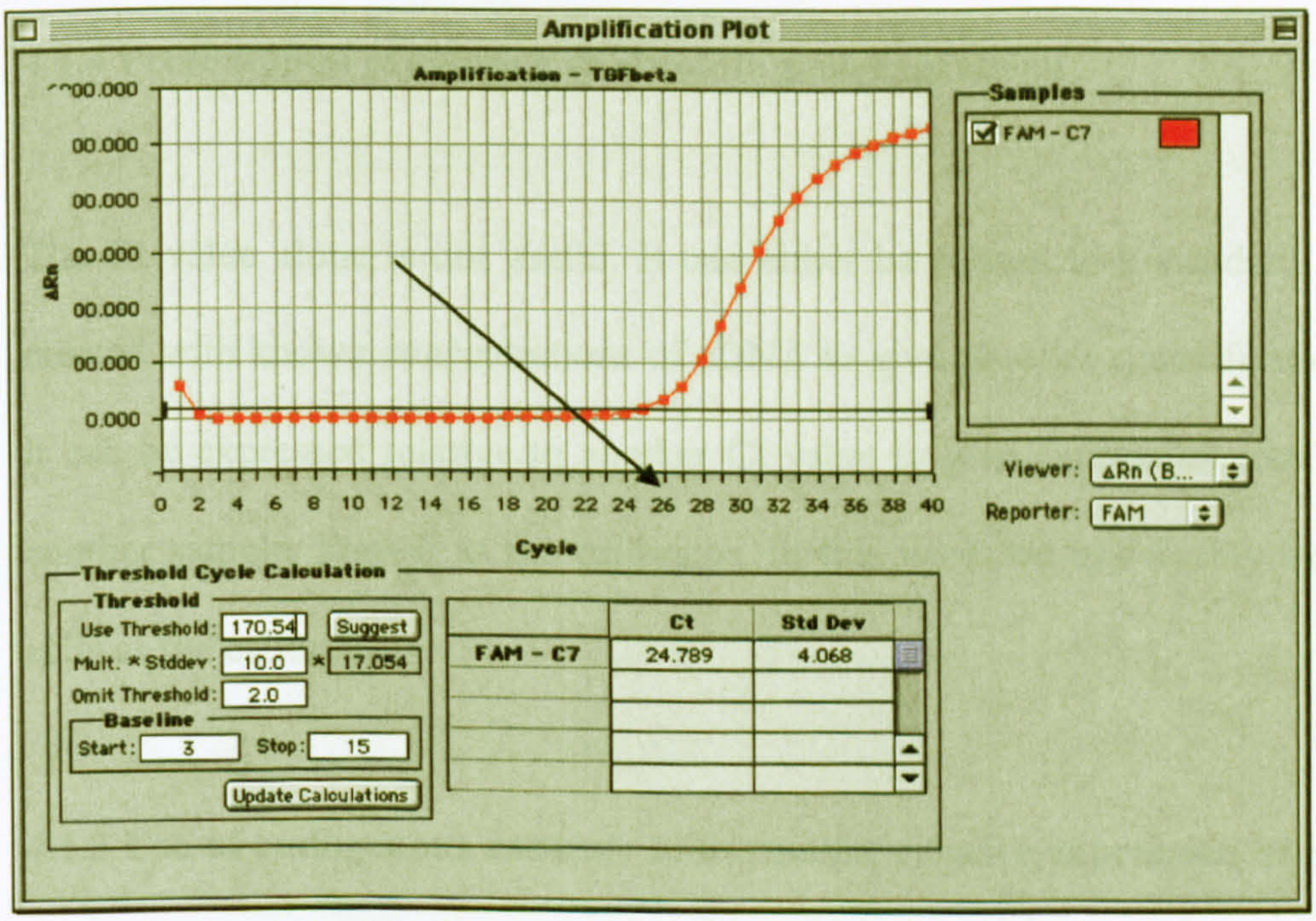


Figure 4.2: Taqman™ software screenshot showing amplification of fluorescence during RT-PCR and the Ct value (24.279) at which fluorescence is first detected.

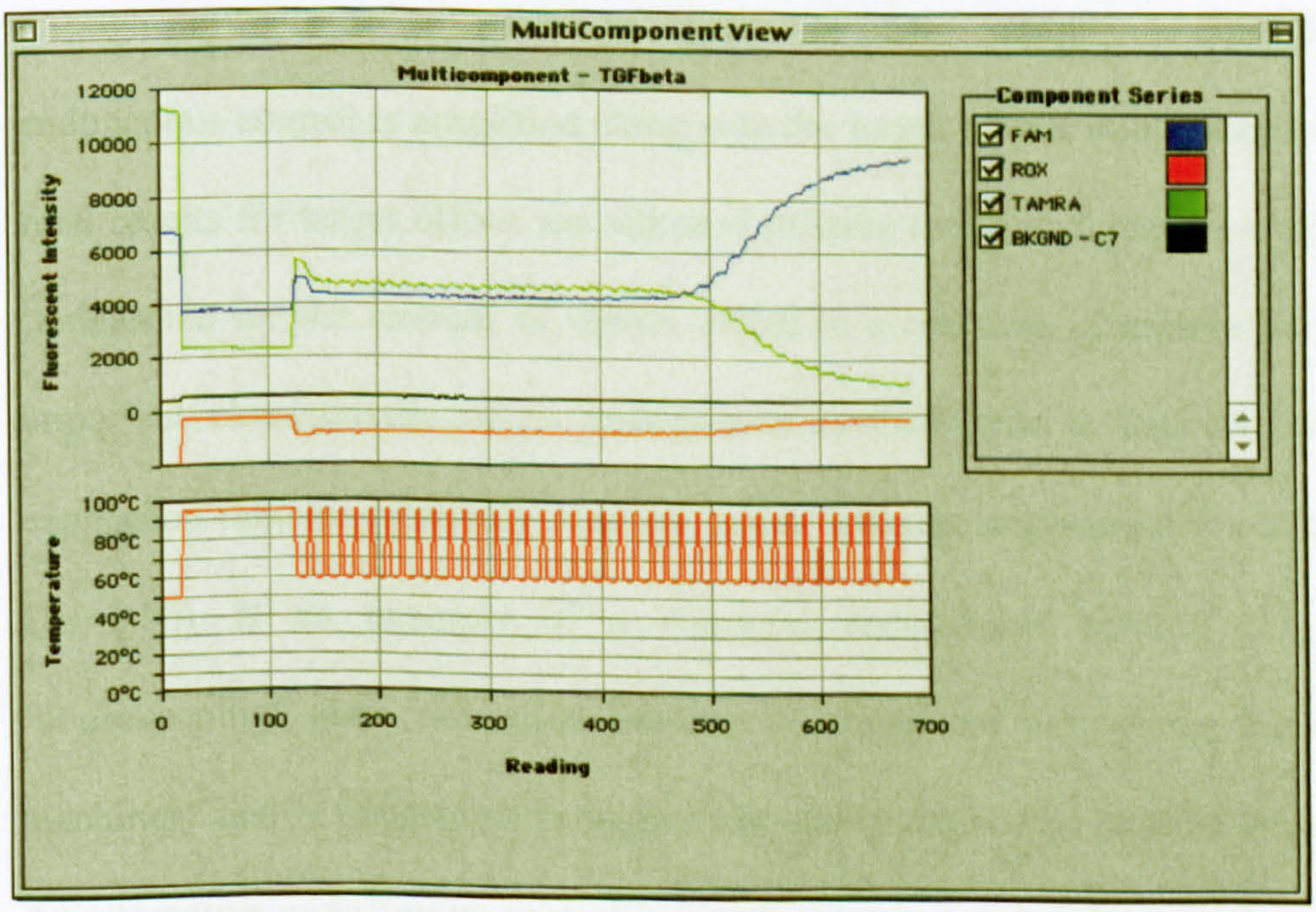


Figure 4.3: Taqman™ software screen shot shows the real time change in FAM (target label) and TAMRA (quencher) detection with respect to PCR heat cycle. Reference dye ROX, to control for detector variation, and background fluorescence are also shown.

4.1.4 Presentation of relative vs absolute gene expression

The Ct value alone is not useful. It can either be related to a standard curve created with known concentrations of cDNA to give absolute quantification, or it can be expressed relative to another Ct value to give expression relative to another sample, known as the calibrator. In this work we use freshly isolated cells as the calibrator.

4.1.5 Use of endogenous controls to normalise relative expression of target cDNA

Accuracy of quantification of the changes in mRNA content can be enhanced through normalisation of results to expression of an endogenous control. The endogenous control is amplified along side the target cDNA in all samples, and final results for target cDNA are adjusted relative to the endogenous control to standardize for the amount of cDNA added to a reaction. Therefore the most important characteristic of an endogenous control gene is that its level of expression remains relatively constant under different experimental conditions. 18s RNA is an example of a common endogenous control, termed a 'housekeeping' gene, so called because it constitutes part of the basic cell machinery and is consequently highly and stably expressed in most situations. An alternative endogenous control in hepatocytes is porphobilinogen deaminase (PBGD), a liver enriched protein that catalyses polymerisation of porphobilinogen molecules. However, no single endogenous control gene always manifests stable expression levels under all experimental conditions and

it is therefore necessary to characterize the suitability of various housekeeping genes to serve as endogenous controls under particular experimental conditions.

4.1.6 Aim of chapter

The studies described in this chapter were designed to investigate the levels of mRNA for various functional enzymes and cytokines in the hepatocyte stellate cell co-culture system. Specifically to:

- Design and validate Taqman™ PCR primer and probe sets for mRNA targets.

- Identify an appropriate method of presenting relative expression of mRNA targets i.e. adjusted to an endogenous control such as 18s mRNA or PBDG mRNA, or as a proportion of total mRNA.

- Apply the technique to investigate the levels of P450 3A, P450 2E1, P450 1A2, HGF and TGFβ_1 mRNA in co-culture.

4.2 Materials and methods

4.2.1 Cell isolation and culture

Cells were cultured as described in chapter 2.

4.2.2 RNA isolation and cleaning

Total RNA was isolated from five day old cultures using Trizol™ (Invitrogen) reagent according to the manufacturer's protocol. The RNA was cleaned using a Minieasy™ kit (Quiagen) and quantified using a Nanodrop™ Spectrophotometer. Quality of the RNA prep was verified by gel electrophoresis.

4.2.3 Reverse transcription reaction

The reverse transcription reaction was performed with Taqman™ RT reagents (Applied Biosystems) according to the manufacturer's instructions. Typically 500 ng to 1 μg total RNA was obtained, and mixed with the appropriate ratio of RT buffer, $MgCl_2$, deoxynucleotide triphosphates, random hexamers, RNase inhibitor and multiscribe reverse transcriptase.

4.2.4 PCR

PCR primers and probes used for quantitation of P450 3A, 1A2, 2E1 and HGF cDNA were designed using Primer Express™ Software (Applied Biosystems) and blasted using the NCBI database. The P450 3A probe picks up both 3A1 and 3A23. Primer and probe combinations were optimized (Table 4.1). The 18s and PBGD primer/probe sets were bought from Roche and used as specified in the manufacturers instructions. The probes were labeled with a 5′-reporter fluorescent dye (6-carboxyfluorescein) and a 3′-quencher dye (6-carboxy-tetramethyl-rhodamine). PCR reaction mixtures were made up according to the manufacturer's instructions in 25 µl triplicates for each target cDNA, using 12.5 µl platinum qPCR supermix UDG™ (Applied Biosystems), the specified primer/probe concentrations (Table 2.1), and 10 ng of cDNA. Positive controls were derived from freshly isolated hepatocytes. Amplification was carried out in 96-well thermal cycler plates (Alpha Labs) in an ABI Prism 7700 Sequence Detection System™ (Applied Biosystems). cDNA standard curves were created using dilution of freshly isolated hepatocyte cDNA, and sample values were compared to this calibration curve and converted to a percentage of freshly isolated cell expression.

4.2.5 Data handling and experimental design

Data is expressed as mRNA content relative to freshly isolated cells. Co-cultures are expressed relative to a co-culture made of freshly isolated hepatocytes and pre-cultured stellate cells in co-culture, and mono-culture is

expressed relative to a freshly isolated Percoll™ purified hepatocyte pellet. N numbers are given for each individual experiment in the results. Charts are shown with error bars representing the standard error of the mean.

Table 4.1

3A1/3A23	Forward Primer	5' TTATGCTCTTCACCGTGATCCA 3'	900 nmol
	Reverse Primer	5' AATGCTGCCCTTGTTCTCCTT 3'	900 nmol
	Probe	5' CTGAACCTTTCTGGGCGAAATTCCTCA 3'	200 nmol
1A2	Forward Primer	5' CAACATTGTCAATGACATCTTTGGA 3'	900 nmol
	Reverse Primer	5' CCGTGGCTGCCGATCTC 3'	50 nmol
	Probe	5' CAATCACCGTGTCCAGCTCCTCATGAA 3'	200 nmol
2E1	Forward Primer	5' GGATCCAGCTTTACAATAACTTTGC 3'	300 nmol
	Reverse Primer	5' GTCCAGTGACTGAAGGTGTTCCT 3'	50 nmol
	Probe	5'TTTTCAAGTGTGTACTGTTTTATTTCAGACA CATTTTTCA 3'	25 nmol
$TGF\beta_1$	Forward Primer	5' AACAAGGTCTGGACTCACATGTTC 3'	900 nmol
	Reverse Primer	5' CGTCTGGCTCCCAGAAGATATG 3'	900 nmol
	Probe	5'CGGTGTAAATCCTCCATATTCTTGTCCCAC AT 3'	200 nmol
HGF	Forward Primer	5' AACAAGGTCTGGACTCACATGTTC 3'	900 nmol
	Reverse Primer	5' CGTCTGGCTCCCAGAAGATAT G 3'	900 nmol
	Probe	5'CGGTGTAAATCCTCCATATTCTTGTCCCAC AT 3'	200 nmol

Table 4.1: The designed Taqman© RT-PCR primer and probe sequences for the cDNA targets and their final concentrations in the reaction mixtures.

4.3 Results

4.3.1 PCR primer and probe optimisation and validation

In a perfect system every PCR cycle will result in a doubling of cDNA i.e. 100% efficiency. In reality, primer and probe concentrations are optimised to achieve between 90 and 110% efficiency. As the increase in cDNA is exponential, a plot of log starting cDNA concentration against the Ct value gives a straight line, the gradient of which corresponds to the efficiency. Figure 4.4 shows a demonstration graph of Ct values plotted against log cDNA concentration for 90, 100 and 110% efficiency and the corresponding gradients. This defines the range within which the gradients of the validation graphs for each primer probe set must fall. Figure 4.5 shows an example validation graph including gradient and correlation coefficient for the P450 3A primer and probe set; table 4.2 shows this validation data for the other primer probe sets.

Figure 4.4

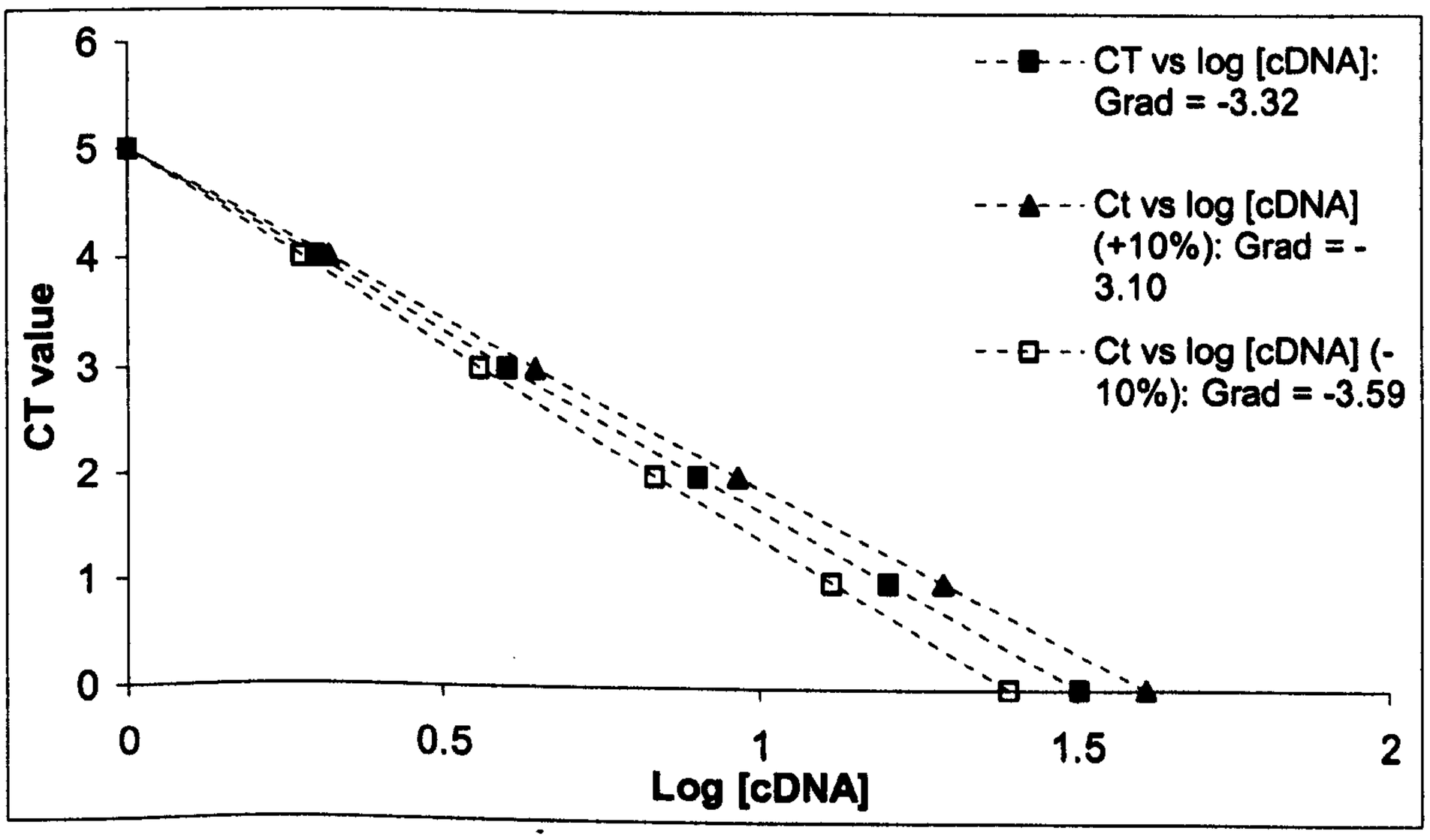


Figure 4.4: A demonstration graph showing gradients of CT value vs log cDNA concentration for hypothetical 90%, 100% and 110% PCR efficiency.

Figure 4.5

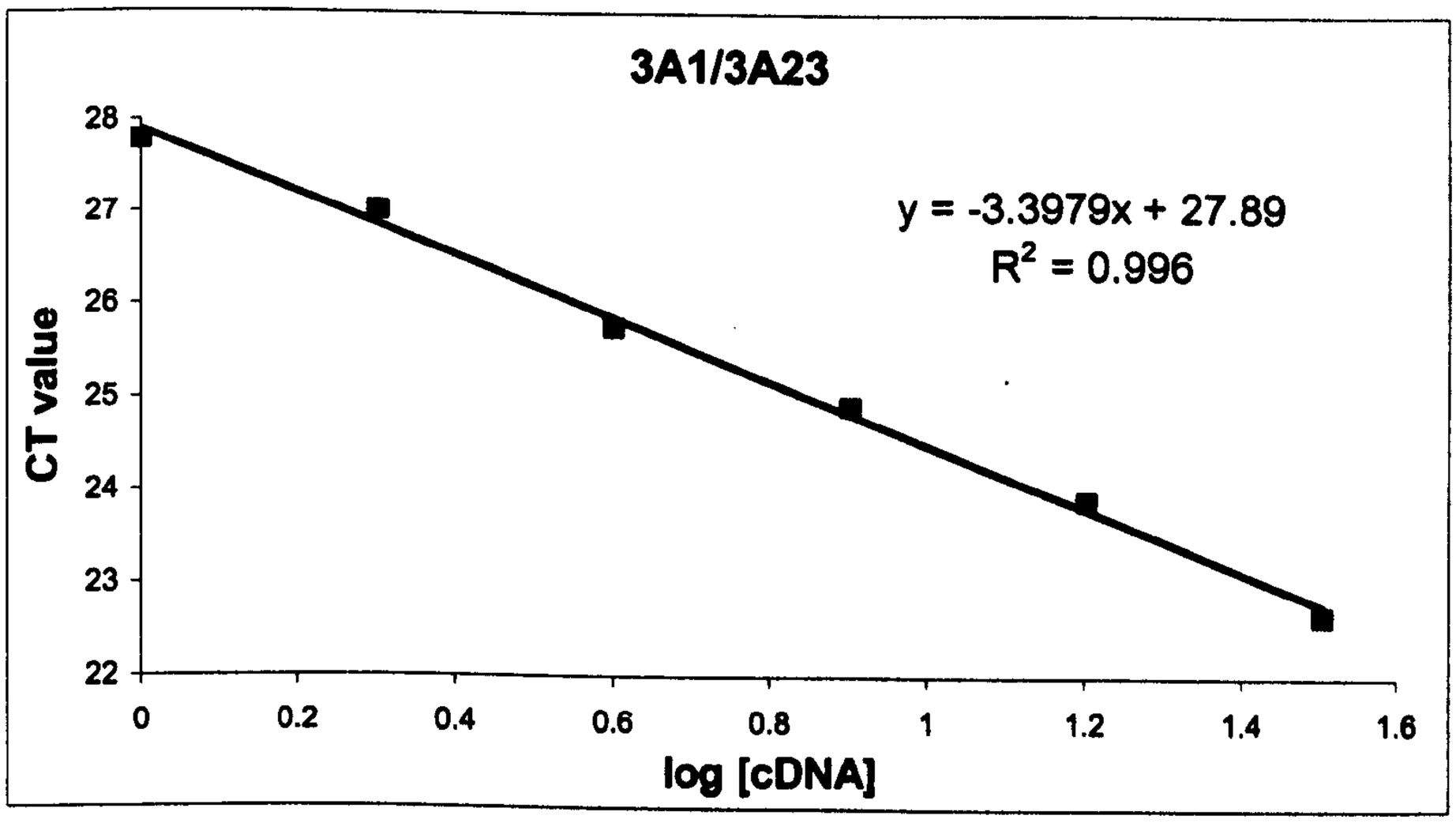


Table 4.2

	Gradient	% PCR efficiency	Correlation coefficient
PBGD	-3.05	110.4	0.999
18s	-3.37	98.0	0.998
3A1/3A23	-3.40	96.8	0.996
2E1	-3.39	97.2	0.972
1A2	-3.34	99.3	0.983
HGF	-3.50	93.0	0.993
$TGF\beta_1$	-3.68	87.1	0.968

Figure 4.5: An example validation graph for the PCR primer and probe set for P450 3A1/3A23 cDNA.

Table 4.2: PCR efficiency data for other similarly validated cDNA primer and probe sets.

4.3.2 Endogenous control expression

18s RNA and PBGD mRNA were investigated as potential endogenous controls. An endogenous control must be stably expressed over time and different experimental conditions to provide a reliable normalising value. However, 18s RNA Ct values became increasingly erratic with time in culture. At the start of culture Ct values were approximately 10 for both mono and co-cultures. After 2 days in culture Ct values for co-cultures were approximately 12 compared with 15-20 for mono-cultures. $P \leq 0.05$ for differences between co-culture and mono-culture on PLA (Tukey's post-test) but statistical confidence was lower than this for the difference between TCP mono-culture and co-culture. After 5 days in culture Ct values were about 15-20 for co-culture compared with negligible values for mono-culture (erratic Ct values above 30) (figure 4.6). The variation of 18s RNA with respect to both time and culture conditions makes it doubly unsuitable as an endogenous control in this instance. Expression of PBGD mRNA was not detected after 4 days in culture. Consequently, data was not normalised to an endogenous control and was therefore expressed relative to total RNA.

Figure 4.6

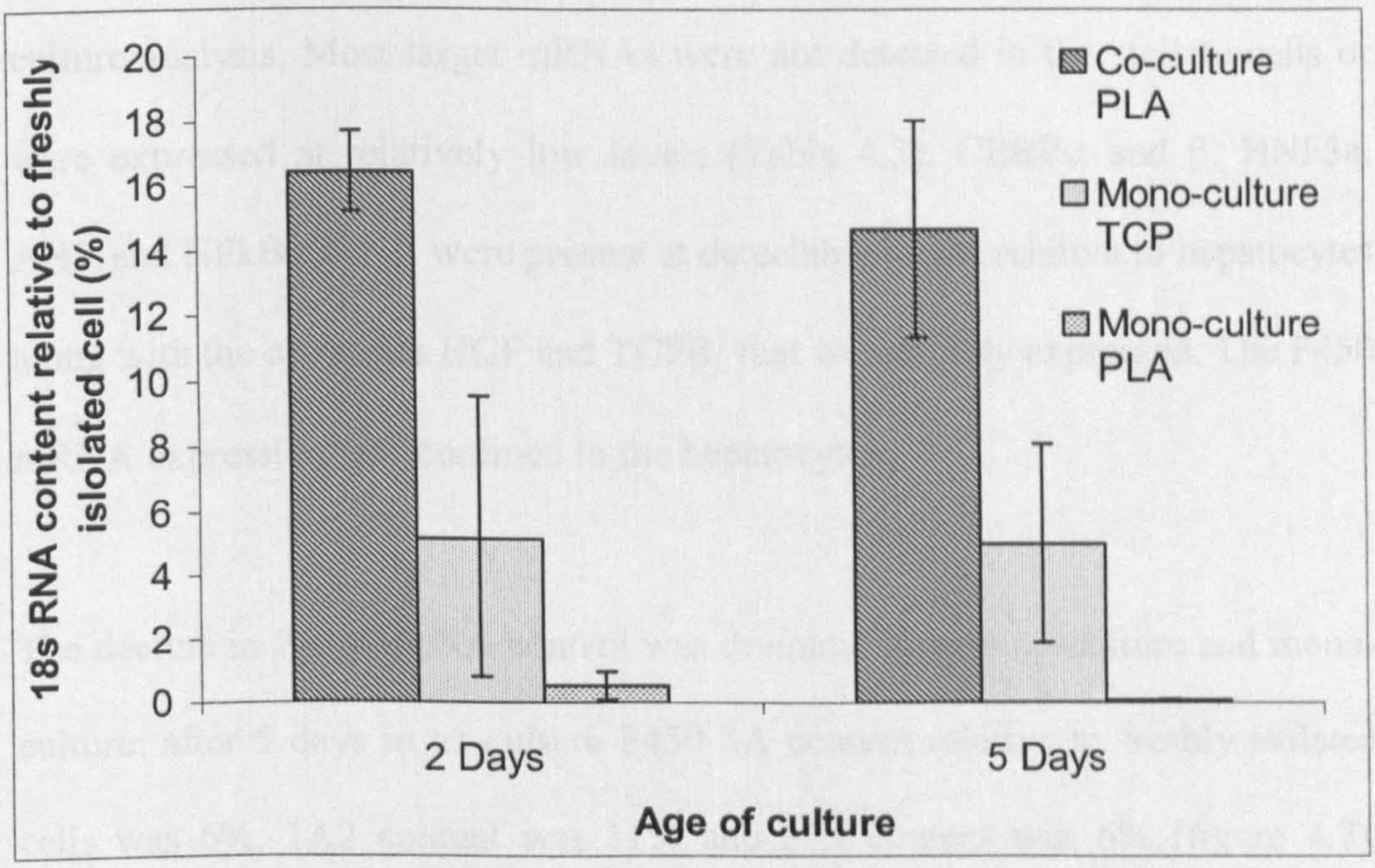


Figure 4.6: The decrease in expression of 18s RNA in cells maintained in hepatocyte stellate cell co-culture or hepatocyte mono-culture on TCP or PLA culture surfaces (N=3).

4.3.3 Liver enriched gene transcription in mono-cultures and co-culture

Expression of mRNA for three P450 liver enzymes and various liver enriched transcription factors and cytokines were determined in stellate cell mono-cultures relative to freshly isolated hepatocytes. This was to give an indication of the likely cell type responsible for mRNA expression in subsequent co-culture analysis. Most target mRNAs were not detected in the stellate cells or were expressed at relatively low levels (Table 4.3). CEBPα and β, HNF3a, AHR and NFkB mRNA were present at detectable levels relative to hepatocytes along with the cytokines HGF and TGFβ_1 that were highly expressed. The P450 mRNA expression was confined to the hepatocytes.

The decline in P450 mRNA content was dramatic in both co-culture and mono-culture: after 5 days in co-culture P450 3A content relative to freshly isolated cells was 6%, 1A2 content was 11% and 2E1 content was 6% (figure 4.7). However, the content of P450 mRNAs in the mono-culture had declined more than in the co-culture spheroids: P450 3A was 5% of that in co-culture, P450 1A2 content 43% of that in co-culture and P450 2E1 content 41% of that in co-culture (figure 4.8).

No RNA was obtained from 5-day-old mono-cultures on $P_{DL}LA$. The expression of the liver enriched transcription factors was erratic and inconsistent and data is not presented.

Table 4.3

2E1	AHR	CEBPβ	CEBPα	HNF1α	HNF3α
-	0.53	4.32	0.08	-	0.13

HNF3β	HNF4α	NFkB	PXR	3A	1A2
-	-	2.77	-	-	-

HGF	$TGF\beta_1$	IL-6	PDGF
4.57	47.92	-	-

Table 4.3: Expression of markers of hepatocyte functionality and certain cytokines in stellate cells relative to freshly isolated hepatocytes (expression in hepatocytes is 1): Any expression of P450 mRNA detected in co-culture can therefore probably attributable to hepatocytes (N=3).

Figure 4.7

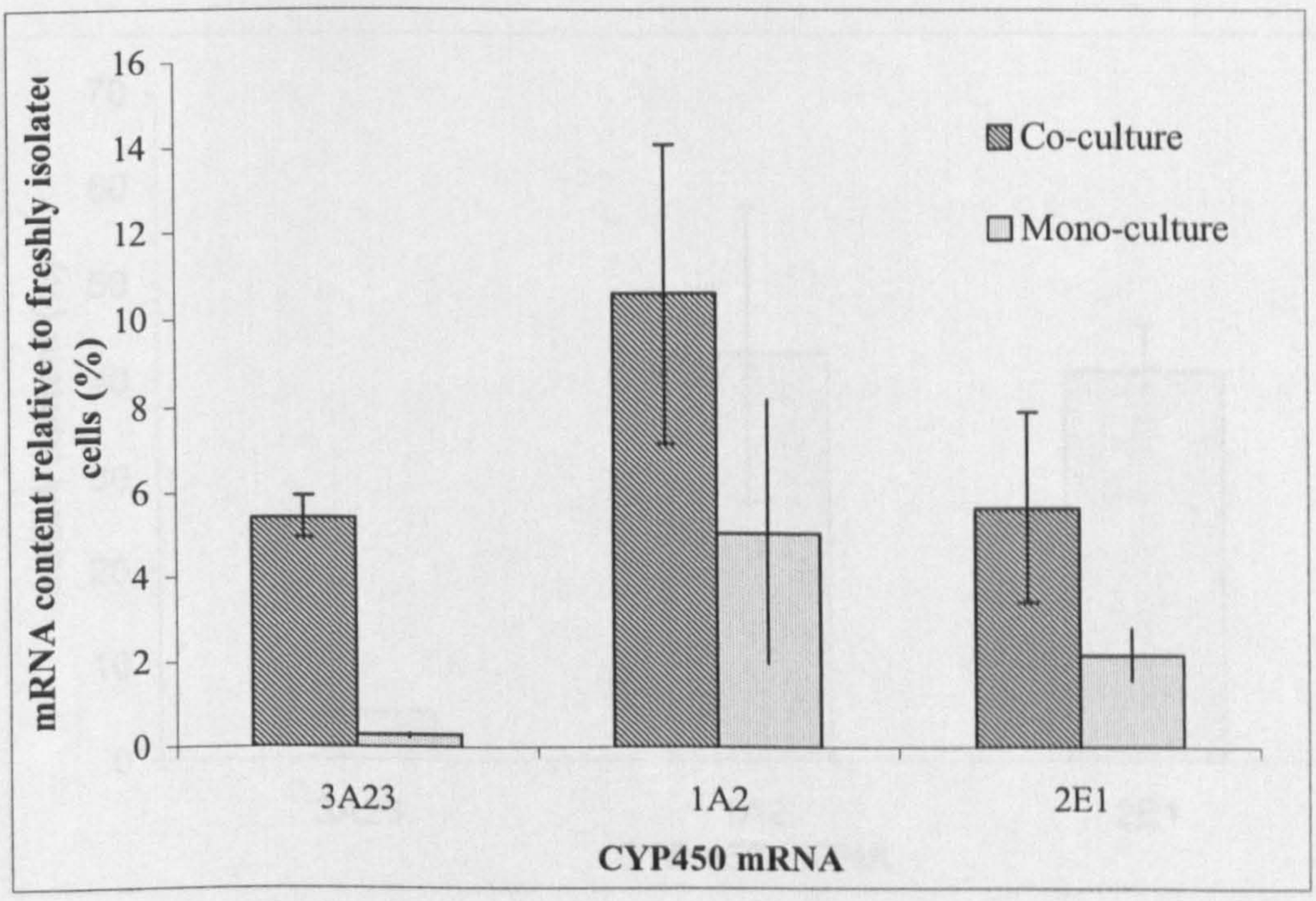


Figure 4.7: The content of three P450 mRNA targets relative to freshly isolated cells after 5 days in hepatocyte stellate cell co-culture on a PLA surface or hepatocyte mono-culture on a TCP surface (N=2).

Figure 4.8

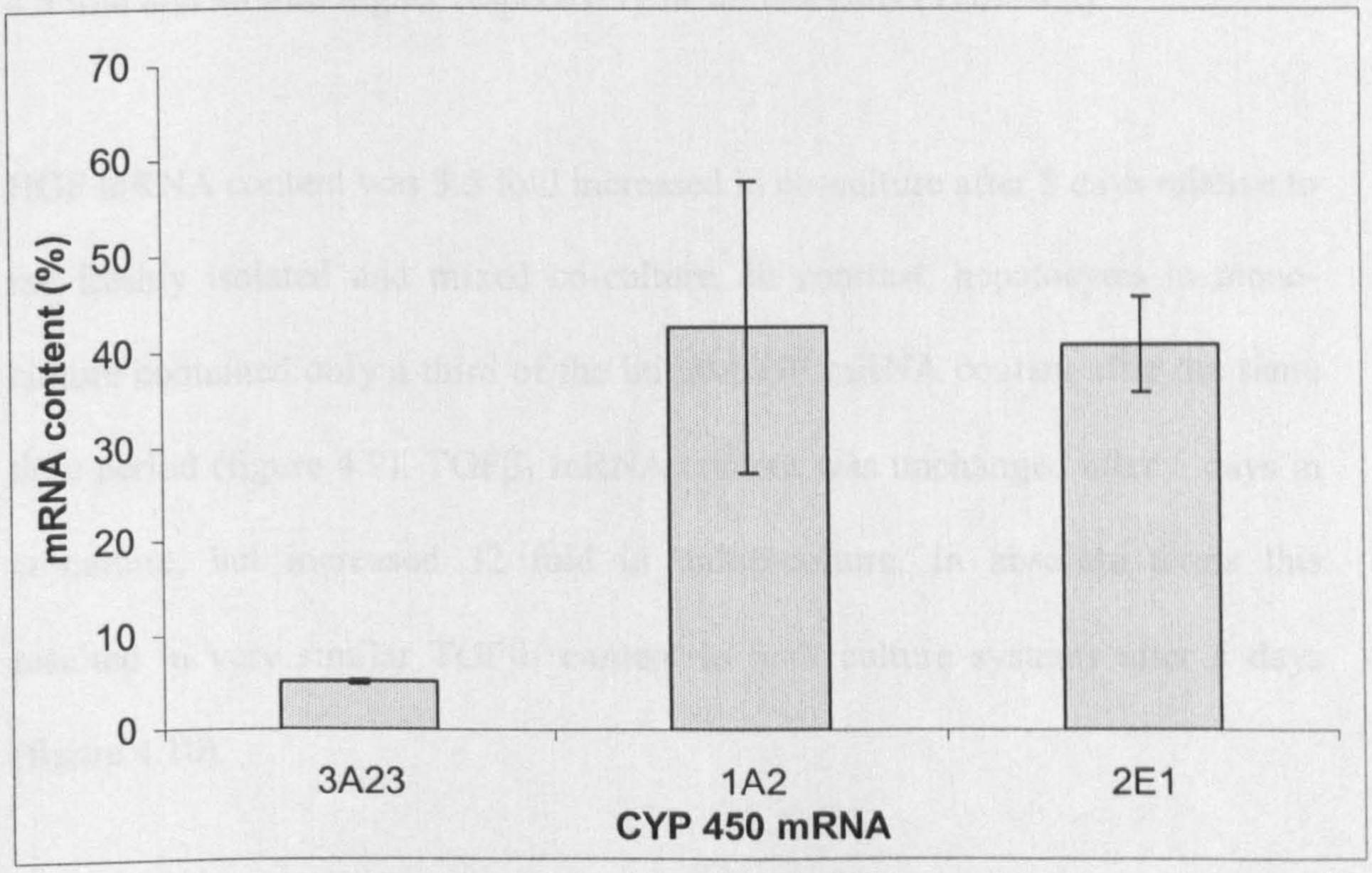


Figure 4.8: The content of three P450 mRNA targets in hepatocytes mono-cultured on TCP relative to hepatocytes and stellate cells co-cultured on PLA after five days (N=2).

4.3.4 Cytokine transcription in mono cultures and co-culture

The stellate cell monoculture was screened for expression of HGF, TGFβ_1, IL-6 and PDGF. HGF and TGFβ_1 were both detected whilst IL-6 and PDGF were not. HGF and TGFβ_1 were therefore investigated in co-culture. HGF and TGFβ_1 were also present in freshly isolated hepatocytes. However, content was 4.5 fold and 48 fold higher respectively in stellate cells (Table 4.3).

HGF mRNA content was 3.5 fold increased in co-culture after 5 days relative to the freshly isolated and mixed co-culture. In contrast, hepatocytes in mono-culture contained only a third of the initial HGF mRNA content after the same time period (figure 4.9). TGFβ_1 mRNA content was unchanged after 5 days in co-culture, but increased 32 fold in mono-culture. In absolute terms this resulted in very similar TGFβ_1 content in both culture systems after 5 days (figure 4.10).

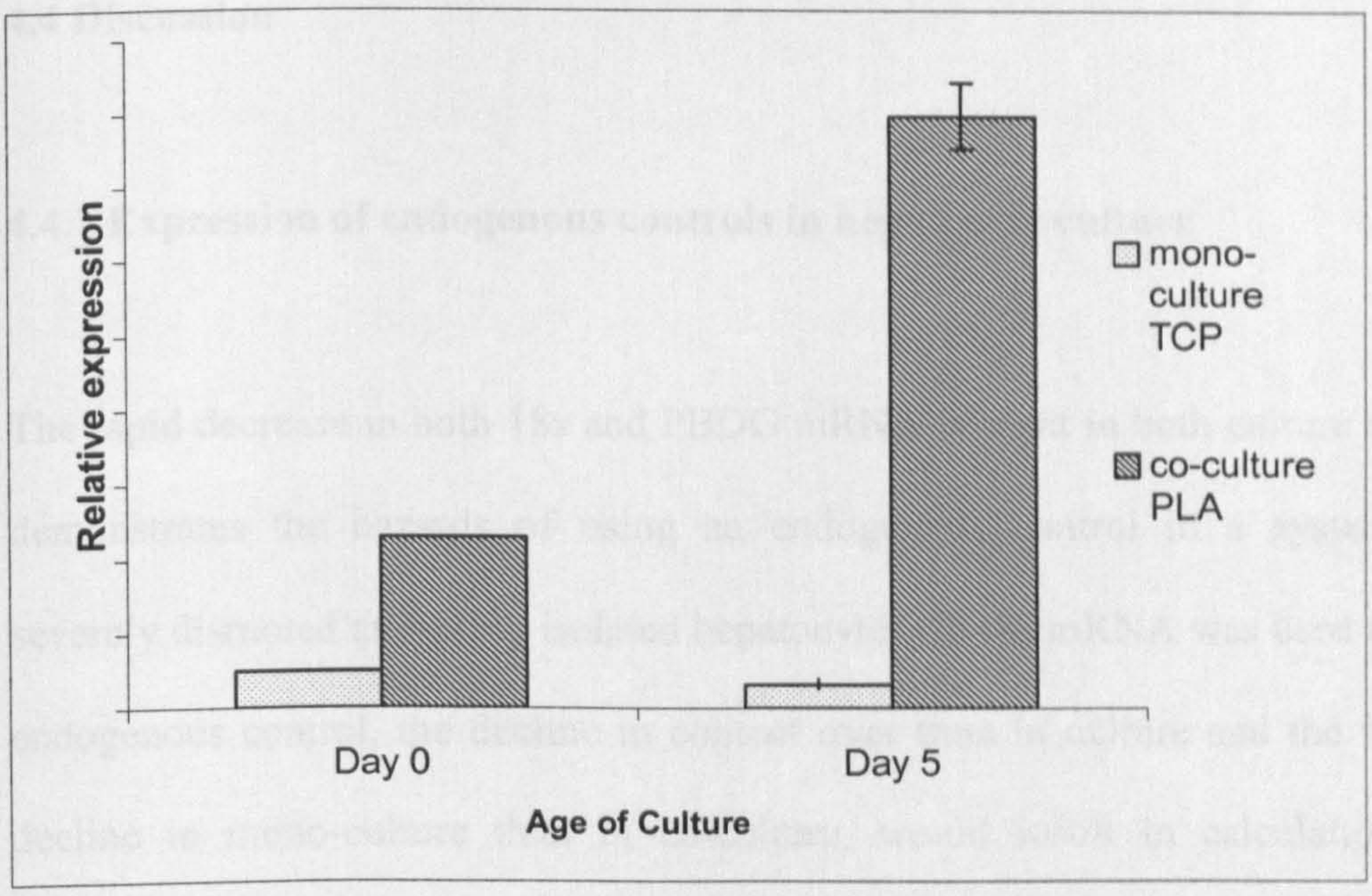


Figure 4.9: The content of HGF mRNA after five days in hepatocyte-stellate cell co-culture or hepatocyte mono-culture on TCP expressed relative to freshly isolated cell cultures.

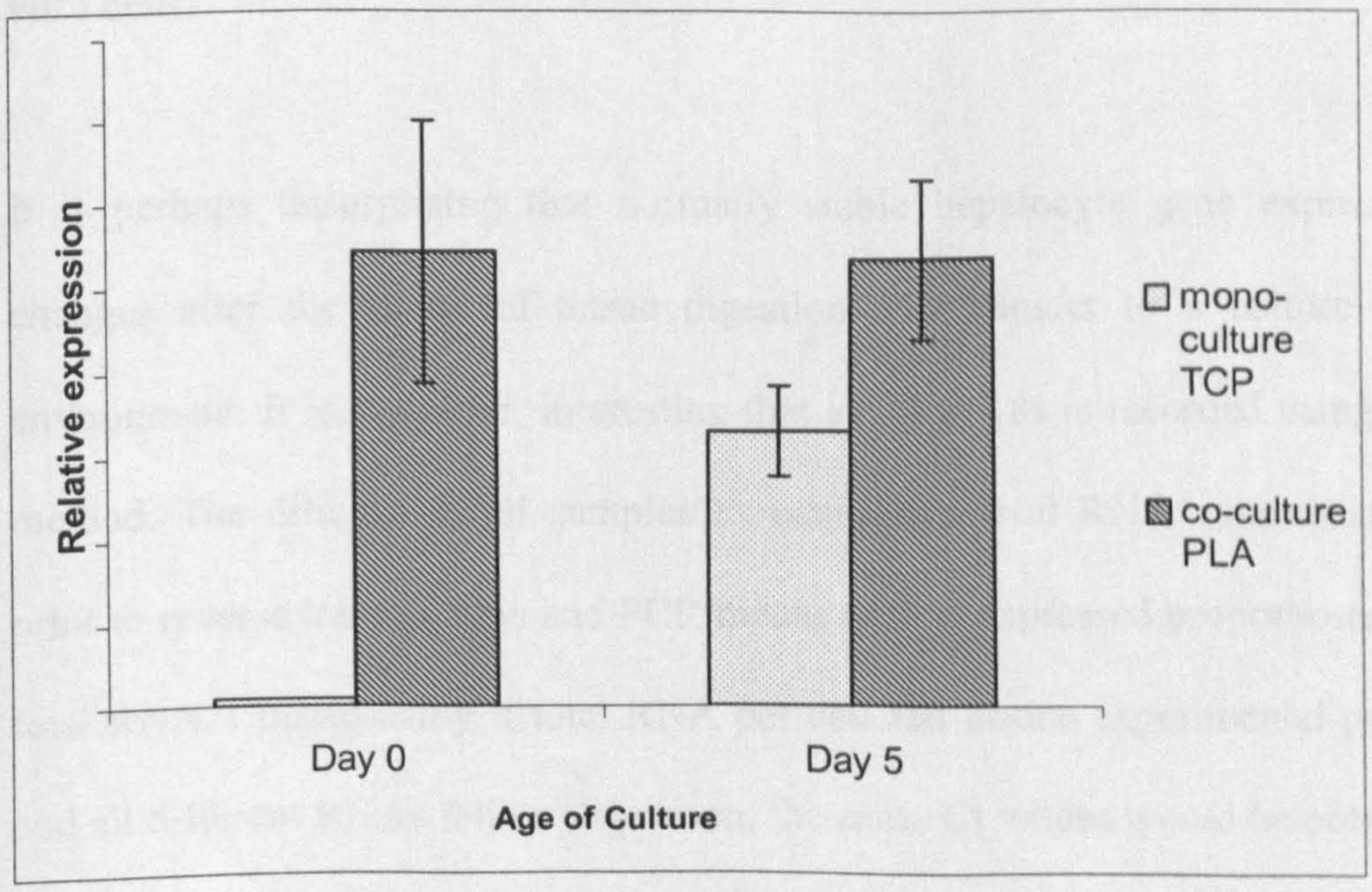


Figure 4.10: The content of TGFβ_1 mRNA after five days in co-culture or mono-culture on TCP expressed relative to freshly isolated cell cultures.

4.4 Discussion

4.4.1 Expression of endogenous controls in hepatocyte culture

The rapid decrease in both 18s and PBDG mRNA content in both culture types demonstrates the hazards of using an endogenous control in a system as severely disrupted as freshly isolated hepatocytes. If 18s mRNA was used as an endogenous control, the decline in content over time in culture and the faster decline in mono-culture than in co-culture, would result in calculation of artificially high levels of expression of the gene of interest in later stages of culture relative to earlier stages and in mono-culture relative to co-culture. The usual stable nature of 18s RNA content emphasises the need for caution when using endogenous controls to identify gene expression in rapidly changing in vitro cells.

It is perhaps unsurprising that normally stable hepatocyte gene expression changes after the shock of tissue digestion and transfer to a culture dish environment. It is, however, interesting that a fall in 18s is recorded using this method. The dilution of all samples to equivalent total RNA concentrations prior to reverse transcription and PCR means data is expressed proportionate to total RNA. Consequently if total RNA per cell fell in one experimental group, and all different RNAs fell in proportion, the same Ct values would be obtained as would be obtained from an alternative sample if everything remained unchanged at initial levels. Therefore, a fall in 18s must imply a rise, or a slower fall, of other RNAs. A possible explanation for this result is a large

synthesis of certain RNAs in response to the shock of isolation. In this case, 18s expression would appear to have fallen as a proportion of the whole. This is supported by reports that a majority of genes are up regulated in culture; compared to those genes down regulated, four times as many genes' expression increased by more than 6 fold in a number of culture systems (Boess *et al*, 2003). Genes involved in both apoptosis and proliferation are significant amongst those up regulated. A further hazard in interpreting the data is identified by reports of variable mRNA stability in hepatocytes in vitro post isolation (Hodgkinson *et al*, 2000). The content of mRNA is not, therefore, a strict guide to transcription but the net effect of a change in transcriptive and degradative processes.

These results do not invalidate the studies, but certainly require that interpretation is cautious, and clearly invalidate the use of either 18s or PBDG as an endogenous control. Therefore, results are not related to any internal standard and simply show whether the mRNA of interest represents a smaller or larger proportion of total RNA.

4.4.2 Complexities of attributing gene expression in co-culture

Analysis of gene expression in co-culture is problematic due to uncertainties regarding the cell source of mRNA. Although stellate cell phenotype probably changes in response to the co-culture environment, analysing cells in mono-culture is the best indicator for allowing cell expression to be attributed to either cell type in co-culture without developing complex cell separation protocols.

The lack of or low expression of P450 mRNA in stellate cell mono-culture makes it unlikely that stellate cells are the source in co-culture. However this is not the case for HGF and TGFβ_1 expression (see section 4.4.4).

Co-culture analysis is further complicated by the uncertainty of changes in cell populations during culture. In mono-culture hepatocyte populations reduce and stellate cells increase. If this occurred in co-culture, expression of a hepatocyte specific gene relative to total RNA using the methods described would be artificially small.

4.4.3 Liver enriched gene transcription in hepatocyte – stellate cell co-culture and hepatocyte mono-culture

The hepatocyte – stellate cell co-culture environment supports a higher maintenance of P450 1A2, 2E1 and 3A mRNA expression than a hepatocyte mono-culture environment. Expression of the results proportional to the total RNA discounts the possibility that this could be due to greater cell survival in the co-culture system. The best comparison in the literature is provided by a microarray study (Boess *et al*, 2003) that reported 18% of initial P450 3A1 and complete disappearance of 3A2 mRNA expression after 5 days in sandwich culture. The probe used in this work is sensitive to both these isoforms and reports about 6% expression after 5 days in co-culture. Therefore in terms of P450 3A expression the co-culture spheroids are probably not greatly dissimilar to sandwich culture after 5 days. The greater support of P450 3A expression relative to mono-culture compared with P450 1A2 or 2E1 expression (figure

4.8) is particularly encouraging as this is the most labile of the mRNAs studied. Although in these studies a single time point does not allow comment on the pattern of P450 mRNA loss, all literature (in the absence of inducers) reports a rapid fall, followed by extinction in standard culture, or by a low plateau in some modified culture systems (Boess *et al*, 2003, Hodgkinson, *et al* 2000; Fujita *et al*, 2005). The results are therefore hypothesised to represent expression during this reported plateau phase.

The data obtained in attempting to validate an endogenous control shows a rapid depletion of 18s RNA and PBDG mRNA. The decline in 18s expression indicates that basic cellular machinery deteriorates as fast as the liver enriched mRNAs. The slightly slower deterioration in co-culture suggests a modest braking effect on cell dedifferentiation. The 18s data also supports the P450 activity data in indicating that in the absence of stellate cells hepatocytes are most unstable under non-adherent conditions i.e. on a PLA surface.

Initial investigation of a range of liver enriched transcription factors generated no meaningful data. Inconsistency was observed with respect to culture time and culture systems. Unfortunately, although the co-culture environment is demonstrated to preserve function, it is still highly unstable. The results suggest that the disruption to the complex network controlling the expression of P450 mRNA is too severe to study in this system. It would be worth revisiting this if the system can be made more stable.

4.4.4 Cytokine expression

The content of HGF and TGFβ_1 in both hepatocyte and stellate cell mono-cultures prevents expression of these cytokines being attributed to a single cell type in co-culture. However, the increase in HGF mRNA content in co-culture to higher than that seen in either hepatocyte or stellate cell mono-culture indicates the co-culture environment is stimulating up regulation of HGF transcription in at least one cell type. Although up regulation of TGFβ_1 transcription is clear in mono-culture hepatocytes, net expression does not change significantly in co-culture. However, the initial higher levels in co-culture than hepatocyte mono-culture due to stellate TGFβ_1 expression could mask a number of scenarios i.e. any increase or decrease in expression from either cell type with a net effect of maintained expression.

Irrespective of cell source these cytokines have interesting implications in a culture system. TGFβ_1 has been credited with enhancing hepatocyte specific function in a 3T3-hepatocyte co-culture model (Chia *et al*, 2005). However, similarly high TGFβ_1 levels in monoculture as well as stellate cell co-culture suggests that this cannot be the sole factor in this case. The level of HGF could explain the observation that more hepatocytes enter S-phase in co-culture than in mono-culture (Lewis, 2003). The high expression of HGF and TGFβ_1 could be regarded as a post-traumatic response to isolation from irretrievably deteriorating cells, or alternatively, could be considered a small part of a more comprehensive regenerative environment (Fausto *et al*, 1995). Cytokines will

inevitably be an important component of regulating hepatocytes in an in vitro environment and the use of stellate cells as a co-culture source of cytokines is an alternative to ad hoc addition of purified cytokines to incubation media. This cell combination could potentially be stimulating other beneficial cytokines and raises the possibility of a self-regulating or user regulated system. For example, $TGF\beta_1$ depletion via antisense mRNA or antibody may reduce $TGF\beta_1$ mediated attenuation of the HGF/TGFα response with beneficial effects on replication and cell viability.

4.4.5 Conclusions

The co-culture model demonstrates prolonged elevated levels of P450 mRNAs, and has an effect on regulatory cytokine production. Unfortunately, the disruption to cellular machinery is too severe after isolation to use housekeeping genes to normalise mRNA expression, or to investigate the complexity of transcriptional regulation underlying the P450 mRNA levels. Although N=2 is too small to allow statistical analysis of P450 differences, the differences observed are substantial, repeated across all three P450 mRNAs and support the functional data in chapter 3. Gene array studies may be a method to further illuminate the control of expression in the spheroids and would allow interesting comparisons between spheroid gene expression profiles and regenerating liver, fibrotic liver and other liver states.

CHAPTER 5

Hepatocyte - Stellate Cell Co-culture Spheroids as a Model of Liver Toxicity

5.1 Introduction

Hepatotoxicity is a major cause of drug failure. This is due in large part to the idiosyncratic nature of liver toxicity and the differences between humans and preclinical species. A reliable high throughput toxicological screen is needed for early stage drug development to reduce the rate of late stage drug candidate attrition, improve drug safety, reduce animal use and improve assay reproducibility. A range of improved predictive in vitro models is being developed with these objectives.

5.1.1 In vitro models of hepatotoxicity

Hepatocytes in vitro rapidly lose toxicological sensitivity. In particular, metabolism mediated toxicity sensitivity is reduced in a matter of hours due to dedifferentiation and loss of P450 enzyme activity (Guillouzo, 1998). It has been suggested that this is associated with a pro-inflammatory response inherent to the collagenase method of cell isolation and is therefore a very early problem in the process of liver culture (Paine and Andreakos, 2004).

Success has been limited in developing systems that will identify complex or non-acute hepatic toxicity. Most trials involve using the prolonged function culture models discussed in previous chapters. The epithelial cell hepatocyte co-culture model can respond to repeated toxic inflammatory stimuli (Conner *et al,* 1990) and detects chronic low dose amitriptyline toxicity at concentrations not toxic over a 24 hour exposure period. However, it does not similarly detect

clonidine or malotilate toxicity and so lacks reliability (Ratanasavanh *et al,* 1988). Hep G2 and primary hepatocyte spheroid systems are both reliable indicators of hepatotoxicity for a number of compounds such as D-galactosamine, propranolol, diclofenac, and paracetamol if a range of functional and enzyme leakage endpoints are used (Xu *et al,* 2003). Spheroid systems also have altered triglycerides and raised cholesterol and esters suggesting a potential role in the study of fatty liver (Bollard *et al,* 2002). However, spheroids are reported to be less sensitive to the toxic effects of the well known hepatotoxin methotrexate than are cells in monolayer (Walker *et al,* 2000). Liver slices are an important liver toxicity model due to the maintenance of liver structure and cell type ratios; they can identify toxicity when more than one cell type is involved e.g. Kupffer cell mediated toxicity (Ishiyama *et al,* 1995) and can give information on lobular localisation of damage (Moronvalle-Halley *et al,* 2005). Liver slices also have an 80% correlation of gene expression in response to diverse toxins relative to the in vivo situation. However, these responses are far less sensitive and of lower magnitude than in vivo assays and are not significantly better correlated than isolated cells (Jessen *et al,* 2003). The development of bioreactors with graduated environments has achieved detection of acute toxicity and modelled lobular localisation of cell death (Allen *et al,* 2005). Such flow-through reactors also demonstrate potential for high throughput non-invasive measurement of toxicity (Deglmann *et al,* 2002). All these systems, despite individual strengths, currently lack the in vivo like responses and reliability required for a robust assay.

5.1.2 Assays of toxicity

Assays designed to identify toxic responses have a range of potential end points. Detection of enzyme leakage (e.g. LDH) is a crude measure of cell death that detects when the cell membrane has been compromised; an alternative and often earlier indication of cytotoxicity is mitochondrial activity (Kikkawa *et al,* 2005). There are also non-acutely lethal but equally important toxic effects, such as steatosis, cholestasis, and genotoxicity, that require different endpoints. In vitro culture models have different strengths in detecting different toxic endpoints and relatively simple models sometimes suffice. For example, the change in total protein content of Hep G2 cultures is a good predictor of acute toxicity (Dierickx *et al,* 2005). Simple short term culture systems can also identify some forms of chronic toxicity through displaying early markers that can be correlated with a later toxic effect. Examples include accumulation of pericanalicular F-actin and an increase in cytosolic free calcium in hepatocyte couplets associated with cholestatic agents (Thibault *et al,* 1994), caspase 3 (amongst others) activation associated with apoptotic agents (Gomez-Lechon *et al,* 2002), and micronucleus induction associated with liver mutagens and carcinogens (Muller-Tegethoff *et al,* 1997). More complex culture models become necessary when chronic toxicity lacks reliable early markers or involves cell-cell interactions.

5.1.3 Aims

The studies described in this chapter were designed to provide a preliminary assessment of the suitability of the hepatocyte-stellate cell co-culture spheroids as a model of acute and chronic toxicity. Specifically, to:

- Use LDH leakage to ascertain the sensitivity of primary hepatocytes to the hepatotoxic mitochondrial oxidation inhibitors DDC and amiodarone.

- Compare cell death in hepatocyte mono-cultures with that in hepatocyte-stellate cell co-culture spheroids due to chronic exposure to sub-acutely lethal concentrations of DDC.

- Compare cell death in hepatocyte and stellate cell mono-cultures with that in hepatocyte-stellate cell co-culture spheroids after 7 days due to acute exposure to a range of DDC and amiodarone concentrations.

5.2 Materials and Methods

5.2.1 Cell isolation and culture

Rat cells were isolated as described in chapter 2 and cultured in 24 well plates; 200 000 Hepatocytes or 100 000 stellate cells were used per well for respective mono-cultures and 200 000 hepatocytes combined with 100 000 stellate cells for co-culture.

5.2.2 LDH assay

The LDH assay was conducted using a commercially available kit (Roche applied science – Cytotoxicity detection kit (LDH)). Supernatant was removed from the cells and centrifuged to remove any cells/spheroids and then refrigerated. Any pelleted cells/spheroids were lysed using 0.5 ml Triton-X 100 (1%) in assay medium that was then transferred to lyse the remaining cells in the culture well. The assay was conducted in a 96 well plate using 100 µl of assay supernatant or cell lysate and 100 µl of freshly prepared Diaphorase, NAD^+, Iodotetrazolium chloride and sodium lactate mixture (from kit). Incubation was 30 minutes. Formazan formation (directly related to LDH content) was measured by detecting absorbance in a plate reader at 490 nm. Percentage cell death was calculated by dividing the LDH activity value from the supernatant by the LDH activity value of the supernatant and remaining cell lysate combined and multiplying by 100.

5.2.3 Experimental design

To determine LD_{50}, cells were plated for 2 hours on TCP before a change to media containing either DDC or amiodarone (0, 10, 20, 30, 50, and 100 μM concentrations in duplicate) and incubated over 24 hours followed by the LDH cytotoxicity assay.

To study chronic exposure to DDC, hepatocytes were co-cultured or mono-cultured for 2 hours or 24 hours before addition of media containing sub lethal 4 μM or 15 μM DDC concentrations respectively. At each media change the LDH assay was conducted on duplicate wells at each concentration and fresh DDC containing media was added.

To determine the sensitivity of 7 day old cells to DDC and amiodarone, hepatocytes were co-cultured or mono-cultured for 7 days before a change to media containing either DDC (0, 40, 80, 120, 160 and 200 μM concentrations in duplicate) or amiodarone (0, 20, 40, 60, 80, and 100 μM concentrations in duplicate) and incubated over 24 hours followed by the LDH cytotoxicity assay.

To determine the sensitivity of stellate cells in mono-culture to DDC or amiodarone, stellate cells were cultured for three weeks before being plated into 24 well plates. 24 hours after plating, media was changed to contain DDC or amiodarone (0, 10, 20, 30, 50, and 100 μM concentrations in duplicate) and cells were incubated over 24 hours followed by the LDH cytotoxicity assay.

5.3 Results

Dose-cell death curves were created for 24 hour exposure of 2 hour old primary hepatocyte mono-cultures on TCP to amiodarone and DDC. LD50 was approximately 90 μM for DDC and 43 μM for amiodarone (figures 5.1 and 5.3). The LDH method of assessing cell viability was supported by microscopy showing increased cell granularity with increased compound concentrations (figures 5.2 and 5.4).

The dose-cell death curve was used to establish low lethality concentrations of DDC to use for chronic exposure. Exposure of mono-culture and co-culture cells over 7 days to 4 μM and 15 μM DDC did not lead to any discernible increase in cytotoxicity relative to non-exposed mono-cultures and co-cultures (figure 5.5).

The response of 7 day old mono-cultures and co-cultures to the toxic compounds DDC and amiodarone was assessed with reference to freshly isolated cells. The mono-cultured cells did not produce a dose-cell death curve, with approximately 40% of remaining cells dieing between day 7 and 8 irrespective of DDC or amiodarone concentration. Cell death was far lower in co-culture, approximately 5% without toxin. This percentage increased with increasing concentration of amiodarone, but only rose to a maximum of 13% cell death at concentrations that were lethal to over 90% of freshly isolated hepatocytes. Cell death also increased with addition of DDC, rising to 20% in

response to relatively low concentrations, but a typical dose response effect was not observed (figure 5.6).

The viability of mono-cultured stellate cells after exposure to a range of concentrations of DDC and amiodarone was assessed (figure 5.7). Over 80% cell death occured when stellate cells were exposed to 25 μM amiodarone and over 60% cell death with 50 μM DDC. Increasing amiodarone concentration above this level to 100 μM has little effect on stellate cell death whilst increasing DDC levels to 200 μM results in a gradual increase up to 90% cell death.

Figure 5.1a

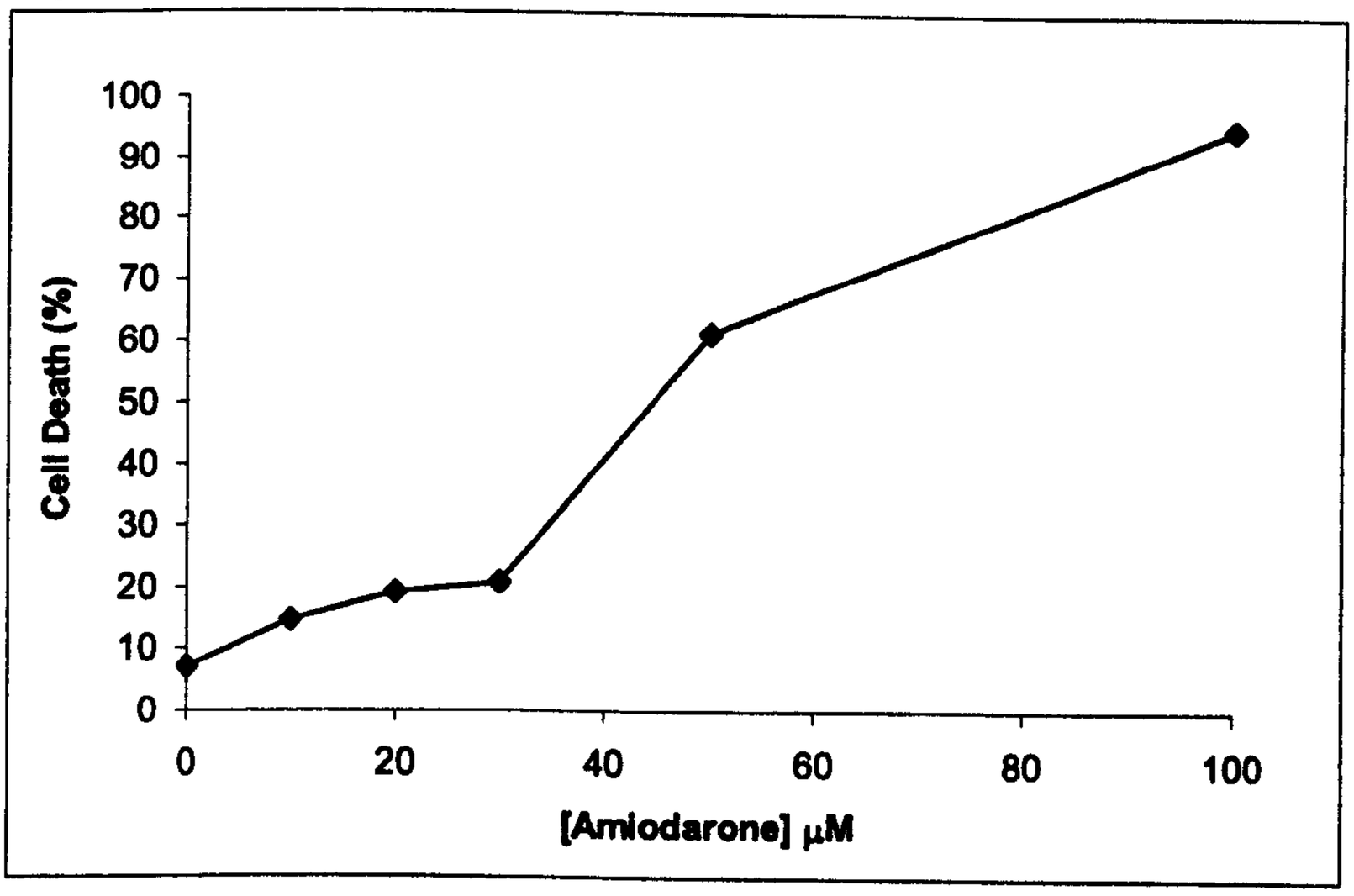


Figure 5.1b

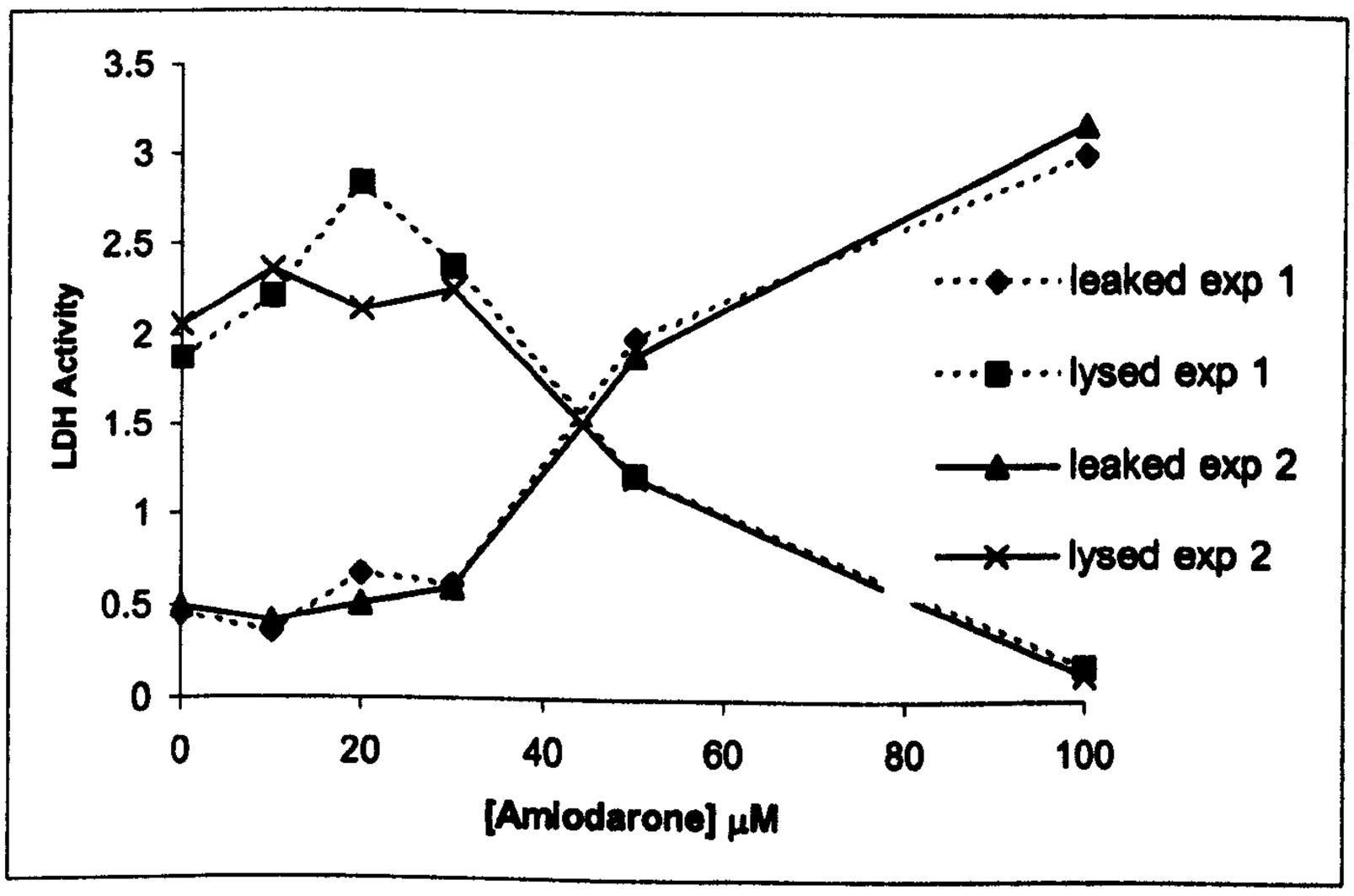


Figure 5.1: (a) A graph of percentage cell death (calculated via leaked LDH activity divided by total LDH activity) of primary hepatocytes exposed over 24 hours to a range of amiodarone concentrations after 2 hours culture. (b) The leaked and lysed LDH activity are shown separately. LD50 is between 32 μM and 50 μM, estimated 43 μM. (N=2)

Figure 5.2

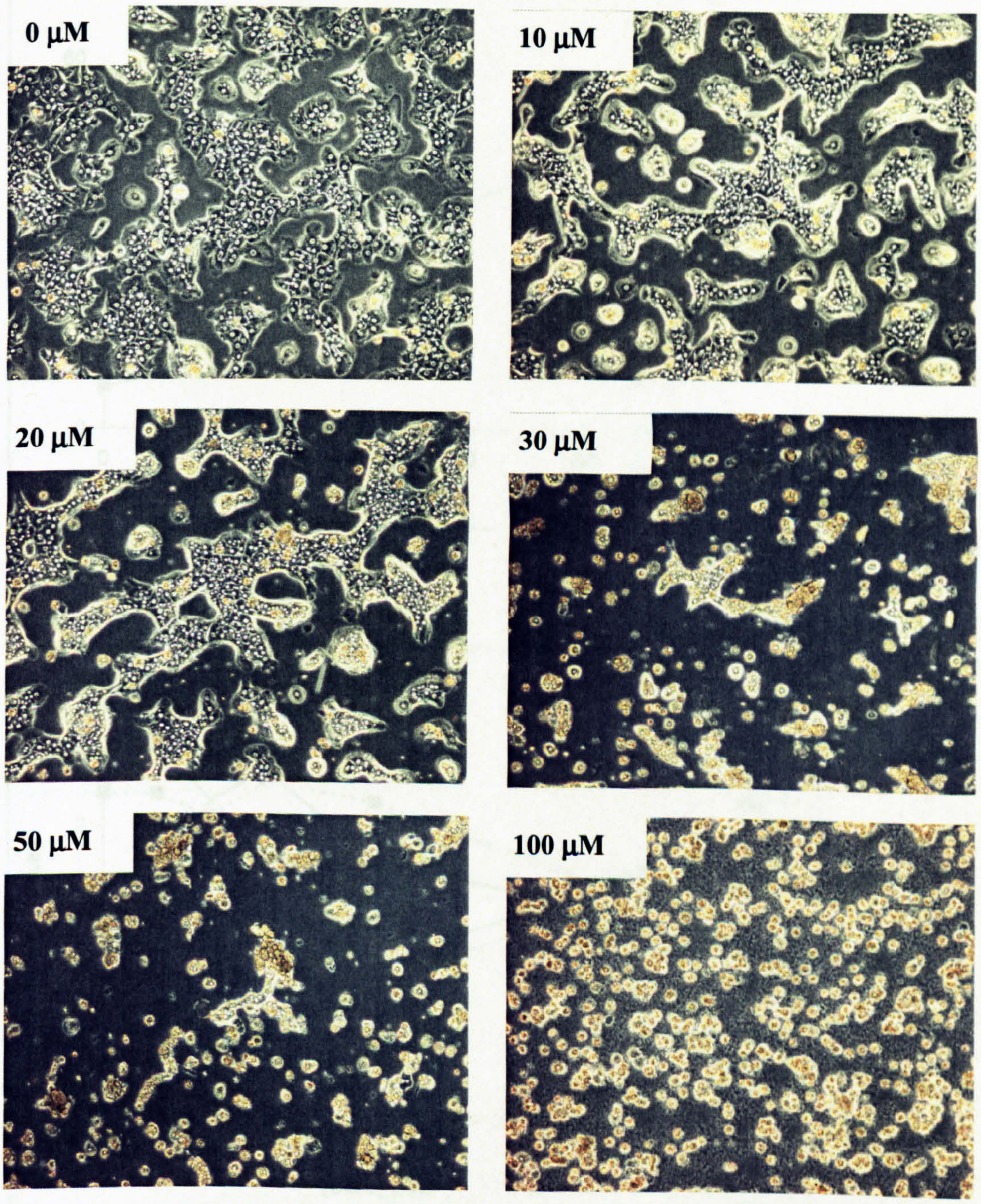


Figure 5.2: Microscopy images support the LDH method of detecting cell death by showing increased cell granularity with increasing amiodarone concentration.

Figure 5.3a

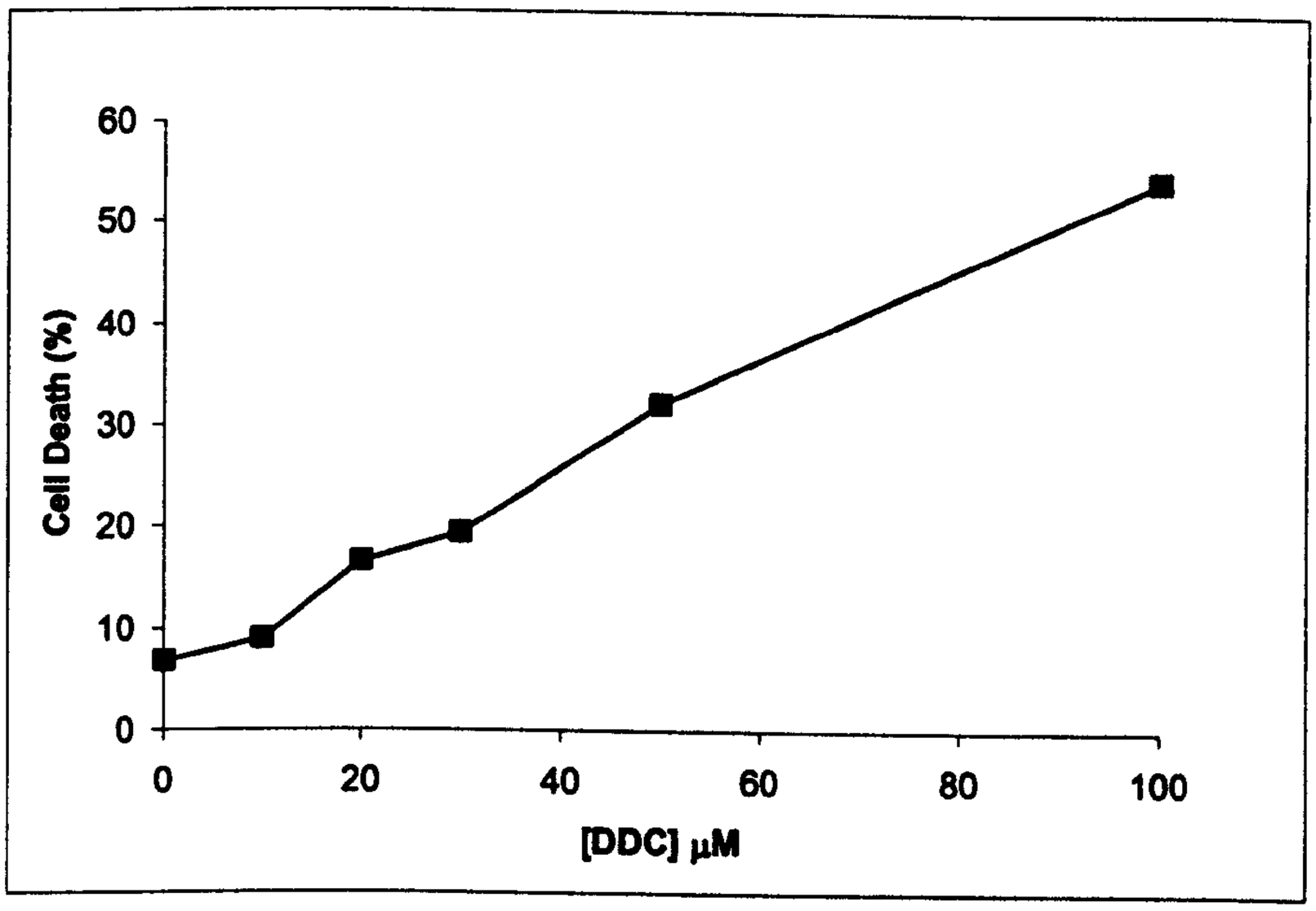


Figure 5.3b

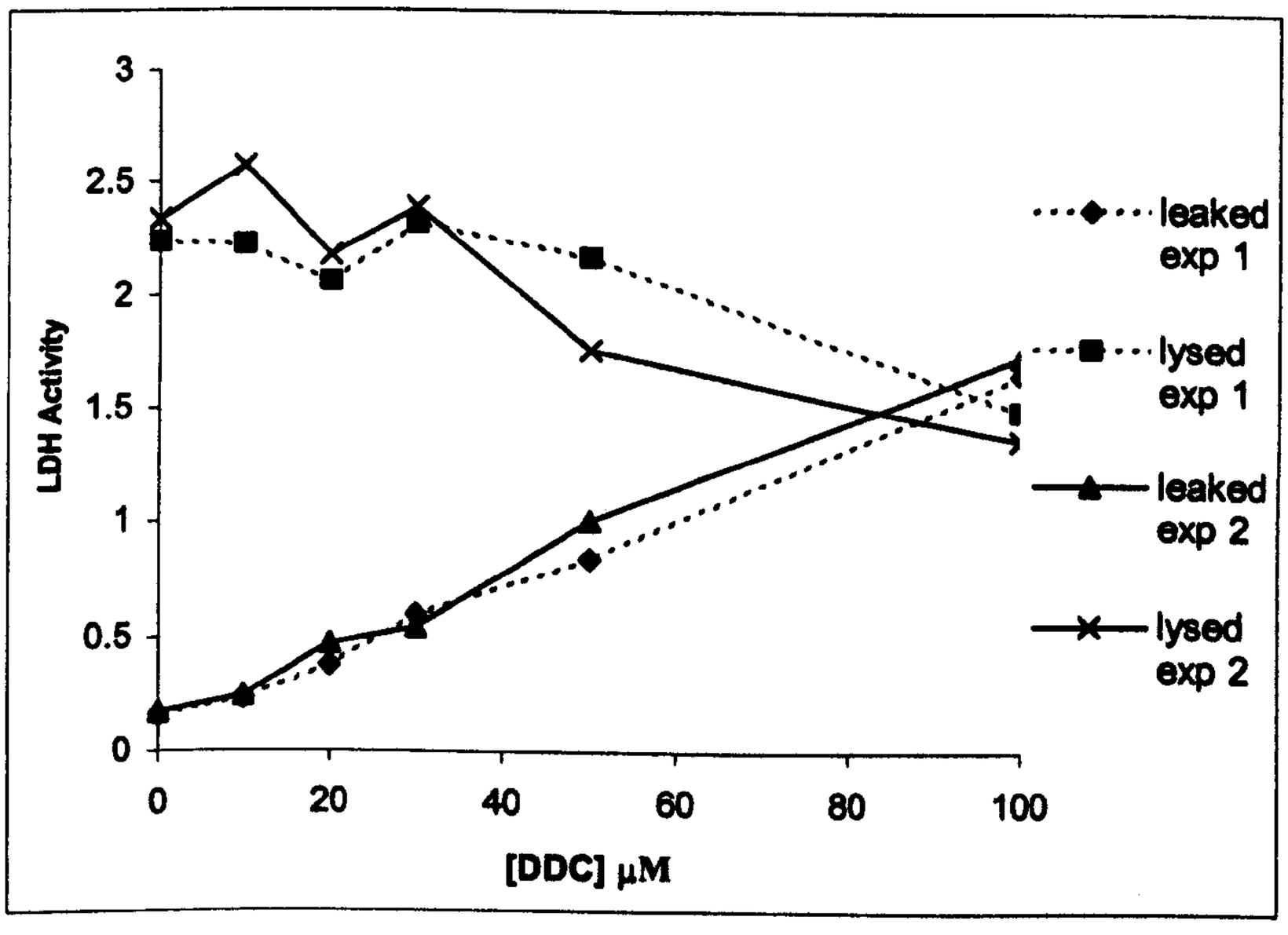


Figure 5.3: (a) A graph of percentage cell death (calculated via leaked LDH activity divided by total LDH activity) of primary hepatocytes exposed over 24 hours to a range of DDC concentrations after 2 hours culture. (b) The leaked and lysed LDH activities are shown separately. LD50 is between 50 μM and 100 μM, estimated 90 μM. (N=2)

Figure 5.4

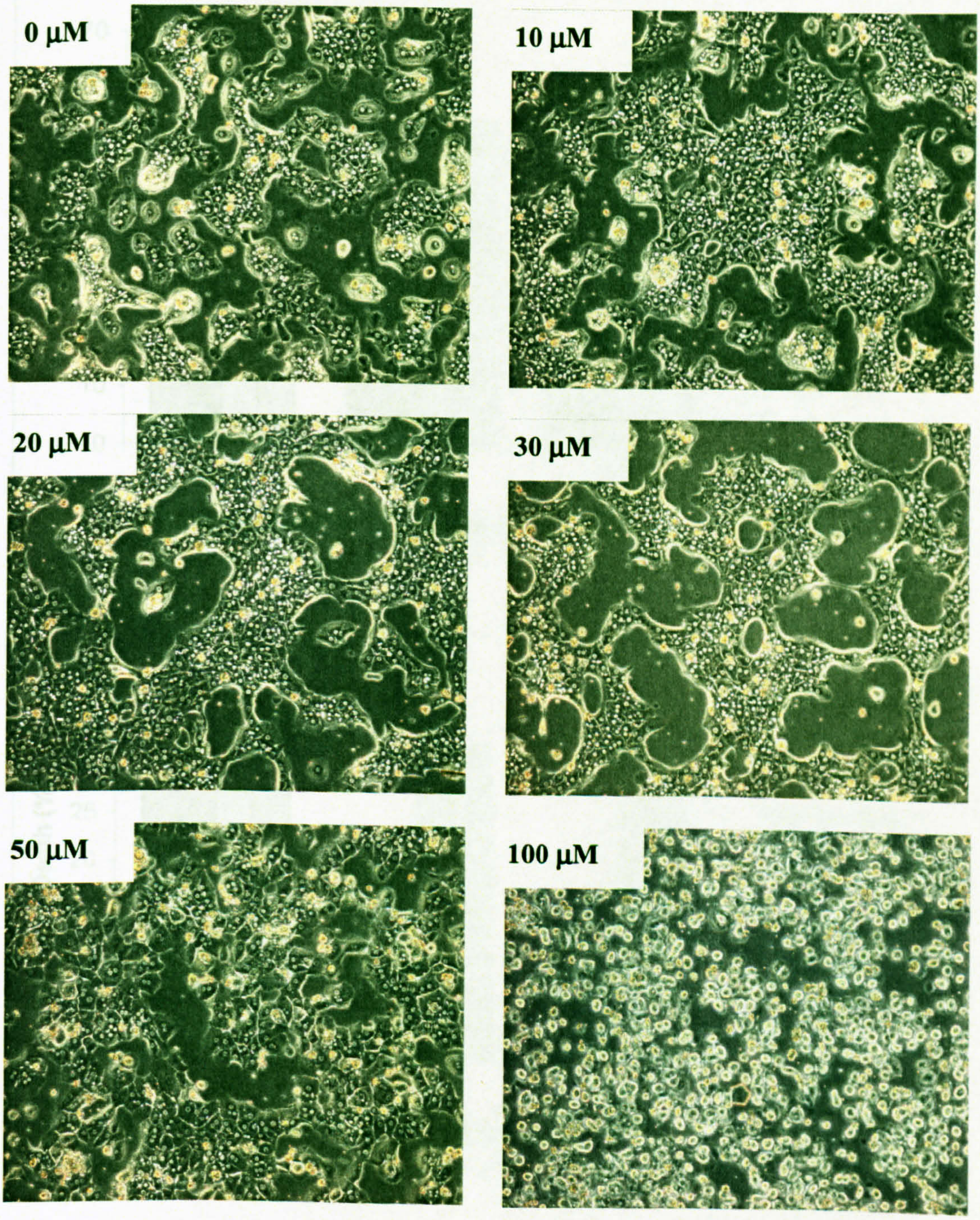


Figure 5.4: Microscopy images support the LDH method of cell death detection showing increasing cell granularity with increasing DDC concentration.

Figure 5.5a

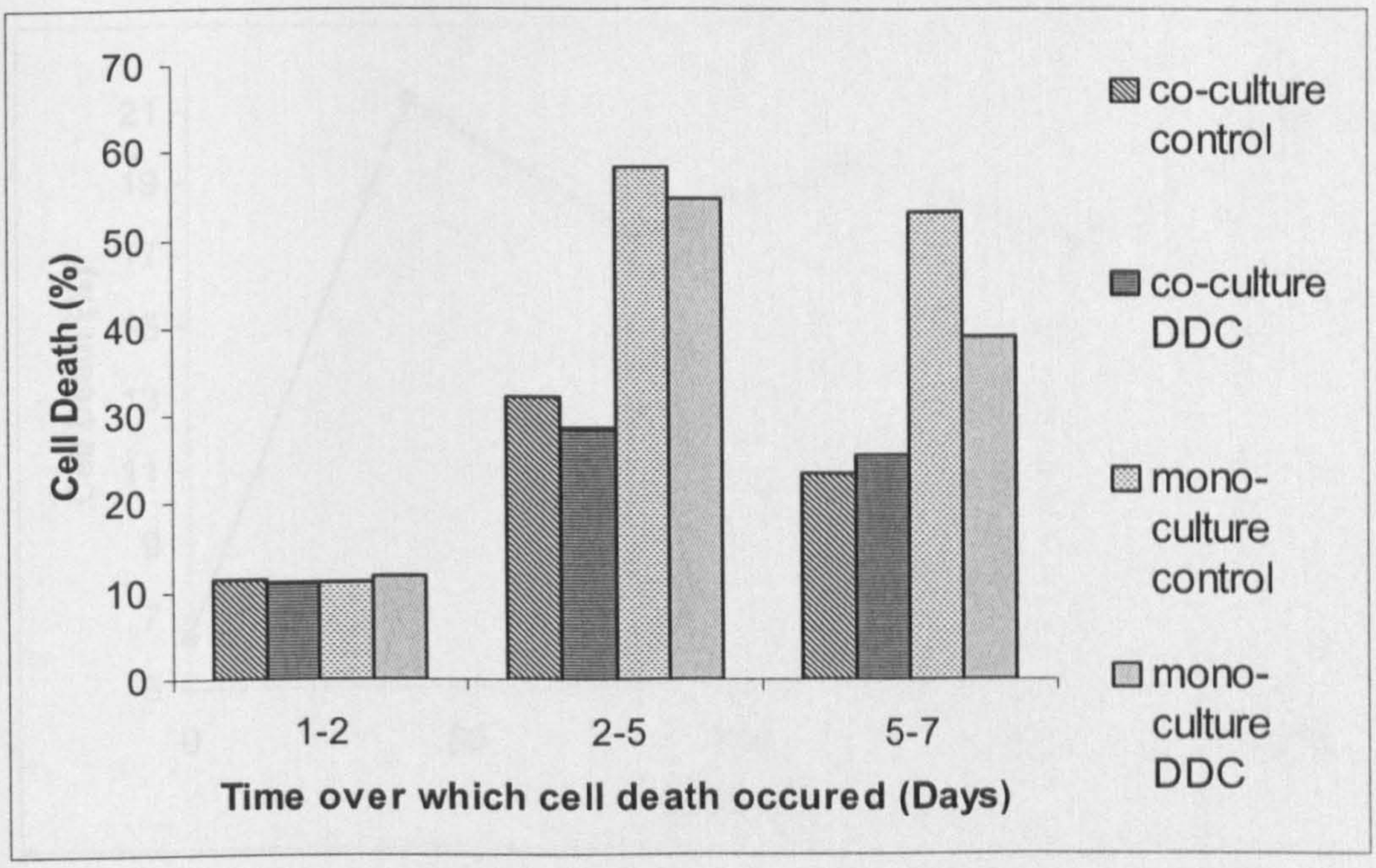


Figure 5.5b

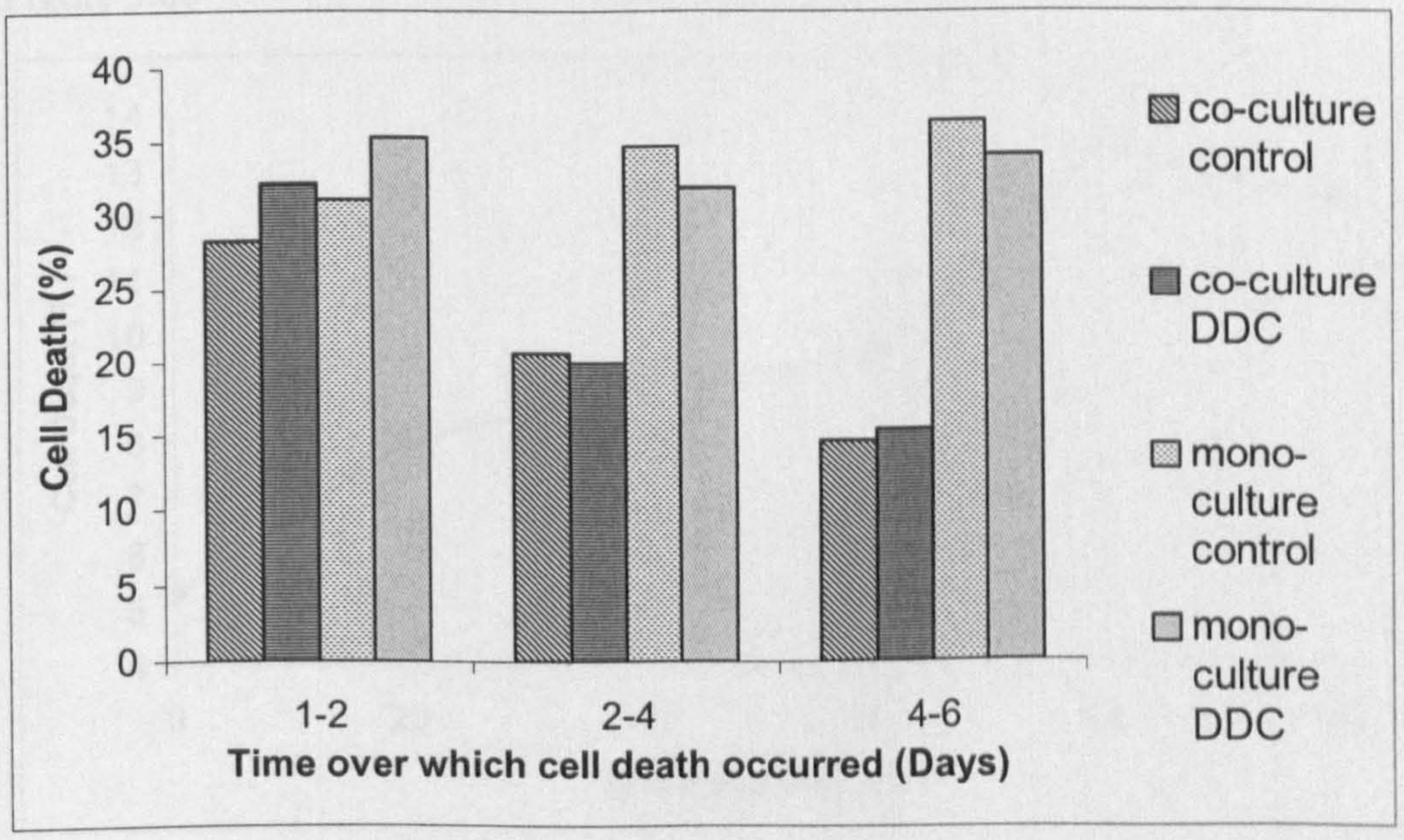


Figure 5.5: (a) Percentage cell death of hepatocytes over three consecutive time periods in culture when exposed to 4 μM DDC from the start of culture. (b) Percentage cell death of hepatocytes in culture exposed to 15 μM DDC after the first 24 hours of culture.

Figure 5.6a

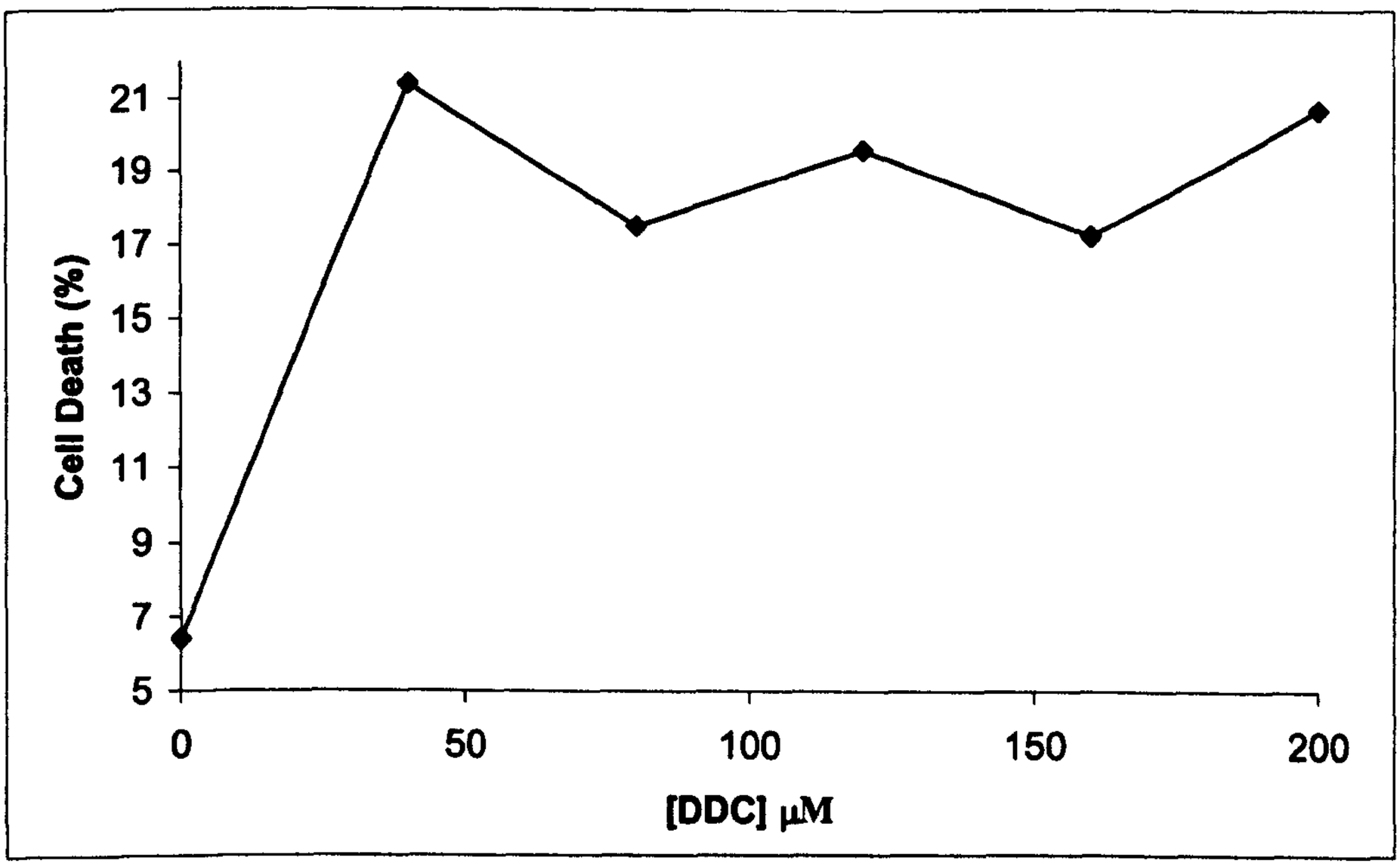


Figure 5.6b

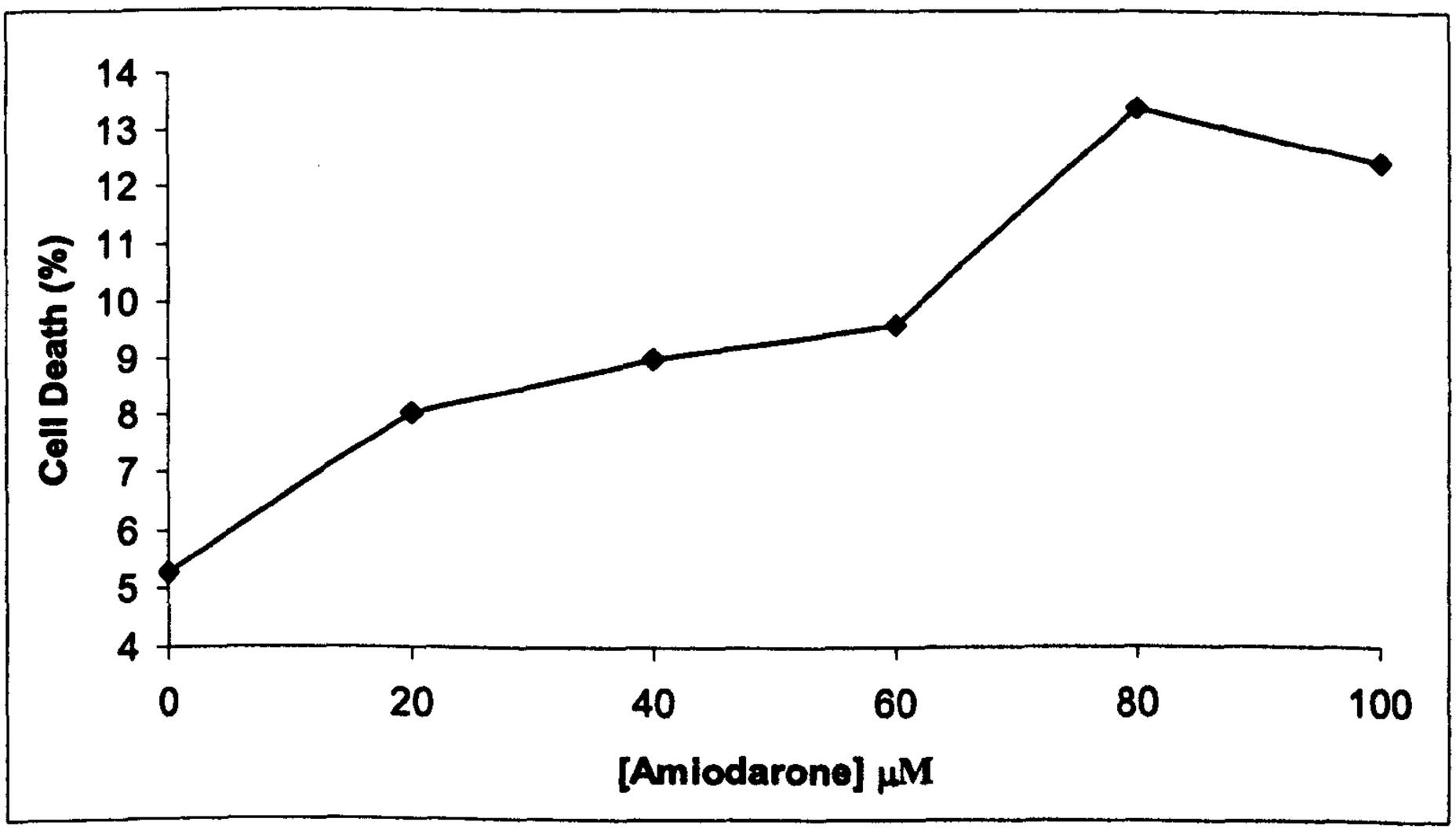


Figure 5.6: (a) Concentration vs cell death graph for exposure of spheroids to a range of DDC concentrations after one week of co-culture (b) Concentration vs cell death graph for exposure of spheroids to a range of amiodarone concentrations after one week of co-culture.

Figure 5.7a

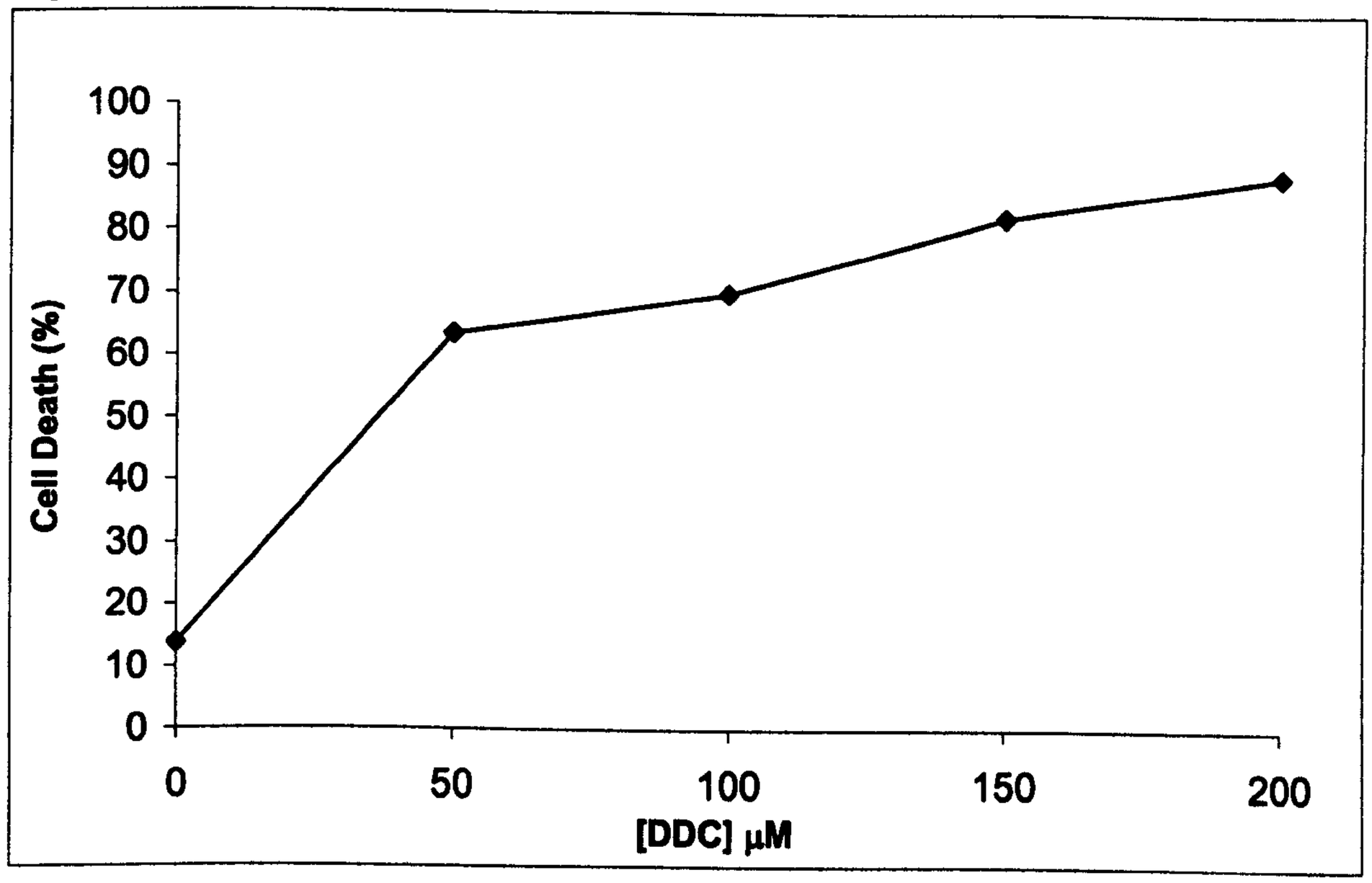


Figure 5.7b

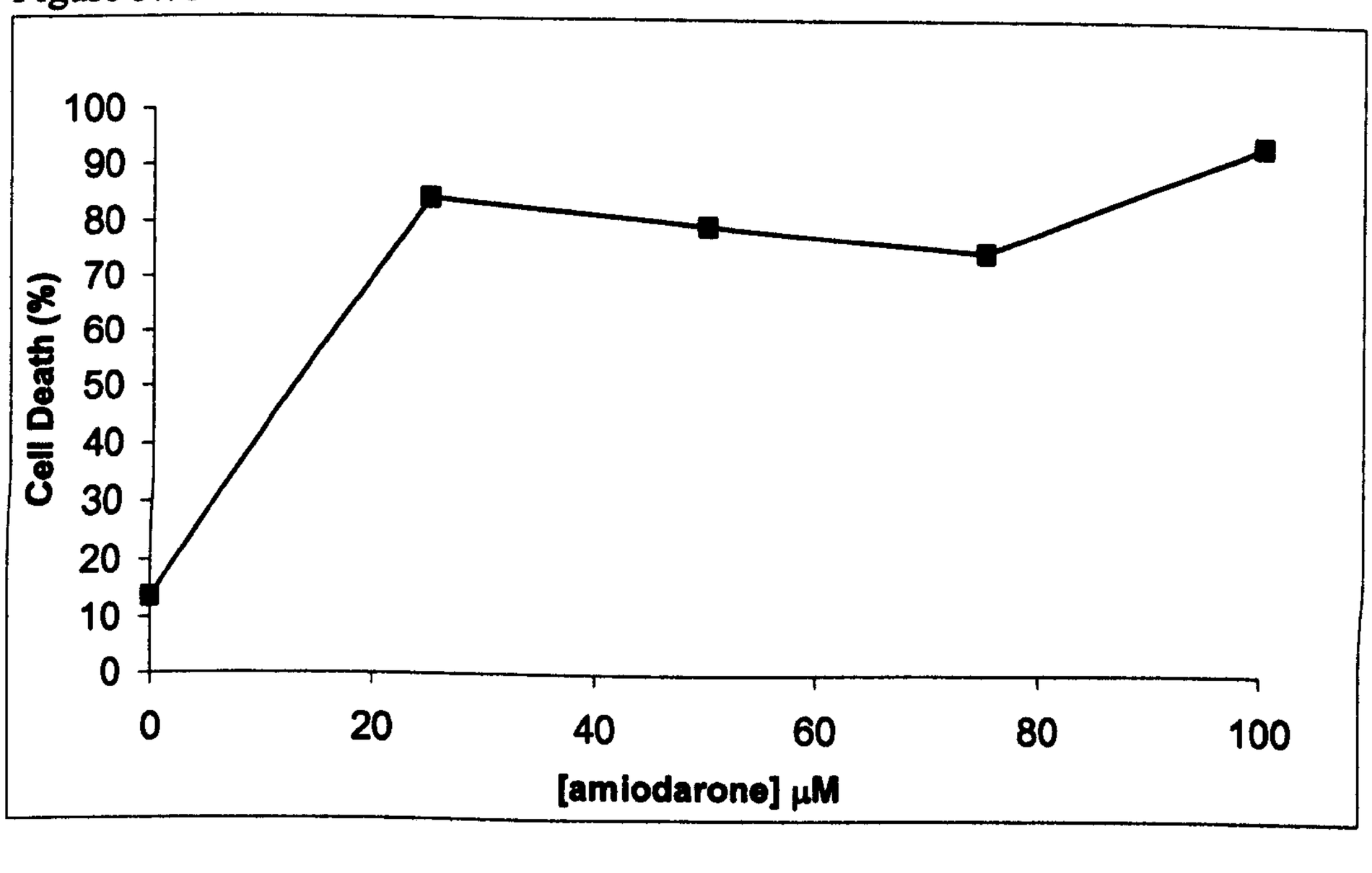


Figure 5.7: (a) A graph of percentage cell death (calculated via leaked LDH activity divided by total LDH activity) of activated stellate cells exposed over 24 hours to a range of DDC concentrations. (b) A graph of percentage cell death (calculated via leaked LDH activity divided by total LDH activity) of activated stellate cells exposed over 24 hours to a range of amiodarone concentrations.

5.4 Discussion

There is a need for reliable in vitro screens for hepatotoxicity. The preliminary studies discussed here were conducted to indicate the suitability of the hepatocyte -stellate cell co-culture system for such a screen. The co-culture is shown to have some benefits relative to hepatocyte mono-culture for detecting two hepatotoxic compounds.

Detection of toxicity after seven days of hepatocyte-stellate cell co-culture represents an improvement over mono-culture, which gave no meaningful results after 7 days. However, the low sensitivity of the response relative to freshly isolated hepatocytes limits the potential usefulness of the model. A potential explanation for this reduced sensitivity is localisation of cell death in certain areas of the spheroids. This could be caused by either poor penetration of the spheroid or cells in the spheroid having micro-environment dependent sensitivity. This would result in a dose response curve that would be diluted by the large proportion of unaffected cells. An alternative explanation could be the sensitivity of stellate cells to the toxic compounds. If stellate cells in co-culture do not change in sensitivity, or increase in number, then over time an increasing proportion of cell death will be due to stellate cell toxicity. The position of the stellate cells on the periphery of the spheroid would make them particularly vulnerable to a toxin in the media. This would similarly result in a dose response curve with a low maximal response proportionate to the amount of the total cell population being affected. These are general issues that would need to be considered in any spheroid model of hepatocyte toxicity or a model that incorporates a non-parenchymal cell.

This system needs to be improved in a number of respects to be an effective in vitro toxicological screen. A primary problem is the rapidly changing nature of the system; the cell population, measured en masse, is highly sensitive to toxin when freshly isolated but over the ensuing days becomes rapidly more resistant. A stable state is required for long term studies of toxicity so the sensitivity of the cells is constant. Furthermore, an equivalence of sensitivity to toxin with primary cells would have to be achieved, or, at worst, a validated equivalence factor (there is some evidence that toxic responses to short high dose exposure in vitro correlate well with long low dose exposure toxicity in vivo (O'Brien *et al,* 2004) indicating an equivalence factor may be workable). Also, the spheroid morphology and resultant heterogenous cell environment needs to be better understood i.e. if the drug did penetrate the spheroids would the cells at different depths respond in the same way. A mitochondrial respiration inhibitor may be less toxic to deeper less active cells, a P450 activated toxin may be more toxic to deeper cells (lower oxygen levels have been suggested to lead to greater P450 expression), but these cells may conversely be exposed to lower concentrations of toxin. Furthermore, in co-culture the impact of various non-parenchymal cells needs to be understood both in terms of their effect on hepatocyte responses to toxic substances as well as their own sensitivity to toxins.

There are other toxic endpoints that would need to be considered to draw a conclusive view of the potential of the hepatocyte-stellate cell co-culture spheroid as a model of toxicity. However, the preliminary results suggest its use

in detecting mitochondrial toxicity is limited. Unfortunately, the complexities that improve the longevity and the function of in vitro hepatocyte culture models such as this currently present barriers to simple assays and validation.

CHAPTER 6

General Discussion and Conclusion

6.1 Project overview

There is a need for in vitro hepatocyte culture systems that maintain an in vivo-like phenotype to facilitate the development of a range of industrial and therapeutic solutions. In this thesis, a study of the formation, morphology and function of hepatocyte-stellate cell co-culture spheroids is reported. This novel 3D co-culture is the first to use a non-parenchymal cell type to actively promote an organised hepatocyte aggregate morphology via control of culture surface properties. The activity of a number of important P450 enzymes and expression of P450 mRNAs are prolonged relative to hepatocytes in 2D monoculture.

6.2 The basis of improved cytochrome P450 function and mRNA expression in hepatocyte-stellate cell co-culture

The problem of hepatocyte de-differentiation and death in vitro can be framed in three simple alternative scenarios.

- Hepatocytes removed from essential paracrine, blood borne or structural in vivo environmental factors rapidly de-differentiate and die for lack of these stimuli.
- Cells do not lack a signal to maintain differentiated function but receive a de-differentiation stimulus from the in vitro culture environment.

- Cells are irretrievably committed to de-differentiate and die during the cell isolation process.

The relative longevity achieved in certain culture systems makes the latter scenario unlikely, although it could occur for a proportion of cells creating a hostile environment for cell survival. The diversity of in vitro culture conditions that suffer similar dedifferentiation fates questions the second scenario unless the dedifferentiation stimulus was common to all culture environments. The most likely scenario seems to be that the hepatocytes require an in vitro environment containing the appropriate stimuli to support differentiated function. The hepatocyte-stellate cell co-culture model supports this hypothesis through providing an environment with many in vivo-like hepatic attributes and corresponding benefits in function.

A general mechanism by which co-culture supports hepatocyte function has not been elucidated. Co-culture may enhance function through soluble mediator production (Chia *et al*, 2005), ECM production (Kudryavtseva *et al*, 2003), cell contact (Bhatia *et al*, 1998), promoting a favourable morphology (Kudryavtseva *et al*, 2003), or simply through the manner of aggregation (Hasebe *et al*, 2005). Studies that have tried to separate these effects in co-culture are in disagreement, probably because they use variable cell types and are too simplistic. A combination of factors may be required to elicit an effect or different factors may be synergistic.

This work has demonstrated a number of factors that may be responsible for the co-culture effect in the hepatocyte-hepatic stellate cell system. Stellate cell production of HGF, $TGF\beta_1$ and various other soluble mediators may influence hepatocyte function. $TGF\beta_1$ production has previously been credited for the 3T3 fibroblast mediated maintenance of hepatocyte function (Chia *et al*, 2005) but is also profibrotic (Nakamura *et al*, 2000), perhaps explaining the ECM deposition and activated stellate cell attributes of the model. The correlation of viability with aggregate formation indicates that either an individual or combination of factors such as cell contact, morphology or ECM environment are important in preventing cell death. A further potential mechanism by which stellate cells could enhance hepatocyte function is through loss of retinol content in vitro, presumably into the culture media, which may act via RXR/RAR nuclear receptors to enhance hepatocyte function. Although the co-culture spheroid model is beneficial in both longevity and function of hepatocytes, some aspects of the system may reduce function. Possible modifications to investigate this are discussed in section 6.5.

6.3 Comparing the hepatocyte-stellate cell co-culture to alternative models

It is important to be able to determine if a given in vitro hepatocyte culture model performs better than those previously reported. This is difficult due to significant inter-laboratory variation (Ringel *et al*, 2002). This variation partially derives from the range of different species and culture techniques used. Cells are isolated from different sexes of different sources, including porcine, human, rat, monkey and others, that differ in many factors such as the prevalence of P450

enzymes. Minor differences in culture technique such as cell handling, media supplementation (particularly with respect to the addition of dexamethasone or DMSO), cell density, cell purity, cell culture substrate and many others can all have major effects on cell phenotype (Vandenberghe *et al*, 1992). Even if media is defined at the start of culture, hepatocytes degrade additives and produce more of their own at a rate dependent on other variables such as cell density and media volume (Nakamura *et al*, 1983) resulting in non-comparability of individual variables after a short period. In more complex systems such as co-cultures, variability in the purity of the initial cell populations may lead to variation in phenotypic changes in either cell type over the culture period, adding more sources of discrepancy. A further barrier to comparison is the variety of functional end points, particularly with P450 function. These diverse assays differ in specificity and sensitivity; some measure the net activity of a range of different P450 enzymes that contribute in different proportions to the final metabolic products. The complexity of the pattern of dedifferentiation also means that equivalent time points are not directly comparable but must be viewed over the full period of dedifferentiation. The pattern of dedifferentiation of transcription factors underlying this response is probably even more complex.

If results are to be compared between laboratories a good set of validated controls is important as a relative benchmark for the activity of a novel culture system. However, no standard exists and good controls are difficult to achieve. For example, a standard monolayer monoculture control is morphologically different from a co-culture spheroid so distinguishing between the affects of co-culture or cell aggregation independently is impossible. Attempts to overcome

this morphological variable by alternative methods of forming cell aggregates, such as agitating monoculture cells, inherently introduce more variables. Consequently, in these studies multiple comparator culture types were used for the functional assays.

A further complication in defining the success of enhanced function hepatocyte cultures is the variation in hepatocyte phenotype through the zones of the lobule in vivo. Different culture systems may produce phenotypically different hepatocytes, but may be equally valid models of different lobular zones. The literature suggests that it is easier to maintain blood protein production than P450 activity. It is possible that high oxygen tension, an almost universal feature of in vitro culture systems, promotes a perilobular phenotype with low P450 activity and high blood protein production.

As a consequence of the comparative difficulties described above, it is only possible to conclude that qualitatively the hepatocyte-stellate cell co-culture model is comparable to other advanced models in creation of both a complex ultra structure and functional support. However, in the aspects investigated, it is better than simple monolayer mono-culture systems.

6.4 Potential applications of the hepatocyte stellate cell co-culture

The functional attributes of the hepatocyte stellate cell co-culture may be valuable in the development of a bioartificial liver system or in vitro metabolic screen. However, the functional advantages are not sufficient in this model to

independently address either of these issues. The system has potential as a model of either healthy or diseased liver tissue. The viability, in vivo like structure and ECM deposition by stellate cells is evidence of recreation of an in vivo like environment for hepatocyte support, but the level of deposition suggests the spheroids may be equally appropriate as a model of fibrosis. The ultra structure and fat storage may allow cholestasis and steatosis respectively to be investigated. The system also shows some promise as a post trauma model or a model of hepatic regeneration. Stellate cells are integral to both of these processes and the ECM deposition and high level of HGF mRNA found in the co-culture are both features of the regenerative liver associated with hepatectomy or severe injury. The spheroids have some attributes that are required for a toxicological or metabolism screen. However, the morphology of the system would potentially limit accessibility of interior cells for maintenance or test compounds and lead to gradients of cell phenotype. The toxicological sensitivity and longevity of function would also both need improving.

6.5 Potential improvements to the hepatocyte-stellate cell co-culture model

The viability and functional benefits of the hepatocyte-hepatic stellate cell co-culture system are identified only after two days in culture. This correlates with the aggregation of the cells, although it could also be attributable to accumulation of soluble mediators or other forms of gene expression. In either case, the speed and manner of formation of the aggregates appears critical. Optimisation by using an alternative faster method of forced aggregation to rapidly establish cell contact, or by pre-culturing stellate cells in the co-culture

environment to allow soluble mediator/ECM accumulation, could potentially achieve these benefits far earlier in the culture process. Also, the support of hepatocytes by cell contact and morphology in this model are closely linked to the method of formation. The final non-homogenous distribution of stellate cells resulting from their role in aggregation may result in a different distribution than is optimal for functional support of the hepatocytes. Similarly, the contractile morphology that promotes aggregation may be less effective at functional maintenance than the quiescent stellate cell phenotype. This could be investigated using manipulation of aggregation to change the cell distributions or two stellate populations, one quiescent and one activated. This may also avoid the formation of a peripheral ECM barrier.

Although the viability results indicate cell contact is important to prevent cell death, the spheroid morphology could have some drawbacks i.e. the peripheral distribution of the stellate cells, formation of an ECM capsule and high density of cells in the co-culture may not be optimal. However, the spheroid morphology would create a nutrient and oxygen gradient similar to that in the hepatic lobule which could be advantageous in developing an in vivo like variety of cell phenotypes. However, to investigate this the co-culture interaction should be explored without the spheroid structure. A stellate cell hepatocyte bi-layer could still support 3D structural aspects but would have fewer complexities in terms of nutrient availability. Also, a bi-layer separated by a semi permeable membrane would create a system reminiscent of hepatocyte plates in vivo.

6.6 Conclusion

The hepatocyte-hepatic stellate cell co-culture system supports a higher level of differentiated hepatocyte function than hepatocytes cultured alone. The mechanism of aggregate formation facilitates retention of differentiated function and leads to a complex structure, some aspects of which are in vivo-like, and some of which are associated with liver pathology. Although the hepatocyte stellate cell interaction alone appears unlikely to be sufficient to produce a useful model, it offers a tool that, in combination with other techniques, may lead to more successful complex culture systems.

REFERENCES

Abu-Absi SF, Friend JR, Hansen LK, Hu WS. Structural polarity and functional bile canaliculi in rat hepatocyte spheroids. *Exp Cell Res*. 2002. 274(1):56-67.

Abu-Absi SF, Hu WS, Hansen LK. Dexamethasone effects on rat hepatocyte spheroid formation and function. *Tissue Eng*. 2005. 11(3-4):415-426.

Akiyama TE, Gonzalez FJ. Regulation of P450 genes by liver-enriched transcription factors and nuclear receptors. *Biochim Biophys Acta*. 2003. 1619(3):223-234.

Akrawi M, Rogiers V, Vandenberghe Y, Palmer CN, Vercruysse A, Shephard EA, Phillips IR. Maintenance and induction in co-cultured rat hepatocytes of components of the cytochrome P450-mediated mono-oxygenase. *Biochem Pharmacol*. 1993. 45(8):1583-1591.

Allen JW, Khetani SR, Bhatia SN. In vitro zonation and toxicity in a hepatocyte bioreactor. *Toxicol Sci*. 2005. 84(1):110-119.

Ang SL, Wierda A, Wong D, Stevens KA, Cascio S, Rossant J, Zaret KS. The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development*. 1993. 119:1301-1315.

Arnaud A, Fontana L, Angulo AJ, Gil A, Lopez-Pedrosa JM. Proliferation, functionality, and extracellular matrix production of hepatocytes and a liver stellate cell line: a comparison between single cultures and cocultures. *Dig Dis Sci*. 2003. 48(7):1406-1413.

Asami O, Ihara I, Shimidzu N, Shimizu S, Tomita Y, Ichihara A, Nakamura T. Purification and characterization of hepatocyte growth factor from injured liver of carbon tetrachloride-treated rats. *J Biochem*. 1991. 109(1):8-13.

Auth MK, Okamoto M, Ishida Y, Keogh A, Auth SH, Gerlach J, Encke A, McMaster P, Strain AJ. Maintained function of primary human hepatocytes by

cellular interactions in coculture: implications for liver support systems. *Transpl Int.* 1998. 11 Suppl 1:S439-443.

Auth MK, Woitaschek D, Beste M, Schreiter T, Kim HS, Oppermann E, Joplin RE, Baumann U, Hilgard P, Nadalin S, Markus BH, Blaheta RA. Preservation of the synthetic and metabolic capacity of isolated human hepatocytes by coculture with human biliary epithelial cells. *Liver Transpl.* 2005. 11(4):410-419.

Baffet G, Loyer P, Glaise D, Corlu A, Etienne P-L, Guguen-Guillouzo. Distinct effects of cell-cell communication and corticosteroids on the synthesis and distribution of cytokeratins in cultured rat hepatocytes. *J Cell Sci.* 1991. 99:609-615.

Battle T, Maguire T, Moulsdale H, Doyle A. Progressive maturation resistance to microcystin-LR cytotoxicity in two different hepatospheroidal models. *Cell. Biol. Toxicol.* 1999. 15(1):3-12.

Baumhueter S, Courtois G, Crabtree GR. A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the beta fibrinogen gene promoter as HNF-1. *EMBO J.* 1988. 7:2485-93.

Begue JM, Guguen-Guillouzo C, Pasdeloup N, Guillouzo A. Prolonged maintenance of active cytochrome P-450 in adult rat hepatocytes co-cultured with another liver cell type. *Hepatology.* 1984. 4(5):839-842.

Bhadriraju K, Hansen LK. Hepatocyte adhesion, growth and differentiated function on RGD-containing proteins. *Biomaterials.* 2000. 21:267-272.

Bhandari RNB, Riccalton LA, Lewis AL, Fry JR, Hammond AH, Tendler SJB, Shakesheff KM. Liver tissue engineering: A role for co-culture systems in modifying hepatocyte function and viability. *Tissue eng.* 2001; 7:345-357.

Bhatia SN, Balis UJ, Yarmush ML, Toner M. Probing heterotypic cell interactions: hepatocyte function in microfabricated co-cultures. *J Biomater Sci Polym Ed.* 1998; 9(11):1137-60.

Boess F, Kamber M, Romer S, Gasser R, Muller D, Albertini S, Suter L. Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared to the in vivo liver gene expression in rats: possible implications for toxicogenomics use of in vitro systems. *Toxicol Sci.* 2003.73(2):386-402.

Bollard ME, Xu J, Purcell W, Griffin JL, Quirk C, Holmes E, Nicholson JK. Metabolic profiling of the effects of D-galactosamine in liver spheroids using (1)H NMR and MAS-NMR spectroscopy. *Chem Res Toxicol.* 2002. 15(11):1351-1359.

Brieva TA, Moghe PV. Functional engineering of hepatocytes via heterocellular presentation of a homoadhesive molecule, E-cadherin. *Biotechnol Bioeng.* 2001. 76:295-302.

Burr AW, Toole K, Chapman C, Hines JE, Burt AD. Anti-hepatocyte growth factor antibody inhibits hepatocyte proliferation during liver regeneration. *J Pathol.* 1998. 185(3):298-302.

Burra P, Tomat S, Conconi MT, Macchi C, Russo FP, Parnigotto PP, Naccarato R, Nussdorfer GG. Acellular liver matrix improves the survival and functions of isolated rat hepatocytes cultured in vitro. *Int J Mol Med.* 2004. 14(4):511-515.

Cassiman D, Roskams T, van Pelt J, Libbrecht L, Aertsen P, Crabbe T, Vankelecom H, Denef C. Alpha B-crystallin expression in human and rat hepatic stellate cells. *J Hepatol.* 2001. 35(2):200-207

Cassiman D, Libbrecht L, Desmet V, Denef C, Roskams T. Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. *J Hepatol.* 2002. 36(2):200-209.

Cereghini S. Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.* 1996. 10:267-282.

Chia SM, Lin PC, Yu H. TGF-beta1 regulation in hepatocyte-NIH3T3 co-culture is important for the enhanced hepatocyte function in 3D microenvironment. *Biotechnol Bioeng.* 2005. 89(5):565-573.

Clement B, Guguen-Guillouzo C, Campion JP, Glaise D, Bourel M, Guillouzo A. Long-term co-cultures of adult human hepatocytes with rat liver epithelial cells: modulation of albumin secretion and accumulation of extracellular material. *Hepatology.* 1984. 4:373-380.

Conner J, Vallet-Collom I, Daveau M, Delers F, Hiron M, Lebreton JP, Guillouzo A. Acute-phase-response induction in rat hepatocytes co-cultured with rat liver epithelial cells. *Biochem J.* 1990. 266(3):683-688.

Costa RH, Grayson DR, Darnell JE Jr. Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. *Mol Cell Biol.* 1989. 9:1415-1425.

Davila JC, Morris DL. Analysis of cytochrome P450 and phase II conjugating enzyme expression in adult male rat hepatocytes. *In Vitro Cell Dev Biol Anim.* 1999. 35(3):120-130.

De Bartolo L, Bader A. Review of a flat membrane bioreactor as a bioartificial liver. *Ann Transplant.* 2001. 6(3):40-46.

Degawa M, Namiki M, Yoshimoto N, Makino M, Iwamoto M, Nemoto K, Hashimoto Y. Constitutive expression of cytochrome p450 genes in newly established rat hepatic cell lines. *J Biochem (Tokyo).* 2003. 133(6):825-831.

Deglmann CJ, Metzger R, Stickel M, Hoerrlein S, Schildberg FW, Koebe HG. A new bioassay including a small scale hepatocyte bioreactor for hepato-mediated toxicity testing in a target cell line. *Int J Artif Organs.* 2002. 25(10):975-984.

Deman JJ, Bruyneel EA, Mareel MM. A study on the mechanism of intercellular adhesion: Effects of neuraminidase, calcium and trypsin on the aggregation of suspended HeLa cells. *J Cell Biol.* 1974. 60:641-652.

Den Otter W, Tuit G. Causes of the zonal distribution of glycogen in the liver acinus after a fat-rich diet. I. Glycogen deposition in the acini of rat livers during normal reversed perfusion. *The Anatomical Record.* 1972. 173(3): 325 – 331.

Dierickx P. Prediction of human acute toxicity by the hep G2/24-hour/total protein assay, with protein measurement by the CBQCA method. *Altern Lab Anim.* 2005. 33(3):207-213.

DiPersio CM, Jackson DA, Zaret KS. The extracellular matrix coordinately modulates liver transcription factors and hepatocyte morphology. *Mol Cell Biol.* 1991. 11(9):4405-4414.

Donato MT, Gomez-Lechon MJ, Castell JV. Drug metabolizing enzymes in rat hepatocytes co-cultured with cell lines. *In Vitro Cell Dev Biol.* 1990. 26(11):1057-1062.

Donato MT, Castell JV, Gomez-Lechon MJ. Co-cultures of hepatocytes with epithelial-like cell lines: expression of drug-biotransformation activities by hepatocytes. *Cell Biol Toxicol.* 1991.7(1):1-14.

Dunn JY, Yarmush ML, Koebe HG, Tompkins RG. Hepatocyte function and extracellular matrix geometry: long-term culture in a sandwich configuration. 1989. *FASEB J.* 3:174-177.

Dvir-Ginzberg M, Elkayam T, Aflalo ED, Agbaria R, Cohen S. Ultrastructural and functional investigations of adult hepatocyte spheroids during in vitro cultivation. *Tissue Eng.* 2004. 10:1806-1817.

Emmanouhl S. Tzanakakis, Linda K. Hansen, Wei-Shou Hu. The role of actin filaments and microtubules in hepatocyte spheroid self-assembly. *Cell Motility and the Cytoskeleton.* 2001. 48(3):175 – 189.

Engl T, Boost KA, Leckel K, Beecken WD, Jonas D, Oppermann E, Auth MK, Schaudt A, Bechstein WO, Blaheta RA. Phosphorylation of hepatocyte growth factor receptor and epidermal growth factor receptor of human hepatocytes can be maintained in a (3D) collagen sandwich culture system. *Toxicol In Vitro.* 2004. 18(4):527-32.

Fausto N, Laird AD, Webber EM. Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. *FASEB J.* 1995. 9(15):1527-1536.

Foliot A, Glaise D, Erlinger S, Guguen-Guillouzo C. Long-term maintenance of taurocholate uptake by adult rat hepatocytes co-cultured with a liver epithelial cell line. *Hepatology.* 1985. 5(2):215-219.

Fontana L, Jerez D, Rojas-Valencia L, Solis-Herruzo JA, Greenwel P, Rojkind M. Ethanol induces the expression of alpha 1(I) procollagen mRNA in a co-culture system containing a liver stellate cell-line and freshly isolated hepatocytes. *Biochim Biophys Acta.* 1997. 1362(2-3):135-144.

Fraslin JM, Kneip B, Vaulont S, Glaise D, Munnich A, Guguen-Guillouzo C. Dependence of hepatocyte-specific gene expression on cell-cell interactions in primary culture. *EMBO J.* 1985. 4(10):2487-2491.

Fujita M, Spray DC, Choi H, Saez J, Jefferson DM, Hertzberg E, Rosenberg LC, Reid LM. Extracellular matrix regulation of cell-cell communication and

tissue-specific gene expression in primary liver cultures. *Prog Clin Biol Res.* 1986. 226:333-360.

Fujita M, Spray DC, Choi H, Saez JC, Watanabe T, Rosenberg LC, Hertzberg EL, Reid LM. Glycosaminoglycans and proteoglycans induce gap junction expression and restore transcription of tissue-specific mRNAs in primary liver cultures. *Hepatology*. 1987. 7:1S-9S.

Fujita S, Ueda Y, Ko IK, Paek HJ, Sajiki T, Ikai I, Yamaoka Y, Ikada Y, Iwata H. Functional evaluation of bioartificial liver using RT-PCR. *Biomed Mater Eng.* 2005. 15(3):211-218.

Fukuda J, Mizumoto H, Nakazawa K, Kajiwara T, Funatsu K. Hepatocyte organoid culture in elliptic hollow fibers to develop a hybrid artificial liver. Int J Artif Organs. 2004. 27(12):1091-1099.

Gokhale MS, Bunton TE, Zurlo J, Yager JD. Cytochrome P450 isoenzyme activities in cultured rat and mouse liver slices. *Xenobiotica.* 1997. 27(4):341-355.

Gomez-Lechon MJ, O'Connor E, Castell JV, Jover R. Sensitive markers used to identify compounds that trigger apoptosis in cultured hepatocytes. *Toxicol Sci.* 2002. 65(2):299-308.

Guillouzo A, Delers F, Clement B, Bernard N, Engler R. Long term production of acute-phase proteins by adult rat hepatocytes co-cultured with another liver cell type in serum-free medium. *Biochem Biophys Res Commun.* 1984. 20(2):311-317.

Guillouzo A. Liver cell models in *in vitro* toxicology. *Environmental health perspectives*. 1998. 106 (Suppl. 2); 511-532.

Hanada S, Kayano H, Jiang J, Kojima N, Miyajima A, Sakoda A, Sakai Y. Enhanced *in vitro* maturation of subcultivated fetal human hepatocytes in three

dimensional culture using poly-L-lactic acid scaffolds in the presence of oncostatin M. *Int J Artif Organs*. 2003. 26(10):943-51.

Harimoto M, Yamato M, Hirose M, Takahashi C, Isoi Y, Kikuchi A, Okano T. Novel approach for achieving double-layered cell sheets co-culture: overlaying endothelial cell sheets onto monolayer hepatocytes utilizing temperature-responsive culture dishes. *J Biomed Mater Res*. 2002. 62(3):464-70.

Hasebe Y, Okumura N, Koh T, Kazama H, Watanabe G, Seki T, Ariga T. Formation of rat hepatocyte spheroids on agarose. *Hepatol Res*. 2005. 32:89-95.

Hatzis P, Talianidis I. Regulatory mechanisms controlling human hepatocyte nuclear factor 4α gene expression. *Mol Cell Biol*. 2001. 21:7320-7330.

Higashiyama S, Noda M, Kawase M, Yagi K. Mixed-ligand modification of polyamidoamine dendrimers to develop an effective scaffold for maintenance of hepatocyte spheroids. .*J Biomed Mater Res*. 2003. 64(3):475-82.

Hodgkinson CP, Wright MC, Paine AJ. Fibronectin-mediated hepatocyte shape change reprograms cytochrome P450 2C11 gene expression via an integrin-signaled induction of ribonuclease activity. *Mol Pharmacol*. 2000. 58(5):976-981.

Hoebe KH, Witkamp RF, Fink-Gremmels J, Van Miert AS, Monshouwer M. Direct cell-to-cell contact between Kupffer cells and hepatocytes augments endotoxin-induced hepatic injury. *Am J Physiol Gastrointest Liver Physiol*. 2001. 280(4):G720-728.

Hou DX, Arimura M, Fukuda M, Oka T, Fujii M. Expression of cell adhesion molecule and albumin genes in primary culture of rat hepatocytes. *Cell Biol Int*. 2001. 25:239-244.

Ijima H, Murakami S, Matsuo T, Takei T, Ono T, Kawakami K. Enhancement of liver-specific functions of primary rat hepatocytes co-cultured with bone marrow cells on tissue culture-treated polystyrene surfaces. *J Artif Organs.* 2005. 8(2):104-109.

Ikeda H, Nagoshi S, Ohno A, Yanase M, Maekawa H, Fujiwara K. Activated rat stellate cells express c-met and respond to HGF to enhance TGF beta expression and DNA synthesis. *Biochem Biophys Res Commun.* 1998. 250(3):769-75.

Ishiyama H, Ogino K, Hobara T. Role of Kupffer cells in rat liver injury induced by diethyldithiocarbamate. *Eur J Pharmacol.* 1995. 292(2):135-141.

Ito A, Takizawa Y, Honda H, Hata K, Kagami H, Ueda M, Kobayashi T. Tissue engineering using magnetite nanoparticles and magnetic force: heterotypic layers of cocultured hepatocytes and endothelial cells. *Tissue Eng.* 2004. 10:833-840.

Jacob A, Budhiraja S, Reichel RR. The HNF3α transcription factor is a primary target for retinoic acid action. *Exp Cell Res.* 1999. 250:1-9.

Jakowlew SB, Mead JE, Danielpour D, Wu J, Roberts AB, Fausto N. Transforming growth factor-beta (TGF-beta) isoforms in rat liver regeneration: messenger RNA expression and activation of latent TGF-beta. *Cell Regul.* 1991. 2(7):535-548.

Jang YY, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. *Nat Cell Biol.* 2004. 6(6):532-539.

Jefferson DM, Reid LM, Giambrone MA, Shafritz DA, Zern MA. Effects of dexamethasone on albumin and collagen gene expression in primary cultures of adult rat hepatocytes..*Hepatology.* 1985. 5:14-20.

Jessen BA, Mullins JS, De Peyster A, Stevens GJ. Assessment of hepatocytes and liver slices as in vitro test systems to predict in vivo gene expression. *Toxicol Sci.* 2003. 75(1):208-222.

Johnson PF. In Liver gene transcription factors (Tronche F, Yaniv M. eds) 1994. RG Lands Co. Austin Texas.

Jung JO, Gwak GY, Lim YS, Kim CY, Lee HS. Role of hepatic stellate cells in the angiogenesis of hepatoma. Korean J Gastroenterol. 2003. 42 (2):142-8.

Kaihara S, Kim S, Kim BS, Mooney DJ, Tanaka K, Vacanti JP. Survival and function of rat hepatocytes cocultured with nonparenchymal cells or sinusoidal endothelial cells on biodegradable polymers under flow conditions. *J Pediatr Surg.* 2000. 35(9):1287-1290.

Kan P, Miyoshi H, Ohshima N. Effects of shear stress on metabolic function of the co-culture system of hepatocyte/nonparenchymal cells for a bioartificial liver. *ASAIO J.* 1998. 44(5):M441-4.

Kan P, Miyoshi H, Ohshima N. Perfusion of medium with supplemented growth factors changes metabolic activities and cell morphology of hepatocyte-nonparenchymal cell coculture. *Tissue Eng.* 2004. 10(9-10):1297-1307.

Kang IK, Kim GJ, Kwon OH, Ito Y. Co-culture of hepatocytes and fibroblasts by micropatterned immobilization of beta-galactose derivatives. *Biomaterials.* 2004. 25(18):4225-4232.

Kaufmann PM, Fiegel HC, Kneser U, Pollok JM, Kluth D, Rogiers X. Influence of pancreatic islets on growth and differentiation of hepatocytes in co-culture. *Tissue Eng.* 1999. 5(6):583-596.

Kern A, Bader A, Pichlmayr R, Sewing KF. Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochem Pharmacol.* 1997. 54(7):761-772.

Khetani SR, Szulgit G, Del Rio JA, Barlow C, Bhatia SN. Exploring interactions between rat hepatocytes and nonparenchymal cells using gene expression profiling. *Hepatology.* 2004. 40(3):545-554.

Kikkawa R, Yamamoto T, Fukushima T, Yamada H, Horii I. Investigation of a hepatotoxicity screening system in primary cell cultures --"what biomarkers would need to be addressed to estimate toxicity in conventional and new approaches?". *J Toxicol Sci.* 2005. 30(1):61-72.

Kligerman AD, Strom SC, Michalopoulos G. Sister chromatid exchange studies in human fibroblast-rat hepatocyte co-cultures: a new in vitro system to study SCEs. *Environ Mutagen.* 1980. 2(2):157-165.

Koide N, Sakaguchi K, Koide Y, Asano K, Kawaguchi M, Matsushima H, Takenami T, Shinji T, Mori M, and Tsuji T. Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other non-adherent environments. *Exp. Cell Res.* 1990. 186: 227-235.

Kudryavtseva EI, Engelhardt NV. Requirement of 3D extracellular network for maintenance of mature hepatocyte morphology and suppression of alpha-fetoprotein synthesis in vitro. *Immunol Lett.* 2003. 15;90(1):25-31.

Lange C, Bassler P, Lioznov MV, Bruns H, Kluth D, Zander AR, Fiegel HC. Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. *World J Gastroenterol.* 2005. 11(29):4497-4504.

Lavery DJ, Schibler U. Circadian transcription of the cholesterol 7 alpha hydroxylase gene may involve the liver-enriched bZIP protein DBP. *Genes Dev*. 1993. 7:1871-1884.

Lewindon PJ, Pereira TN, Hoskins AC, Bridle KR, Williamson RM, Shepherd RW, Ramm GA. The role of hepatic stellate cells and transforming growth factor-beta(1) in cystic fibrosis liver disease. Am J Pathol 2002 160(5):1705-15.

Lewis AL. Flow cytometric analysis of hepatocyte proliferation in vitro. PhD Thesis, University of Nottingham. 2003.

Li C, Locker J, Wan YJ. RXR-mediated regulation of the alpha-fetoprotein gene through an upstream element. *DNA Cell Biol.* 1996. 15:955-963.

Liu JK, DiPersio CM, Zaret KS. Extracellular signals that regulate liver transcription factors during hepatic differentiation in vitro. *Mol Cell Biol*. 1991. 11:773-84.

Liu X, Zhang Z, Yang L, Chen D, Wang Y. Inhibition of the activation and collagen production of cultured rat hepatic stellate cells by antisense oligonucleotides against TGF beta1 is enhanced by cationic liposome delivery. *Hua Xi Yi Ke Da Xue Xue Bao*. 2000. 31(2):133-135.

Liu X, Zhang J, Zhang Y. Effects of platelet-derived growth factor on the proliferation of hepatic stellate cells and their expressions of genes of collagens and platelet-derived growth factor. *Zhonghua Bing Li Xue Za Zhi*. 2000(b). 29(1):27-29.

Loreal O, Levavasseur F, Fromaget C, Gros D, Guillouzo A, Clement B. Cooperation of Ito cells and hepatocytes in the deposition of an extracellular matrix in vitro. *Am J Pathol.* 1993. 143(2):538-544.

Loyer P, Cariou S, Glaise D, Bilodeau M, Baffet G, Guguen-Guillouzo C. Growth factor-dependence of entry and progression through G1 and S phases of adult rat hepatocytes in vitro. *J Biol Chem*. 1996. 271:11484-11492.

Luk JM, Wang PP, Lee CK, Wang JH, Fan ST. Hepatic potential of bone marrow stromal cells: Development of in vitro co-culture and intra-portal transplantation models. *J Immunol Methods*. 2005. [Epub ahead of print]

Mabuchi A, Mullaney I, Sheard PW, Hessian PA, Mallard BL, Tawadrous MN, Zimmermann A, Senoo H, Wheatley AM. Role of hepatic stellate cell/hepatocyte interaction and activation of hepatic stellate cells in the early phase of liver regeneration in the rat. *J Hepatol*. 2004. 40(6):910-6.

Maher JJ, Bissell DM. Cell-matrix interactions in liver. *Semin Cell Biol*. 1993. 4(3):189-201.

Malik R, Selden C, Hodgson H. The role of non-parenchymal cells in liver growth. *Cell and Dev Biol*. 2002. 13:425-431.

Martinez-Hernandez A, Amenta PS. The hepatic extracellular matrix. I. Components and distribution in normal liver. *Virchows Arch A Pathol Anat Histopathol*. 1993. 423(1):1-11.

Martinez-Hernandez A, Amenta PS. The hepatic extracellular matrix. II. Ontogenesis, regeneration and cirrhosis. *Virchows Arch A Pathol Anat Histopathol*. 1993. 423:77-84.

Mesnil M, Fraslin JM, Piccoli C, Yamasaki H, Guguen-Guillouzo C. Cell contact but not junctional communication (dye coupling) with biliary epithelial cells is required for hepatocytes to maintain differentiated functions. *Exp Cell Res*. 1987. 173(2):524-533.

Michalopoulos G, Pitot HC. Primary culture of parenchymal liver cells on collagen membranes. *Exp Cell Res*. 1975. 94:70-80.

Mischoulon D, Rana B, Bucher NL, Farmer SR. Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBP alpha) gene expression during hepatocyte proliferation in the regenerating liver and in culture. *Mol Cell Biol.* 1992. 12:2553-2560.

Mitsue S, Hamanoue M, Tanabe G, Ogura Y, Yoshidome S, Aikou T, Nakamura T. Expression of HGF and TGF-beta 1 mRNA after partial hepatectomy in rats with liver cirrhosis. *Surg Today.* 1995. 25(3):237-243.

Moghe PV, Berthiaume F, Ezzell RM, Toner M, Tompkins RG, Yarmush ML. Culture matrix configuration and composition in the maintenance of hepatocyte polarity and function. *Biomaterials.* 1996. 17:373-385.

Morin O, Normand C. Long-term maintenance of hepatocyte functional activity in co-culture: requirements for sinusoidal endothelial cells and dexamethasone. *J Cell Physiol.* 1986. 129(1):103-110.

Moronvalle-Halley V, Sacre-Salem B, Sallez V, Labbe G, Gautier JC. Evaluation of cultured, precision-cut rat liver slices as a model to study drug-induced liver apoptosis. *Toxicology.* 2005. 207(2):203-214.

Muller D, Glockner R, Rost M, Steinmetzer P. Monooxygenation, cytochrome P450-mRNA expression and other functions in precision-cut rat liver slices. *Exp Toxicol Pathol.* 1998. 50(4-6):507-513.

Muller-Tegethoff K, Kersten B, Kasper P, Muller L. Application of the in vitro rat hepatocyte micronucleus assay in genetic toxicology testing. *Mutat Res.* 1997. 392(1-2):125-138.

Nakamura T, Yoshimoto K, Nakayama Y, Tomita Y, Ichihara A. Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell--cell contact and cell membranes. *Proc Natl Acad Sci U S A.* 1983. 80(23):7229-7233.

Nakamura T, Ichihara A. Control of growth and expression of differentiated functions of mature hepatocytes in primary culture. *Cell Struct Funct.* 1985. 10:1-16.

Nakamura T, Sakata R, Ueno T, Sata M, Ueno H. Inhibition of transforming growth factor beta prevents progression of liver fibrosis and enhances hepatocyte regeneration in dimethylnitrosamine-treated rats. *Hepatology*. 2000. 32(2):247-255.

Nakshatri H, Chambon P. The directly repeated RG(G/T)TCA motifs of the rat and mouse cellular retinol-binding protein II genes are promiscuous binding sites for RAR, RXR, HNF-4, and ARP-1 homo- and heterodimers. *J Biol Chem.* 1994. 269:890-902.

Naughton BA, Sibanda B, Weintraub JP, San Roman J, Kamali V. A stereotypic, transplantable liver tissue-culture system. *Appl Biochem Biotechnol.* 1995. 54(1-3):65-91.

Nozato E, Shiraishi M, Nishimaki T. Up-regulation of hepatocyte growth factor caused by an over-expression of transforming growth factor beta, in the rat model of fulminant hepatic failure. *J Surg Res.* 2003. 115(2):226-234.

O'Brien PJ, Chan K, Silber PM. Human and animal hepatocytes in vitro with extrapolation in vivo. *Chem Biol Interact.* 2004. 150(1):97-114.

Oakley F, Trim N, Constandinou CM, Ye W, Gray AM, Frantz G, Hillan K, Kendall T, Benyon RC, Mann DA, Iredale JP. Hepatocytes express nerve growth factor during liver injury: evidence for paracrine regulation of hepatic stellate cell apoptosis. *Am J Path.* 2003. 163(5):1849-58.

Oe S, Lemmer ER, Conner EA, Factor VM, Leveen P, Larsson J, Karlsson S, Thorgeirsson SS. Intact signaling by transforming growth factor beta is not

required for termination of liver regeneration in mice. *Hepatology*. 2004. 40(5):1098-1105.

Okamoto M, Ishida Y, Keogh A, Strain A. Evaluation of the function of primary human hepatocytes co-cultured with the human hepatic stellate cell (HSC) line LI90. *Int J Artif Organs*. 1998. 21(6):353-359.

Paine AJ, Andreakos E. Activation of signalling pathways during hepatocyte isolation: relevance to toxicology in vitro. *Toxicol In Vitro*. 2004. 18(2):187-193.

Papadimitriou D, Arkadopoulos N, Kouskouni E, Tsarouxa N, Demesticha T, Kousi T, Drakopoulou M, Mitrou S, Vassileiou J, Katsorhis T, Patargias T. Hepatocyte function during experimental use of a bioartificial liver. *Transplant Proc*. 2004. 36(6):1741-1743.

Phaneuf D, Moscioni AD, LeClair C, Raper SE, Wilson JM. Generation of a mouse expressing a conditional knockout of the hepatocyte growth factor gene: demonstration of impaired liver regeneration. *DNA Cell Biol*. 2004. 23(9):592-603.

Piechocki MP, Toti RM, Fernstrom MJ, Burk RD, Ruch RJ. Liver cell-specific transcriptional regulation of connexin 32. *Biochim Biophys Acta*. 2000. 1491:107-122.

Pollok JM, Kluth D, Cusick RA, Lee H, Utsunomiya H, Ma PX, Langer R, Broelsch CE, Vacanti JP. Formation of spheroidal aggregates of hepatocytes on biodegradable polymers under continuous-flow bioreactor conditions. *Eur J Pediatr Surg*. 1998. 8(4):195-199.

Powers MJ, Janigian DM, Wack KE, Baker CS, Beer Stolz D, Griffith LG. Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. *Tissue Eng*. 2002. 8(3):499-513.

Qiu Y, Krishnan V, Pereira FA, Tsai SY, Tsai MJ. Chicken ovalbumin upstream promoter-transcription factors and their regulation. *J Steroid Biochem Mol Biol*. 1996. 56:81-85.

Ratanasavanh D, Beaune P, Baffet G, Rissel M, Kremers P, Guengerich FP, Guillouzo A. Immunocytochemical evidence for the maintenance of cytochrome P-450 isozymes, NADPH cytochrome C reductase, and epoxide hydrolase in pure and mixed primary cultures of adult human hepatocytes. *J Histochem Cytochem. 1986. 34(4):527-533.*

Ratanasavanh D, Baffet G, Latinier MF, Rissel M, Guillouzo A. Use of hepatocyte co-cultures in the assessment of drug toxicity from chronic exposure. *Xenobiotica*. 1988.18(6):765-771.

Raucy JL, Mueller L, Duan K, Allen SW, Strom S, Lasker JM. Expression and induction of CYP2C P450 enzymes in primary cultures of human hepatocytes. *J Pharmacol Exp Ther*. 2002. 302:475-482.

Reid LM. Stem cell biology, hormone/matrix synergies and liver differentiation. *Curr Opin Cell Biol.* 1990. 2(1):121-130.

Reid LM, Fiorino AS, Sigal SH, Brill S, Holst PA. Extracellular matrix gradients in the space of Disse: relevance to liver biology. *Hepatology*. 1992. 15(6):1198-1203.

Riccalton-Banks L, Bhandari R, Fry J, Shakesheff KM. A simple method for the simultaneous isolation of stellate cells and hepatocytes from rat liver tissue. *Molecular and Cellular Biochemistry*. 2003; 248:97-102

Riccalton-Banks L, Liew C, Bhandari R, Fry J, Shakesheff KM. Long-term culture of functional liver tissue: Three-dimensional co-culture of primary hepatocytes and stellate cells. *Tissue engineering*. 2003(b). 9:401-410.

Ries K, Krause P, Solsbacher M, Schwartz P, Unthan-Fechner K, Christ B, Markus PM, Probst I. Elevated expression of hormone-regulated rat hepatocyte functions in a new serum-free hepatocyte-stromal cell coculture model. *In Vitro Cell Dev Biol Anim.* 2000. 36(8):502-512.

Ringel M, Oesch F, Gerl M, Klebach M, Quint M, Bader A, Bottger T, Hengstler JG. Permissive and suppressive effects of dexamethasone on enzyme induction in hepatocyte co-cultures. *Xenobiotica.* 2002. 32(8):653-666.

Ringel M, von Mach MA, Santos R, Feilen PJ, Brulport M, Hermes M, Bauer AW, Schormann W, Tanner B, Schon MR, Oesch F, Hengstler JG. Hepatocytes cultured in alginate microspheres: an optimized technique to study enzyme induction. *Toxicology.* 2005. 206(1):153-167.

Rojkind M, Gatmaitan Z, Mackensen S, Giambrone MA, Ponce P, Reid LM. Connective tissue biomatrix: it's isolation and utilization for long-term cultures of normal rat hepatocytes. *J Cell Biol.* 1980. 87:255-263.

Rojkind M, Novikoff PM, Greenwel P, Rubin J, Rojas-Valencia L, Camos de Carvalho A, Stockert R, Spray D, Hertzberg EL, Wolkoff AW. Characterization and functional studies on rat liver fat storing cell line and freshly isolated hepatocyte coculture system. *Am. J. Pathol.* 1995. 146;1508-1520.

Roskelley CD, Srebrow A, Bissell MJ. A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr Opin Cell Biol.* 1995. 7(5):736-747.

Runge D, Runge DM, Jager D, Lubecki KA, Beer Stolz D, Karathanasis S, Kietzmann T, Strom SC, Jungermann K, Fleig WE, Michalopoulos GK. Serum-free, long-term cultures of human hepatocytes: maintenance of cell morphology, transcription factors, and liver-specific functions. *Biochem Biophys Res Commun.* 2000. 5; 269:46-53.

Samson CM, Schrum LW, Bird MA, Lange PA, Brenner DA, Rippe RA, Behrns KE. Transforming growth factor-beta1 induces hepatocyte apoptosis by a c-Jun independent mechanism. *Surgery*. 2002. 132(3):441-449.

Sanchez A, Alvarez AM, Pagan R, Roncero C, Vilaro S, Benito M, Fabregat I. Fibronectin regulates morphology, cell organization and gene expression of rat fetal hepatocytes in primary culture. *J Hepatol*. 2000. 32:242-250.

Sato F, Mitaka T, Mizuguchi T, Mochizucki Y, Hirata K. Effects of nicotinamide-related agents on the growth of primary rat hepatocytes and formation of small hepatocyte colonies. *Liver* 1999. 19:481-488.

Sato M, Suzuki S, Senoo H. Hepatic stellate cells: unique characteristics in cell biology and phenotype. *Cell Struct Funct.* 2003. 28:105-12.

Schmeichel KL, Bissell MJ. (2003) Modeling tissue-specific signaling and organ function in three dimensions. *J Cell Sci.* **116**, 2377-88.

Schuetz EG, Li D, Omiecinski CJ, Muller-Eberhard U, Kleinman HK, Elswick B, Guzelian PS. Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. *J Cell Physiol.* 1988. 134:309-323.

Seglen PO (1976) Preparation of isolated rat liver cells. *Methods in Cell Biology* 19, 29-83.

Semler EJ, Ranucci CS, Moghe PV. Mechanochemical manipulation of hepatocyte aggregation can selectively induce or repress liver-specific function. *Biotechnol. Bioeng.* 2000. 69:359-69.

Shen H, Huang GJ, Gong YW. Effect of transforming growth factor beta and bone morphogenetic proteins on rat hepatic stellate cell proliferation and trans-differentiation. *World J Gastroenterology*. 2003. 9(4):784-7.

Sigal SH, Brill S, Fiorino AS, Reid LM. The liver as a stem cell and lineage system. *Am J Physiol.* 1992. 263(2 Pt 1):G139-148.

Skrtic S, Wallenius V, Ebkberg S, Brenzel A, Gressner AM, Jansson JO. Hepatocyte-stimulated expression of hepatocyte growth factor (HGF) in cultured rat hepatic stellate cells. *J Hepatol.* 1999. 30:115-24

Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK, Parkinson A. Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. *Arch Biochem Biophys.* 1987. 15. 255:27-41

Sonderfan AJ, Arlotto MP, Parkinson A. Identification of the cytochrome P-450 isozymes responsible for testosterone oxidation in rat lung, kidney, and testis: evidence that cytochrome P-450a (P450IIA1) is the physiologically important testosterone 7 alpha-hydroxylase in rat testis. *Endocrinology.* 1989. 125:857-66.

Spray DC, Fujita M, Saez JC, Choi H, Watanabe T, Hertzberg E, Rosenberg LC, Reid LM. Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. *J Cell Biol.* 1987. 105(1):541-551.

Stolz DB, Michalopoulos GK. Differential modulation of hepatocyte growth factor-stimulated motility by transforming growth factor beta1 on rat liver epithelial cells in vitro. *J Cell Physiol.* 1998. 175(1):30-40.

Takai KK, Hattori S, Irie S. Type V collagen distribution in liver is reconstructed in co-culture system of hepatocytes and stellate cells; the possible functions of type V collagen in liver under normal and pathological conditions. *Cell Struct Funct.* 2001. 26(5):289-302.

Talamini MA, McCluskey MP, Buchman TG, De Maio A. Expression of alpha2-macroglobulin by the interaction between hepatocytes and endothelial cells in coculture. *Am J Physiol.* 1998. 275(1 Pt 2):R203-11.

Tamura T, Koide N, Hada H, Shiraha H, Tsuji T. Gene expression of liver-specific proteins in hepatocyte spheroids in primary culture. *Acta Med Okayama*. 1995. 49(3):161-7.

Theret N, Musso O, L'Helgoualc'h A, Clement B. Activation of matrix metalloproteinase-2 from hepatic stellate cells requires interactions with hepatocytes. *Am J Pathol*. 1997. 150:51-58.

Thibault N. Scanning laser cytometry: alterations induced by cholestatic agents in isolated rat hepatocyte couplets. *Cell Biol Toxicol.* 1994. 10(5-6):323-328.

Tilles AW, Baskaran H, Roy P, Yarmush ML, Toner M. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. *Biotechnol Bioeng*. 2001. 73(5):379-389.

Tomiya T, Ogata I, Fujiwara K. Transforming growth factor alpha levels in liver and blood correlate better than hepatocyte growth factor with hepatocyte proliferation during liver regeneration. *Am J Pathol.* 1998 153(3):955-961.

Tomiya T, Ogata I, Yamaoka M, Yanase M, Inoue Y, Fujiwara K. The mitogenic activity of hepatocyte growth factor on rat hepatocytes is dependent upon endogenous transforming growth factor-alpha. *Am J Pathol.* 2000. 157(5):1693-1701.

Torok E, Pollok JM, Ma PX, Kaufmann PM, Dandri M, Petersen J, Burda MR, Kluth D, Perner F, Rogiers X. Optimization of hepatocyte spheroid formation for hepatic tissue engineering on three-dimensional biodegradable polymer within a flow bioreactor prior to implantation. *Cells Tissues Organs*. 2001. 169(1):34-41.

Tronche F, Yaniv M. HNF1, a homeoprotein member of the hepatic transcription regulatory network. *Bioessays*. 1992. 14:579-587.

Tsuda Y, Kikuchi A, Yamato M, Nakao A, Sakurai Y, Umezu M, Okano T. The use of patterned dual thermoresponsive surfaces for the collective recovery as co-cultured cell sheets. Biomaterials. 2005. 26(14):1885-1893.

Tsukamoto H. Cytokine regulation of hepatic stellate cells in liver fibrosis. *Alcohol Clin Exp Res*. 1999. 23(5):911-916.

Umek RM, Friedman AD, McKnight SL. CCAAT-enhancer binding protein: a component of a differentiation switch. *Science*. 1991. 251:288-292.

Uyama N, Shimahara Y, Kawada N, Seki S, Okuyama H, Iimuro Y, Yamaoka Y. Regulation of cultured rat hepatocyte proliferation by stellate cells. *J Hepatol*. 2002. 36:590-599.

Vandenberghe Y, Tee L, Rogiers V, Yeoh G. Transcriptional- and post-transcriptional-dependent regulation of glutathione S-transferase expression in rat hepatocytes as a function of culture conditions. *FEBS Lett.* 1992. 313(2):155-159.

Walker TM, Rhodes PC, Westmoreland C. The differential cytotoxicity of methotrexate in rat hepatocyte monolayer and spheroid cultures. *Toxicol In Vitro*. 2000. 14(5):475-485.

Walker TM, Woodrooffe AJ. (2001) Cytochrome P450 activity in control and induced long-term cultures of rat hepatocyte spheroids. *Toxicol In Vitro*. 15(6):713-9.

Wan YJ, Wang L, Wu TC. Dexamethasone increases the expression of retinoid X receptor genes in rat hepatoma cell lines. *Lab Invest.* 1994. 70:547-552.

Wang XJ, Hodgkinson CP, Wright MC, Paine AJ. Temperature-sensitive mRNA degradation is an early event in hepatocyte de-differentiation. *Biochem J.* 1997. 328(Pt 3):937-944.

Washizu J, Berthiaume F, Chan C, Tompkins RG, Toner M, Yarmush ML. Optimization of rat hepatocyte culture in citrated human plasma. *J Surg Res.* 2000. 93(2):237-246.

Washizu J, Berthiaume F, Mokuno Y, Tompkins RG, Toner M, Yarmush ML. Long-term maintenance of cytochrome P450 activities by rat hepatocyte/3T3 cell co-cultures in heparinized human plasma. *Tissue Eng.* 2001. 7(6):691-703.

Waxman DJ, Lapenson DP, Aoyama T, Gelboin HV, Gonzalez FJ, Korzekwa K. Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Arch Biochem Biophys.* 1991. 290:160-6.

Waxman DJ. P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch Biochem Biophys.* 1999. 369(1):11-23.

Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos.* 2003. 31(8):1035-1042.

Xu J, Ma M, Purcell WM. Characterisation of some cytotoxic endpoints using rat liver and HepG2 spheroids as in vitro models and their application in hepatotoxicity studies. I. Glucose metabolism and enzyme release as cytotoxic markers. *Toxicol Appl Pharmacol.* 2003.189(2):100-111.

Yamazaki H, Shimada T. Progesterone and testosterone hydroxylation by cytochromes P450 2C19, 2C9, and 3A4 in human liver microsomes. *Arch Biochem Biophys.* 1997. 1;346:161-9.

Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K, Xu L, Eng F, Afdhal N, Kalluri R. Liver fibrosis: insights into migration of hepatic stellate cells in response to extracellular matrix and growth factors. *Gastroenterology.* 2003. 124(1):147-59.

Yoffe B, Darlington GJ, Soriano HE, Krishnan B, Risin D, Pellis NR, Khaoustov VI. Cultures of human liver cells in simulated microgravity environment. *Adv Space Res.* 1999. 24(6):829-836.

Zeilinger K, Sauer IM, Pless G, Strobel C, Rudzitis J, Wang A, Nussler AK, Grebe A, Mao L, Auth SH, Unger J, Neuhaus P, Gerlach JC. Three-dimensional co-culture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions. *Altern Lab Anim.* 2002. 30(5):525-538.

Zeilinger K, Holland G, Sauer IM, Efimova E, Kardassis D, Obermayer N, Liu M, Neuhaus P, Gerlach JC. Time course of primary liver cell reorganization in three-dimensional high-density bioreactors for extracorporeal liver support: an immunohistochemical and ultrastructural study. *Tissue Eng.* 2004. 10:1113-1124.