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| **Title** | Roles of p53 in hepatocytes |
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| **Qualification** | PhD |
| **Year** | 1998 |

# The roles of p53 in hepatocytes

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*Submitted for the degree of PhD*

**University of Edinburgh, 1997**

# Declaration

The written composition of this thesis is my own work. The work described in this thesis was planned and performed by myself.

Christopher O. C. Bellamy

July 1997

# Acknowledgements

I wish to thank the Cancer Research Campaign for the Gordon Hamilton Fairley CRC Clinical Fellowship award that funded this project; also my supervisors, David Harrison and Andrew Wyllie, for their advice, enthusiasm and encouragement, not to mention David's ever-ready coffee supply.

Many people have given of their time, knowledge and friendship, and so helped to enrich the time spent on this research. In particular, Sandrine Prost, Alan Clarke, Derek Bishop, John Virth and Bob Morris have advised on labwork.

I dedicate this thesis to my mum and dad.

# ABSTRACT

Despite good evidence for p53 dysfunction in human hepatocellular carcinomas, rather little is known of the significance of p53 to normal hepatocytes, or whether p53 dysfunction has consequences that could be relevant to early hepatocarcinogenesis. Therefore, the consequences of targeted p53-deficiency in hepatocytes were examined for regulation of proliferation, apoptosis, ploidy and responses to DNA-damaging agents. In order to complement and extend observations made in vivo, a method for primary culture of mouse hepatocytes was established and evaluated. A modified form of Chee's medium gave superior culture preservation compared with other proprietary media tested, and allowed serum-free culture, providing hepatocyte selectivity and fully-defined conditions of culture under which the effects of specific cytokines could be tested.

p53-deficiency was silent in normal liver and did not affect progression from diploidy to polyploidy in the aging liver. However, in primary culture the absence of p53 resulted in increased hepatocyte proliferation indices and decreased sensitivity to proliferation inhibition by TGFβ. Moreover, p53-deficient murine hepatocytes continued to survive and proliferate under conditions of minimal trophic support that led to growth arrest and apoptosis of wild type cells. In vivo, p53-deficient mice had enhanced proliferative responses to both xenobiotic hepatomitogen and CCl4-induced liver necrosis, although lack of persistent proliferation showed that other control mechanisms are important.

There was no simple relationship between p53 and apoptosis following DNA damage, since uv irradiation led to p53-independent apoptosis, even though p53 was stabilized and transcriptionally activated — as assessed with a transiently transfected p53-specific reporter plasmid. By contrast, γ-irradiation injury to hepatocytes failed to produce detectable changes in either p53 immunopositivity or transcriptional transactivation activity. Nevertheless, p53 did couple both uv and γ-irradiation injury to growth arrest, showing that agents producing different forms of DNA damage can act differently through p53, yet produce a common biological response. The observation has implications for how particular dysfunctional mutations of p53 in carcinogenesis could alter hepatocyte responses to different DNA injuries. Abnormal mitoses after γ-irradiation of regenerating p53-null livers demonstrated circumstances where loss of p53-dependent $G_1$ and $G_2$ checkpoints may generate abnormal ploidy. Indeed, the likelihood of mice to develop diffuse hepatic cytological atypia or morpholgically-atypical foci in liver, after a single-dose of a hepatocarcinogen (diethylnitrosamine), was increased with decreasing p53 gene-dose.

Thus p53 becomes important when hepatocytes are released from $G_0$ and stressed, sensitizing them to mitogen and cytokine regulators of cell cycle progression and apoptosis. Hence p53 deficiency is likely to be significant in an environment of persistent regenerative stimuli and unfavorable trophic support, or in the presence of other enabling genetic lesions. This model is relevant to human hepatocarcinogenesis, which almost always occurs against a background of chronic hepatocellular destruction in hepatitis and cirrhosis. In that context, by reducing the need for cytokine support and disabling DNA damage-induced growth arrest, p53 deficiency should facilitate the expansion of preneoplastic clones in chronic liver disease.

In summary, the 4 major findings of this thesis are that in murine hepatocytes (1) p53 sensitises stimulated hepatocytes to cytokine regulators of proliferation and survival; (2) p53 is essential for normal growth arrest, but not apoptosis, after exposure to DNA damaging agents (uv, γ-irradiation); (3) after uv and γ-irradiation there are differences in p53 accumulation and transcriptional activation that suggest multiple pathways to p53-dependent growth arrest after different genotoxic injuries; (4) reduction of p53 gene dose does not regulate normal nuclear polyploidisation, but after exposure to a carcinogen increases the likelihood to develop diffuse nuclear cytological abnormalities and morphologically atypical foci.

# Contents

# Abbreviations

| | |
|---|---|
| 5-AAF | 5-acetylaminofluorene |
| AP-1 | activator protein 1 |
| BrdU | 5-bromodeoxy-uridine |
| CAT | chloramphenicol acetyltransferase |
| $CCl_4$ | carbon tetrachloride |
| CD | cluster of differentiation |
| CMV | cytomegalovirus |
| Con A | concavalin A |
| DAB | diaminobenzidine |
| DDW | double distilled water |
| DEN | diethylnitrosamine |
| Dex | dexamethasone |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediamine tetraacetic acid |
| EGF | epidermal growth factor |
| FBS | foetal bovine serum |
| FITC | fluorescein isothiocyanate |
| HBX | hepatitis B virus protein X |
| HCC | hepatocellular carcinoma |
| HGF | hepatocyte growth factor |
| HSS | hepatic stimulator substance |
| i.p. | intra-peritoneal |
| Ifnγ | γ interferon |
| IGF | insulin-like growth factor |
| IL | interleukin |
| IRF | interferon regulatory factor |
| LB | Luria-Bertani |
| LRF-1 | liver regulatory factor |
| mRNA | messenger ribonucleic acid |
| MTT | 3-(4,5-dimethylyhiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| OD | optical density |
| ONPG | o-nitrophenyl-β-D-galactopyranoside |
| PBS | phosphate-buffered saline |
| PHx | partial hepatectomy |
| pRB | retinoblastoma protein |
| RGC | ribosomal gene cluster |
| SFM | serum free medium |
| STAT | signal transducer and activator of transcription |
| SV40 | simian virus 40 |
| TBE | Tris-Borate-EDTA |
| TCPOBOP | 1,4 bis 2-(3,5dichloropyridyloxybenzene) |
| TE | Tris-EDTA |
| TGFα, TGFβ | transforming growth factor α, β |
| TNFα | tumor necrosis factor α |
| TNFR | TNF receptor 1 |
| uv | ultra violet |
| X-GAL | 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside |

# 1. Introduction

## 1.1.a General introduction

Many tissues grow or atrophy to meet variations in demand, and can restore deficits due to injury. Regulated changes in cell number are critical to these activities, and are achieved by control of proliferation, active cell death (apoptosis), and differentiation into or away from the particular cell type. By contrast, the cancers are a group of diseases characterised by dysregulated increases in cell number, and carcinogenesis is facilitated by genetic lesions that relax the regulation of proliferation or apoptosis. The p53 tumour suppressor gene regulates both proliferation and apoptosis in certain tissues, and is commonly dysfunctional or deleted in human cancer.

The premise of this thesis was that the disease consequences of defective p53 could only be understood for the cell type in which the studies were performed, and that different environmental contexts could alter these consequences. The idea was to attempt to develop a broad understanding of how loss of p53 affected proliferation, apoptosis and carcinogenesis in a cell type relevant to human cancer, under different environmental conditions.
The constraints of tissue specificity and relevance to human cancer required that primary epithelial cells be studied, using the resource of p53-gene-targeted mice. Hepatocytes are particularly suitable and interesting to study, since they are differentiated, yet proliferation-competent, and the regulation of hepatocyte turnover is fundamental to understanding inflammatory and neoplastic liver disease, liver failure and liver carcinogenesis. Moreover there was evidence that defective p53 was common in human liver cancer (although thought to be a late event) but almost no data on the role of p53 in hepatocytes.

My reviews – written during the period of this thesis – of general aspects of apoptosis, p53, and the relationship of p53 to apoptosis and cancer, are included as bound submissions at the back of the thesis, and the contents are not repeated here. Therefore, this chapter focuses on hepatocyte proliferation, apoptosis and carcinogenesis as 3 major themes relevant to this thesis, and specifically considers the roles of the p53 protein in these processes.

## 1.1.b The liver

The adult liver is composed of specialised epithelial cells and non-parenchymal cells (including endothelium, Kupffer cells, Ito cells, fibroblasts). Together, these elements form a structured multicellular community in which no single cell type is autonomous. Receiving 2 blood supplies, the liver carries out unique synthetic, catabolic and regulatory responsibilities that affect many aspects of physiology and are differentially distributed within the liver acinus along the axis of vascular flow, producing zonal heterogeneity of function and response to a signal. Whilst there is functional reserve, this is backed up by

an enormous capacity to regenerate parenchyma, even after repeated insults. Conversely, parenchymal mass decreases during states of decreased physiological demand, by extra apoptosis and reduced generation of hepatocytes.

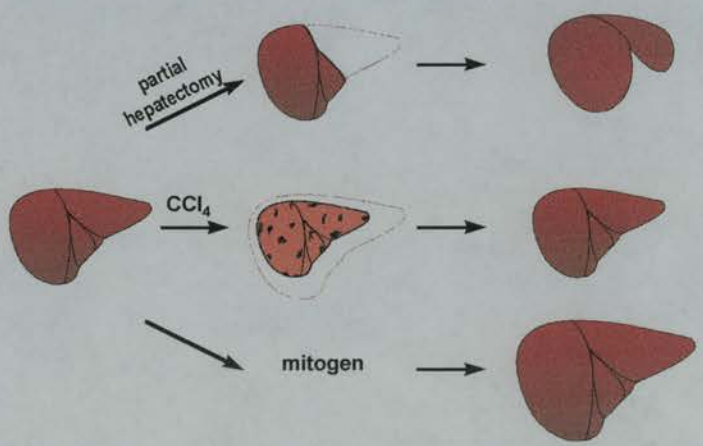
The mechanisms of liver-size homeostasis and plasticity in response to new environmental demands are remarkably unclear. Physical constraints are important, as well as metabolic, hormonal, and genetic factors [1]. The liver normally maintains a constant mass relative to body weight, and may be regulated more by total parenchymal mass or volume than by cell number. This is significant because hepatocytes are of different sizes, according to their ploidy and whether they are binucleate cells — which contribute significantly to the total cell population. Indeed, when the liver returns to normal size after partial excision or after withdrawal of a pharmacological mitogen, the final cell number is not necessarily the same as the original [2-4].

## *1.2 Hepatocyte turnover*

In this section the models commonly used to study hepatocyte proliferation are described, then the regulation of proliferation and apoptosis are considered in turn. It is clear throughout that the complexity is daunting, with factors often ambiguous in role depending on context; these difficulties are compounded by differences between species, strains and data obtained from different systems. Much new data in the field derives from mice, whilst older material is largely from rat experiments, and the differences from humans are often unknown. Moreover, the liver has to maintain differentiated functions during proliferation, and it is difficult in many instances to clearly discriminate adaptive changes during proliferation to those accomplishing the proliferative process.

### 1.2.a Hepatocyte proliferation

There are 4 major models used to study liver proliferation:




*Legend: models of hepatocyte proliferation.*

1. **Continuous basal renewal.** In normal adult rat, one per 2,000–20,000 hepatocytes is in S phase. Mice transgenic for certain hepatocyte mitogens can dysregulate this type of turnover (e.g. TGFα-transgenic mice).

2. **Compensatory hyperplasia after 70% partial hepatectomy** [5]. The 2 major liver lobes are ligated and removed without disturbance to the remainder. The residual liver lobes increase in size to restore liver mass; the original anatomy is not restored and thus regeneration is not an appropriate term. There is no significant inflammation or necrosis, beyond that related to the small surgical incisions to access the peritoneal cavity. Thus, this model does not reflect the circumstances producing hepatocyte proliferation in human disease processes (bar surgical resection). Nevertheless, this is an exceptional model system of semi-synchronised response to a proliferative stimulus: in rats, DNA synthesis begins after 12–16 hours and peaks at about 24 hours. For individual hepatocytes, S phase lasts about 8 hours; $G_2$ 4–6 hours; mitosis 30–60 minutes. In the first 35 hours most hepatocytes undergo one cell cycle; for those entering a second cycle, $G_1$ only lasts 6–8 hours. The majority of liver mass is restored within the first 72 hours, and is completely restored by 7–10 days, after which quiescence resumes. In mice, the peak of DNA synthesis after partial hepatectomy may occur a little later than in rats, at about 36 hours [6,7].

3. **Regeneration after cytotoxic injury** (e.g. viral infection, drug, ischaemia, immune attack, metabolic injury). Hepatocytes are destroyed, with or without non-parenchymal cell damage, and replaced by proliferation of neighbouring hepatocytes. This model recapitulates more closely than partial hepatectomy the pattern of damage-regeneration of human hepatitis and cirrhosis. A difficulty is to discriminate changes specifically related to the insult from those pertaining to the regeneration process.
   A standard version of this model is $CCl_4$-induced hepatocyte necrosis: intraperitoneal injection of $CCl_4$ produces acute swelling and degeneration of hepatocytes in the central regions of lobules (acinar zone 3) [8]. This is followed by necrosis that is fully developed by 24 hours, then by regeneration and restoration of the normal liver anatomy within 3–7 days. By contrast with partial hepatectomy, damaged regions of lobules are repaired, but no new hepatic lobules are formed.
   Unlike the acute necrosis produced by $CCl_4$, some transgenic mice have chronic, low grade hepatocyte destruction that stimulates continuous regeneration (e.g. mice expressing the Hepatitis B surface antigen transgene in hepatocytes [9]).

4. **Primary hyperplasia.** Exogenous mitogen causes hepatocytes to proliferate without the stimulus of tissue loss, by contrast with 2 and 3 above. As a result, liver mass increases. This model is also used to investigate hepatocyte apoptosis, which increases after withdrawal of the mitogen, during the return to normal of liver mass (the regressing liver model).

One further model that is not well developed is that of altered polyploidisation/binucleation. The majority of the postnatal increase in mouse liver weight is not due to increase in cell number, but increased cell size related to binuclearity (through acytokinetic mitosis) and polyploidy [2]. Without these processes the total hepatocyte number would need to be considerably increased to achieve a normal liver mass.

### *1.2.a.1 Hepatocyte neoformation*

Hepatocytes are unusual in that they are differentiated cells, yet capable of proliferating, and they are the normal source of new hepatocytes in adult liver. Hepatocytes are long-lived (over 2 years in rats and mice [10]), so turnover is very low. There is a diurnal variation in proliferation rate, related to feeding [11,12].

The proliferating hepatocytes in normal liver are distributed in a sporadic, fractal manner [13]. They produce microclones of daughter cells that are recruited into adjacent hepatic plates [14]. The fractal pattern of proliferation is retained after partial hepatectomy [15], until sufficiently extreme resection stimulates virtually all residual hepatocytes to undergo practically synchronous DNA synthesis [16]. Indeed, the liver has an enormous capacity to restore itself [17,18]: after serial partial hepatectomies, rodent hepatocytes can undergo 12–20 consecutive cell cycles (potentially producing over 1 million cells from each parent cell) [15,18,19].

A second source of new hepatocytes is multipotential cells around portal tracts and in bile ductules, and whose progeny (oval cells) can differentiate into hepatocytes, biliary epithelium or even intestinal or exocrine pancreatic epithelium. [20]. These multipotential cells may represent facultative stem cells, whose contribution to hepatocyte neoformation is normally negligible but can be made significant if hepatocyte regeneration is prevented — for example by 5-AAF treatment — in combination with a strong stimulus to regenerate, such as partial hepatectomy [20-23]. In these experiments many oval cells die by apoptosis and it is difficult to prove a contribution to hepatocyte formation, but hepatocyte neoformation from oval cells is now accepted by most investigators [10,20,22]. Oval-like cells have been reported in human liver disease [24], however, their significance outside of special experimental circumstances is uncertain.

### *1.2.a.2 Molecular-genetic changes in hepatocytes after partial hepatectomy*

The sequence of events linking excision of liver lobes (partial hepatectomy) to recognition and response initiation by hepatocytes in the remaining lobes is unknown. Nevertheless, specific changes occur in hepatocytes within minutes of PHx that will now be described.

The earliest molecular-genetic events after PHx do not require synthesis of new proteins and are accomplished through the rapid activation of **preexisting cytoplasmic transcription factors** that translocate to the nucleus, bind DNA and transactivate target

genes (defined as primary response genes or immediate early genes). Two candidate transcription factors to initiate immediate early gene transcription in hepatocytes are NF-κB and STAT3.

**NF-κB** activity peaks and subsides to almost normal within the first hour after partial hepatectomy [25]. NF-κB is the prototype of a family of dimeric proteins, formed by products of the *rel* oncogene family. The active form is a p50/NF-κB1–p65/RelA heterodimer, that is activated by dissociation from an IκB inhibitor. Several cytokines or mitogens can activate NF-κB, of which TNFα may be important in liver regeneration [26,27]. The targets of NF-κB after partial hepatectomy are undefined but probably include genes maintaining a differentiated phenotype, such as those involved in gluconeogenesis, also constitutive hepatocyte transcription factors such as the C/EBPs (CAAT enhancer binding proteins) [28,29].

**STAT 3** (signal transducer and activator of transcription) shows increased DNA binding activity within 30 minutes after partial hepatectomy, and this is sustained until 5 hours. STAT 3 may therefore contribute to induction of a subset of immediate early genes that are induced over a prolonged period during $G_1$. IL-6 and TNFα are activators of STAT3 that are likely to be important after partial hepatectomy, and appear to be required for a normal proliferative response [27,30]. STAT 3 is also activated by cytokines such as IL-1, and by the hepatomitogen, EGF [29]. Immediate early gene targets of STAT 3 after partial hepatectomy may include *c-jun*, *c-myc* and *c-fos* — involved in progression through $G_1$.

Hepatocytes induce expression of at least 70 **immediate early genes** within a couple of hours after partial hepatectomy [31]. The transition from quiescence ($G_0$) to early $G_1$ has been defined by the appearance of certain of these gene products. The immediate early genes include members of several transcription factor families (e.g. *jun-fos, LRF-1*, *rel*, nuclear receptors, helix-loop-helix and zinc finger families). The Jun and Fos families are probably important inducers of delayed early gene transcription through the AP-1 (activator protein) transcription factors formed by their products; individual members show different patterns of activity during $G_1$; for example, *c-fos*, *c-jun*, *junB* and *junD* are expressed, but not *fra-1*, *fra-2* or *fosB* [32]. Jun is essential to hepatogenesis — *c-jun*-deficient embryonic stem cells do not contribute to liver in chimaeras, by contrast with all other somatic cell types [33] — and these gene families are probably essential for regulation of hepatocyte proliferation and phenotype during regeneration. Other categories of immediate early gene are still poorly understood, but intriguingly include the hormone and potent hepatomitogen EGF [34]). It is of interest that maximum activity *of c-jun* is probably dependent on early TNFα stimulation of hepatocytes [35], illustrating that external stimuli can both trigger and modulate the immediate early response.

Approximately one third of immediate-early genes induced after partial hepatectomy is induced only in the hepatocytes. A proportion is part of an adaptive response to maintain metabolic homeostasis (e.g. increased gluconeogenesis) in the face of tissue loss, rather than having primary involvement in proliferation. This reflects that for the hepatocyte to

combine dual roles of differentiated function and proliferation requires intimate co-regulation of these activities [36]. Indeed at least some immediate early genes probably serve dual roles, for example IGFBP-1 [29].

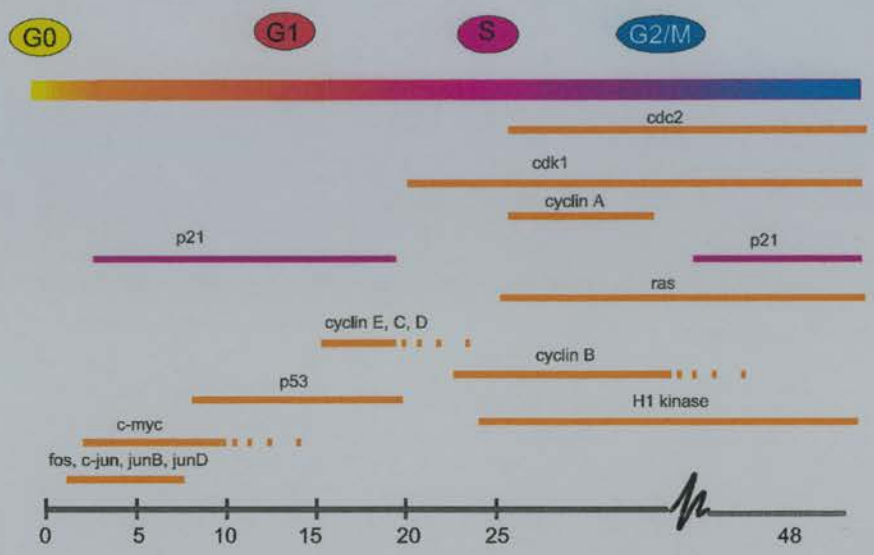



*Figure 1. Gene expression after partial hepatectomy (the bottom axis indicates hours after resection). Sequential activation of genes during $G_1$ provides multiple opportunities for strict control of hepatocyte proliferation, by ultimately regulating activation of $G_1$ cyclins to start the irreversible step of DNA synthesis. Adapted from [7,29,30,37-47].*

Many of the immediate early gene products are themselves transcription factors, so they in turn induce a set of secondary response genes (**delayed early genes**, including *p53*), in an amplifying cascade of transcription events during progression through $G_1$ to S phase. Specific and sequential expression of certain oncogenes and other cell cycle related genes has been defined and correlated with the onset of DNA synthesis and mitosis in the liver remnant (Figure 1). The significance for the regulation of entry to DNA synthesis by hepatocytes will now be discussed.

### *1.2.a.3 Regulation of mitogen responsiveness of hepatocytes*

The identification of specific, sequential gene transcription in the interval between partial hepatectomy and DNA synthesis shaped the idea of regulated progression from quiescence to a point where hepatocytes became irreversibly committed to DNA synthesis. The observation that quiescent hepatocytes in normal liver were refractory to mitogen stimulation of DNA synthesis, but became responsive after stimuli insufficient by themselves to produce significant proliferation (30% hepatectomy or low dose hepatic collagenase perfusion [48,49]), led to the concept of unresponsiveness that needed "priming", and the proposal of **2 key stages** in the pre-replicative period after partial hepatectomy [43,44]: during the first 4 hours (characterised by *c-fos*, *c-jun* and *c-myc*

expression) hepatocytes enter $G_1$ and become "competent" to respond to mitogen stimulation ("**primed**"). Thereafter "**progression**" occurs, controlled by paracrine or autocrine mechanisms, correlating with transit from mid-$G_1$ to S, and marked by increasing *p53* expression. *ras* expression coincides with the start of replicative DNA synthesis and the period to mitosis.

Thus priming was conceived as a permissive process for mitogen responsiveness, whilst commitment to DNA synthesis was assumed to occur during progression. The model allowed for a ready but easily reversed stimulation of hepatocytes into the primed state from which the likelihood of subsequent proliferation was tightly regulated by environmental factors. In culture, hepatocytes become unresponsive to mitogens after a few rounds of cell division, requiring replating or pharmacological agents (e.g. DMSO) to regain sensitivity [50]; this implies a cell internal regulator of the primed state that needs "refreshing" periodically, to maintain a mitogen-responsive state.

Loyer has used a primary culture model, in which similar genetic changes occur as following partial hepatectomy, to define a **mitogen restriction point** in mid-late $G_1$ (42–48 hours after isolation) [51]. Hepatocytes require mitogen (EGF or TGFα) to progress beyond this point and begin DNA synthesis; without mitogen, they growth arrest. Thus commitment to DNA synthesis must occur after the mitogen restriction point.

Although rather more poorly understood than soluble regulators of cell cycle progression, **cell-matrix interactions** through specific receptors (e.g. integrin receptors) regulate priming events in early $G_1$, while alterations in cell shape (itself determined by cell-cell and cell-matrix interactions) may regulate progression through late $G_1$ phase [52,53]. The interactions can have a comitogenic or inhibitory effect on hepatocyte proliferation [52-55]. These factors may be particularly relevant to true regeneration (i.e. tissue damage), in which there are additional but little understood signals such as local electrolyte and metabolic abnormalities, with altered cell-cell and cell-matrix contact [52,56].

### *1.2.a.4 Molecular genetic events in other models of regeneration and primary hyperplasia*

Regenerating liver after **$CCl_4$ injury** shows the same sequence and similar time course of expression of *c-jun*, *c-fos*, *c-myc*, *p53*, *c-Ha ras* and *c-Ki ras* as occurs after partial hepatectomy [41,57-62]. These changes start in the lethally-damaged hepatocytes (in the centers of lobules) 2-3 hours earlier than in the remainder, suggesting that the response to injury overlaps with the immediate early events of proliferation [63]. Kubin reported that *mdm-2* expression peaked 12 hours after $CCl_4$ injury (just before the onset of necrosis) but did not increase after partial hepatectomy, and interpreted the increase as a response in the injured cells to the drug-induced damage [57]. TNFα is an important stimulus of hepatocyte *c-fos* and *c-jun* expression after $CCl_4$ injury — just as after partial hepatectomy — and is necessary for rapid repair and regeneration [26].

Thus, despite the differences in the initial insult, partial hepatectomy and $CCl_4$ treatment have very similar effects on cell cycle-related gene expression in the residual hepatocytes. By contrast, hepatocyte injury producing **oval cell proliferation** (Galactosamine treatment, or 5-AAF pretreatment before $CCl_4$) showed a different pattern of oncogene expression: there was delayed and prolonged expression of *c-myc*, *c-fos* and *c-jun*, despite livers showing similar kinetics of DNA synthesis [62].

Hepatocyte hyperplasia produced by **xenobiotic mitogens** involves different signal transduction and transcriptional events from those after $CCl_4$ or PHx [64,65]. The particular pathways vary, depending on the chemical class of mitogen. Some mitogens may act through non-parenchymal cells, for example by stimulating TNFα release [64,66]. However, in general, whilst hyperplastic DNA synthesis is followed by increased expression of *c-ras*, there is no preceeding increased expression of *c-jun*, *c-fos* or *c-myc*, suggesting that immediate early gene activation is not necessary for hepatocyte cell cycle entry [60,61,64,65,67]. Other differences with post-hepatectomy proliferation include the occurrence of increased apoptosis in hyperplastic growth.

The molecular genetic differences between these models have biological significance: induction of regenerative DNA synthesis is commonly used to fix the DNA damage produced by a liver carcinogen, whereas hyperplastic DNA synthesis cannot fulfill this purpose [68,69] (see section below). Taken together, these models increase understanding of the complexities regulating hepatocyte proliferation, but show a need to select relevant models when attempting to understand human disease processes.

### *1.2.a.5 Late cell cycle events: acytokinetic mitosis and polyploidy*

Hepatocyte binuclearity and polyploidy are features of normal adult liver growth, developing after weaning [2]. In mice, hepatocyte polyploidisation occurs to a relatively high degree, predominantly through repeated rounds of acytokinetic mitosis to produce a binuclear cell, followed by nuclear fusion (thus doubling cell ploidy) in a subsequent cell cycle (Figure 2) [2]. By contrast, regeneration is non-binucleating: after partial hepatectomy, mitoses temporarily become cytokinetic, so the proportion of binuclear cells in the liver remnant decreases. However, average nuclear ploidy increases because cytokinetic mitosis of the binuclear cell population produces mononuclear cells of increased ploidy (Figure 3) [16,64]. Once liver mass is restored, the normal pattern of cell division resumes, and the proportion of binuclear cells slowly increases. By contrast with regeneration, hepatocyte hyperplasia produced by certain xenobiotic mitogens can be accompanied by increased binuclearity (eg. lead nitrate) or increased polyploidisation (e.g. nafenopin) [4].

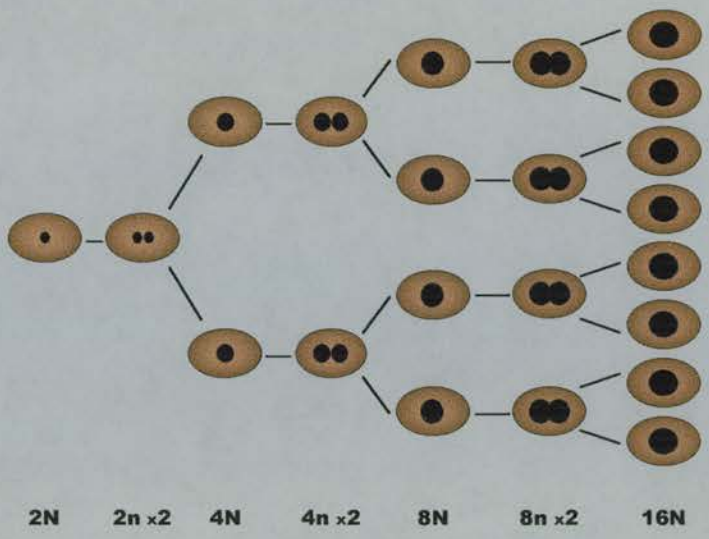



*Figure 2. The evolution of polyploidy in hepatocytes. Acytokinetic mitosis is followed in the next cell cycle by nuclear fusion and cytokinesis to generate two cells of higher ploidy.*

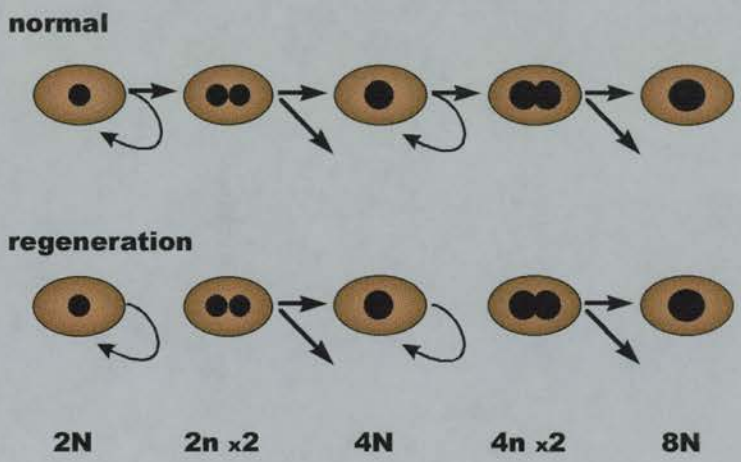



*Figure 3. The change from polyploidising (acytokinetic) to non-polyploidising (cytokinetic) proliferation during liver regeneration.*

Acquisition of a polyploid nuclear DNA content is an irreversible event that involves modulation of centrosome replication, and of the normal couplings of S to M phase and M phase to cytokinesis. The observations described above show that proliferating hepatocytes are capable of reverting to normal cytokinesis, suggesting that polyploidisation is specifically regulated. Binuclear and polyploid hepatocytes are less sensitive than diploid cells to physiological mitogens (EGF, insulin) [70], and are less likely to go through multiple cell cycles during liver regeneration. Thus mice transgenic for hepatic mitogens often show a relative preponderance of diploid cells [71,72]. However,

both the regulation and the significance of polyploidy are still rather poorly understood [73-76]. It is possible that polyploidising growth protects against the development of neoplasia, through increased gene copy number (reducing the consequences of allele mutation) or reduced capacity to proliferate. Indeed, preneoplastic nodules and carcinomas in experimental carcinogenesis, human focal nodular hyperplasia and hepatocellular carcinomas all show a predominance of diploid, non-polyploidising hepatocytes [3,77]. The switch to cytokinetic division during regeneration might preserve the liver's capacity for self-renewal.

### *1.2.a.6 Extracellular regulators of hepatocyte proliferation*

#### Mitogens & comitogens

Critical experiments showed that there are signals producing hepatocyte proliferation after PHx, which:

1. begin within minutes (or, at least, there are electrophysiological [78-80] and biochemical changes in residual hepatocytes).
2. are specific to liver, because no proliferation occurs in other organs (except a small increase in DNA synthesis in exocrine pancreas [81]).
3. are blood borne, because there is concomitant proliferation of hepatocytes placed in other body sites (tissue engrafts [82] and transplants of isolated hepatocytes [83]). Moreover, the blood of normal rats is not mitogenic, but the blood of a rat subjected to partial hepatectomy stimulates DNA synthesis in normal rat liver maintained in parabiotic circulation [84,85].
4. involve blood-borne stimuli of extrahepatic origin, because when rats are maintained in parabiotic circulation, removal of the liver from one rat induces DNA synthesis in the other [84,85].

Although foetal hepatocytes continue to proliferate for a few days in culture [86], primary cultures of adult hepatocytes require mitogen stimulation to proliferate. Culture studies on primary hepatocytes maintained in chemically-defined medium have led to a categorisation of mitogens as "complete" (those that stimulate hepatocyte DNA synthesis) or "co-mitogens" (those that do not themselves stimulate DNA synthesis but that increase the effect of complete mitogens). The term "complete" is slightly misleading since these factors have little effect on hepatocyte proliferation in quiescent liver, but efficiently stimulate DNA synthesis in primed hepatocytes.

Many agents have been reported to stimulate hepatocyte DNA synthesis, but only a few have been shown to be important *in vivo*, and there are conflicting reports or only associative data for several others [87]. Interspecies differences (for example with vasopressin [88,89] and members of the fibroblast growth factor family [90]) account for

some discrepancies, and illustrate the difficulties of cross-species comparisons. Table 1 lists hepatocyte mitogens commonly recognised to be important *in vivo*. Table 2 lists co-mitogens; other comitogens, such as angiotensin II [91], neurotensin [92], hepatopoietin B [93], remain uncharacterised or of uncertain importance *in vivo*, whilst many hormones reported to facilitate DNA synthesis (pineal, oestrogens, glucagon) may only do so as a side-effect of their general stimulation of cellular metabolism [1,87]. Still others, such as IGF-1, have only been shown to stimulate proliferation in co-culture [1].

| **Complete mitogens.** | **References.** |
|---|---|
| Epidermal growth factor (EGF) | (94) |
| Hepatocyte growth factor (HGF) | (72) |
| Transforming growth factor α (TGF α) | (95) |
| Tumour necrosis factor α (TNF α) | (26) |

*Table 1. Complete hepatocyte mitogens*

| **Hepatocyte co-mitogens.** | **References.** |
|---|---|
| Hepatic stimulator substance (HSS) | (1) |
| Insulin | (96) |
| Interleukin 6 (IL6) | (30,97) |
| Noradrenaline | (96) |

*Table 2. Hepatocyte co-mitogens.*

Proliferation signals can be delivered to hepatocytes by hormonal (EGF), paracrine (HGF) or autocrine (TGFα) mechanisms. Of course, these factors do not act in isolation and their effects are titrated against existing gene expression and micro-environmental influences. Transgenic and gene-targeted mice are now a major resource to show how the factors act *in vivo*, and what are the consequences of their dysregulation. This section briefly describes factors thought to be the major players regulating and perhaps initiating hepatocyte proliferation.

**Hepatocyte growth factor (HGF)** is a pleiotropic epithelial cell mitogen and the most potent hepatocyte mitogen. In liver, HGF is secreted by Ito cells and so probably acts in paracrine fashion, binding the specific receptor product of the *c-met* oncogene [98]. During liver regeneration, release of HGF by the Ito cells may be stimulated by the cytokine interleukin-6. HGF injection accelerates regeneration, and after a prolonged infusion even induces proliferation in quiescent liver [99]. Transgenic mice that overexpress HGF have twice the normal baseline rate of hepatocyte proliferation, although little increase in liver:body weight ratio, suggesting a shorter hepatocyte lifespan [72]. The transgenic hepatocytes continuously express *c-jun* and *c-myc*, suggesting that HGF helps to prime hepatocytes, and there is a relative increase in diploid hepatocytes, in keeping with the

increased cell proliferation (see polyploidy above). Indeed, HGF-transgenic mice recover from partial hepatectomy in half the time of controls. Despite increased proliferation, there is no increased susceptibility to hepatocellular carcinoma, in keeping with data that HGF suppresses proliferation of several hepatocellular carcinoma cell lines [100]. Homozygous targeted germline-deletion of *c-met* or HGF is embryonic lethal; however, the embryos show up to 55% reduction in liver size, consistent with a critical role for HGF in hepatogenesis as well as proliferation of differentiated hepatocytes [101].

**Transforming growth factor-α (TGFα) and epidermal growth factor (EGF)** share the same receptor (EGFR) which, like the HGF receptor, is a receptor tyrosine kinase. Young TGFα-transgenic mice have enlarged livers with increased hepatocyte proliferation rates [71]. They also have an increased proportion of diploid hepatocytes — the ploidy population most responsive to growth factor stimulation (see polyploidy above). Like HGF-transgenic mice, the peak of DNA synthesis after partial hepatectomy is increased, suggesting increased numbers of hepatocytes responding (or greater synchrony). By contrast with HGF-transgenic mice, the interval to DNA synthesis is not shortened, suggesting TGFα may not actually prime hepatocytes, but act later in $G_1$.

The hepatomegaly of TGFα-transgenic mice resolves later in life due to increased hepatocyte apoptosis (i.e. shorter lifespan), that may in part relate to the development of chromosomal abnormalities and aneuploidy in a high proportion of hepatocytes. At 12–15 months 75% of TGFα-transgenic mice develop hepatocellular carcinomas, in striking contrast to HGF-transgenic mice. The reason for this difference is not clear. In primary culture, TGFα-transgenic hepatocytes readily replicate continuously without transformation, and maintain a differentiated phenotype [102].

TGFα-null mice show no liver abnormalities and regenerate normally [103,104], presumably reflecting redundancy with EGF. However, the converse does not hold: the major source of circulating EGF in rodents is the salivary gland; excision of the salivary glands or ligation of their venous drainage in rats delays and reduces the peak of DNA synthesis after partial hepatectomy, suggesting that endogenous TGFα secretion is insufficient to compensate [105]. This does not affect immediate-early oncogene expression in the liver remnant (*c-jun*, *c-fos*, *c-myc*), further suggesting that EGFR signalling acts later in $G_1$ to permit DNA synthesis. Interestingly, in chimaeras, EGFR-homozygous-null embryonic stem-cells contribute to formation of hepatocytes in the neonate but not adult [106]. This implies that EGFR signals are required for growth of postnatal but not embryonic liver. As described earlier, EGF is an immediate early gene of hepatocytes, but the significance of this observation is unknown.

**Noradrenaline (Nor).** Stimulation of hepatocyte $\alpha_1$-adrenergic receptors causes downregulation of the growth arrest specific gene *gas-6* in the first 4 hours after partial hepatectomy, and is necessary for the normal peak of DNA synthesis to occur [93,107-109]. Noradrenaline stimulation of hepatocytes is also an effective antagonist of proliferation

inhibitors such as TGF$\beta_1$ or activin [110,111]. Thus, although still little understood, there is good evidence for neuroendocrine modulation of liver regeneration.

**Inflammatory cytokines.** In human liver disease, hepatocyte proliferation usually occurs in a context of inflammatory destruction, in a micro-environment rich in inflammatory cytokines. These molecules might be expected to influence hepatocyte proliferation and the two cytokines thought to be most important to liver regeneration are **tumour necrosis factor-α (TNFα)** and its inducible cytokine, **interleukin-6 (IL6)**. TNFα and IL6 are released by macrophages, monocytes and endothelial cells after tissue injury or exposure to toxins such as bacterial endotoxin [514]. Indeed, normal liver is probably continuously exposed to low concentrations of these cytokines, due to various gut bacterial and other toxins in portal blood. Several lines of evidence show that inflammatory cytokines are important to liver regeneration:

1. Animals with deficient inflammatory or immune environments show retarded liver regeneration: for example, pathogen-free rats, which lack gut bacterial endotoxin [112], or $\beta_2$-microglobulin-deficient mice, which lack MHC class I and CD8 T cells [113]. Mice lacking endotoxin-responsive macrophages show decreased secretion of TNFα and IL-6 after partial hepatectomy, and have retarded liver regeneration [114].

2. IL6-deficient gene-targeted mice have severely impaired liver regeneration, leading to necrosis and liver failure. This is associated with decreased activation of STAT3 and decreased immediate early gene expression (AP-1, myc and cyclin D) after partial hepatectomy. These biochemical and regenerative defects are corrected by a single dose of IL6, which also promotes DNA synthesis in cultured hepatocytes [97]. TNF receptor I-deficient mice have similar defects in activation of transcription factors STAT3 and AP-1 after partial hepatectomy, with reduced hepatocyte DNA synthesis [27]. These defects are partially corrected by IL6 (NFκB remains inactive), illustrating the degree of overlap between cytokine signal pathways.

3. TNFα promotes DNA synthesis in primary cultures of mouse hepatocytes [115,116] and enhances EGF-induced hepatocyte DNA synthesis [117]. *In vivo*, TNFα rapidly activates NF-κB [25], and is necessary for increased expression of immediate early genes (*c-fos, c-jun*) and for increased DNA synthesis after $CCl_4$ injury [26] or partial hepatectomy [35,118]. Intravenous infusion of TNFα into healthy rats increases hepatocyte proliferation and liver mass [119,120].

Taken together, the data suggest that TNFα and IL6 prime hepatocytes for proliferation, and are important for initiation of the regenerative response. Although most reports use a post-hepatectomy model, these cytokines might be expected to be even more relevant to inflammatory-necrotic regeneration such as in lobular hepatitis.

**Signal integration** (Figure 4). Cytokines such as TNFα, IL6, γ- and α-interferon bind different receptor types from the receptor tyrosine kinases binding HGF, EGF and TGFα; however, there are potential common targets for mitogen and cytokine signal transduction pathways: for example, the STAT transcription factors implicated in immediate early gene activation after partial hepatectomy, and the stress-activated protein kinases (e.g. jun kinase) that in some cell types mediate growth arrest responses to cytokines or uv-irradiation [36]. Such cross-talk allows orchestrated reprogramming of hepatocyte gene expression to balance regenerative demands with general metabolic requirements, participation in the local acute phase response to injury, and the need to perform systemic tasks such as synthesis of acute phase proteins [121,122].

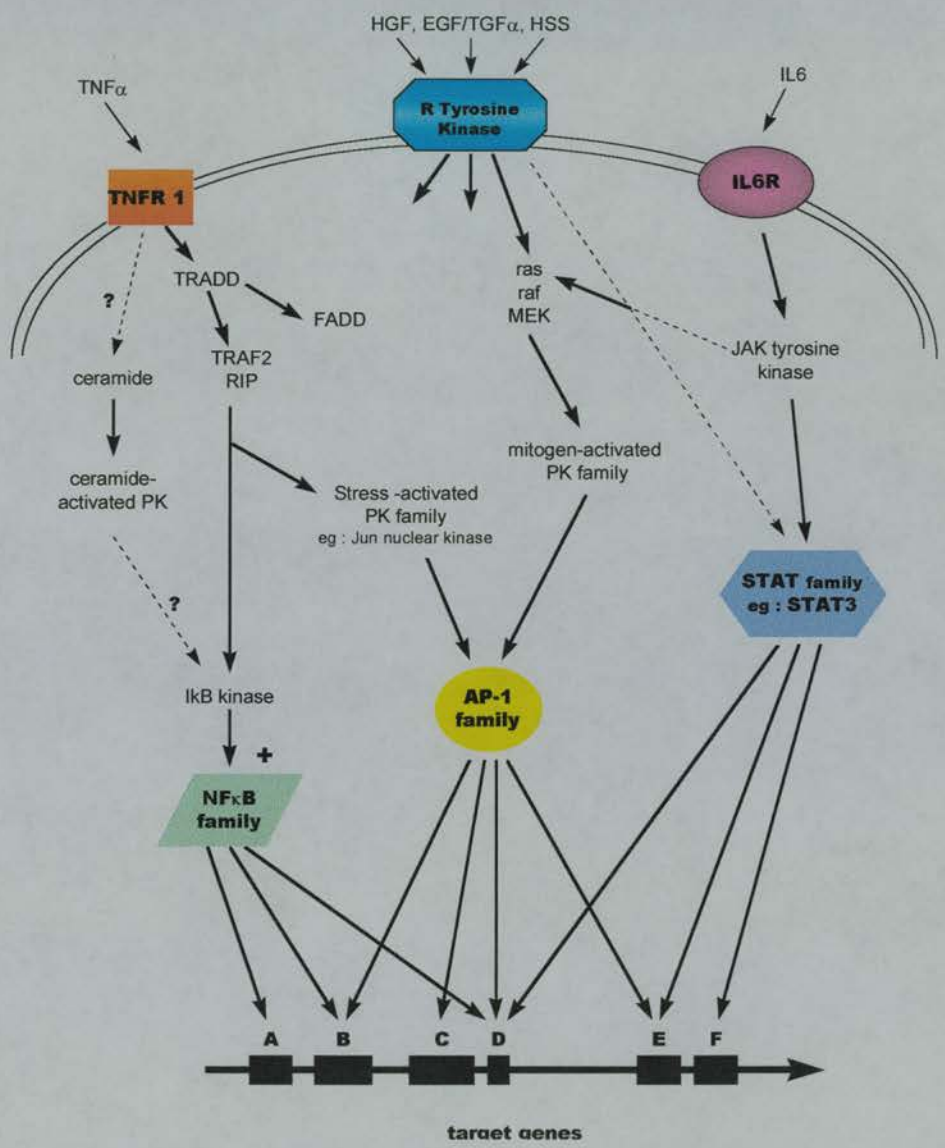



*Figure 4. Cartoon illustrating interaction and overlap between mitogen and cytokine signal transduction pathways to influence expression of overlapping sets of target genes. The overlap allows redundancy between signals, and interactive effects that are qualitatively different from the individual contributions.* [36,122,123].

## Extracellular inhibitors of proliferation (restoration & maintenance of quiescence)

The regenerative response stops abruptly when liver mass has been restored. This has been interpreted to imply an important role for specific extracellular inhibitors of hepatocyte proliferation. At the same time, however, it is usually implicitly assumed that the "default" state of hepatocytes is replicative quiescence and that stimulation is required to cause proliferation. There is thus the expectation that the system returns itself to quiescence when no longer stimulated. In fact, hepatocyte proliferation in the presence of a mitogenic stimulus is self-limited, presumably by cell-intrinsic properties: thus in culture adult primary hepatocytes become refractory to mitogen stimulation after 2–3 rounds of cell division, unless "reprimed" periodically by replating or temporary exposure to pharmacological agents such as DMSO or phenobarbital [50,124]. Investigators have regarded this phenomenon as one of "inadequate" (unphysiological) culture conditions preventing continuous replication; however, the observation is entirely compatible with a simple stimulus (mitogen) producing perturbation (cell cycle) but with inevitable return to the set point by a self-organising system (see below) [125]. Mice transgenic for growth factors demonstrate the same phenomenon: although there may be increased basal proliferation rates, almost all hepatocytes are still *not* proliferating (although capable, as demonstrated after partial hepatectomy); the increased proliferation seen is entirely in keeping with such a self-limited response to mitogen at the individual cell level. Certainly there is no evidence that soluble growth inhibitors are involved in maintaining refractoriness to mitogens in quiescent liver, or are necessary for resumption of quiescence, or indeed are capable of maintaining quiescence after partial hepatectomy, and such hypotheses seem a rather unnecessary overcomplication in this cell type.

It is possible that quiescence in the adult hepatocyte can be understood in the terms of a self-organising system [125]. In a self-organising system, perturbation is followed by oscillations but eventual return to the default state — a so-called *stable set point* or *stable limit cycle* in state space (state space represents all the possible changes in the system). Cell cycle progression and checkpoints are perhaps best understood using such models [125,126]. In hepatocytes, replicative quiescence may represent a stable set point in state space. Stimuli to proliferate produce or represent perturbations that push the system away from the stable state (into cell cycle). This is sufficient to generate some cycles in culture but ultimately quiescence resumes as the system returns to the stable state. There is thus no need for cell-external antiproliferative influences because the oscillations are necessarily self limited. Nevertheless, some situations may demand suppression of proliferation that would otherwise occur, and therefore require that specific extracellular inhibitory influences can be brought to bear. These will now be discussed.

### *Extracellular regulators*

It is of interest that factors that inhibit hepatocyte proliferation all have other important functions in the regenerating or inflamed liver; it is possible that anti-proliferative actions

on hepatocytes are not their primary effect. Moreover, these agents cause only temporary growth arrest in mitogen-stimulated hepatocytes, rather than resumption of quiescence, and none is able to prevent liver regeneration. Table 3 lists factors shown to suppress mitogen-stimulated entry to DNA synthesis in primary culture. At present, only TGFβ$_1$ is widely recognised to be important. Some of these factors will now be discussed.

| Mito-inhibitor. | References. |
|---|---|
| Transforming growth factor β (1,2) | (88) |
| Activin A | (127) |
| Interleukin 1β | (128) |
| Hepatic proliferation inhibitor peptide | (129) |
| γ interferon | (116,130) |

*Table 3. Physiological inhibitors of hepatocyte proliferation.*

### *TGFβ$_1$*

Three TGFβ isoforms are produced in liver, although most is known about TGFβ$_1$. TGFβ$_{1\text{-}3}$ delivery to hepatocytes during liver regeneration was thought to be paracrine [131], however, recent work shows that TGFβ expression can occur in hepatocytes as well as Kupffer cells, hepatic endothelium and Ito cells, and favours autocrine delivery as the major mechanism during liver regeneration [132-134]. Extracellular TGFβ is normally inactive, sequestered by matrix binding proteins that control its bioavailability or complexed to latency associated peptide on cell surfaces; the mechanisms of activation on the hepatocyte surface are multistep and poorly understood, but may involve altered interaction with the IGF-2 receptor [132].

The TGFβ receptors are serine-threonine kinases, but the post-receptor events leading to growth arrest in hepatocytes are not well-defined. In epithelial cell lines, TGFβ$_1$ activates inhibitors of cyclin-dependent kinases (p27$^{kip1}$, p15ink2B and p21$^{WAF1}$) to prevent activation of the G$_1$ cyclins and subsequent Rb phosphorylation [135-138]. This produces growth arrest in late G$_1$ phase. Similar inhibition of pRb phosphorylation has been shown in TGFβ$_1$-treated liver remnants after partial hepatectomy, and induction of p21$^{WAF1}$ expression by TGFβ$_1$ has been demonstrated in primary hepatocytes [7,137,139]. TGFβ$_1$ does not alter hepatic expression of *c-myc* or *p53* [1]; however, in other cell types p53 function is important for effective TGFβ$_1$ growth arrest [140], due to a co-operative but distinct action on p21$^{WAF1}$ [141,142].

TGFβ$_1$ is the most potent inhibitor of hepatocyte DNA synthesis produced by different mitogens, for example, EGF or TNFα [115,143]. *In vivo*, TGFβ$_1$ produces a dose-dependent reduction in the proportion of hepatocytes entering S phase if given 11 hours after (but not if only administered simultaneously with) partial hepatectomy, suggesting it induces growth arrest in late G$_1$, in keeping with an effect on pRb [104,144]. However DNA synthesis is only postponed (from 22 to 72 hours), and cannot be further blocked by more

$TGF\beta_1$. The rise in $\alpha_1$ adrenergic receptor stimulation after partial hepatectomy inhibits the anti-proliferative effects of $TGF\beta_1$ [110].

$TGF\beta_1$ activity is negligable in normal adult liver, suggesting it has no role in maintenance of quiescence. Transgenic mice expressing $TGF\beta_1$ show liver fibrosis, increased hepatocyte apoptosis and increased hepatocyte proliferation. The proliferation is probably secondary to the other hepatic disturbances, but shows that even supraphysiological autocrine and paracrine $TGF\beta_1$ expression is insufficient to stop hepatocyte regeneration. $TGF\beta_1$ homozygous-knockout mice die with systemic inflammatory disease within a couple of weeks of separation from sources of maternal $TGF\beta_1$ (placenta and milk). However there is no drastic change in liver function and no evidence of abnormal proliferation [32]. Thus the role of $TGF\beta_1$ in the downregulation of hepatocyte proliferation, although universally cited as important, may be rather limited and less critical than other activities, for example, liver fibrosis and angiogenesis [132].

### *Activin A*

Activin-A is an autocrine product of hepatocytes that binds a specific receptor and inhibits mitogen-induction of DNA synthesis in primary culture [127]. Hepatocytes (and other cell types) also secrete follistatin, which binds and inhibits the actions of activin-A [111]. The importance of activin-A for hepatocytes *in vivo* is unknown — infusion delays regenerative DNA synthesis, whilst follistatin administration accelerates regeneration after partial hepatectomy, suggesting some endogenous activin activity [145]. Although activin-A is a member of the TGFβ superfamily, it does not compete with TGFβ for receptor binding.

### *Interleukin 1β*

The TNF-inducible cytokine interleukin 1β also decreases mitogen-induced DNA synthesis in culture, although less effectively than $TGF\beta_1$ [128].

### *γ-interferon (γIfn)*

γIfn receptors are expressed by hepatocytes in diseased but not normal liver [146]. γIfn is produced by activated T cells, and may be involved in immune hepatic injury (see hepatocyte apoptosis). In primary culture, γIfn suppresses the induction of hepatocyte DNA synthesis by mitogens [115,130], but whether this is important *in vivo* is not known.

### *Intracellular regulation*

**C/EBPα.** Expression of the C/EBPα transcription factor decreased mitotic activity in a hepatoblastoma cell line, and is reduced during liver regeneration [147]. It is possible that upregulation of C/EBPα is important to restore quiescence, and although little is known about its regulation, p53 or pRb are not required [147]. Quiescence-associated genes such as *gas-6* are downregulated at the start of regeneration, however any role in the restoration of quiescence is not understood [27].

**p21$^{WAF1}$** is a cyclin-dependent-kinase inhibitor that arrests eukaryotic cell cycle progression at multiple points, including late $G_1$. p21$^{WAF1}$ mRNA concentration is very low in normal liver, but increases after partial hepatectomy during $G_1$ and again after S phase [7,148]. The importance of p21$^{WAF1}$ to downregulation of liver growth, at least when expressed at high levels, has been shown in mice bearing a liver-specific p21$^{WAF1}$ transgene [149]. The mice had small livers with hypoplastic lobules and did not show the normal compensatory hyperplasia of remaining lobes after partial hepatectomy. p21$^{WAF1}$ is activated by a variety of upstream signals, including p53, however the post-hepatectomy increase occurs in p53-deficient mice and so is p53-independent [7]. Sawada found that cultured hepatocytes from aged mice were more likely than those from young mice to express p21$^{WAF1}$ protein during late $G_1$ phase, and has suggested that this accounts for the age-related reduction in the capacity of hepatocytes to proliferate [150]. Extracellular stimuli that increase p21$^{WAF1}$ mRNA concentration in cycling hepatocytes include protein deprivation (by a p53-dependent mechanism), TGF$\beta_1$ and activin-A [7].

**In summary**, no extracellular physiological inhibitor can prevent liver regeneration or induce quiescence. Defects in mitogens (eg Brattleboro rat [88], IL-6 deficiency [30]) have more profound consequences for reduced liver regeneration than excessive levels of TGFβ — perhaps illustrating the intrinsic bias of liver homeostasis towards quiescence.

## 1.2.b Hepatocyte apoptosis

Many of the same players are identified as regulators of hepatocyte death, proliferation, proliferation-inhibition and acute phase responses; the context appears to determine how a particular change in stimulation affects hepatocytes. It is therefore relevant that culture studies are assessing hepatocytes in cell cycle, in contrast to studies of hepatocytes *in vivo*, where this is not always the case.

### *1.2.b.1 Occurrence in normal adult liver*

Hepatocyte apoptosis is rare in the normal adult liver and has a prevalence of 0.001-0.005% in mice or rats [151]. The incidence of apoptosis shows a circadian rhythm in rodents, being reduced after feeding [152]. Apoptotic hepatocytes are rapidly engulfed by Kupffer cells, sinusoidal endothelium and adjacent hepatocytes; this is the form in which they are usually identified in tissue sections. The mean duration of histologically appreciable hepatocyte apoptosis was estimated to be 169 minutes (95% CI 137 - 222 minutes) and found to be similar for hepatocytes of preneoplastic foci [153]. The apoptotic hepatocytes are usually identified in the 2 rows of cells closest to the central vein [154,155], but whether this reflects an increased incidence of cell death in centrilobular hepatocytes is not clear. Genetic marker studies show that hepatocytes do not migrate towards the central vein as they age ("streaming liver" hypothesis) [156], therefore suggesting that centrilobular hepatocytes are shorter-lived than their neighbours, or that the kinetics of disposal are different. Alternatively, since Kupffer cells are avid scavengers of apoptotic

bodies [157] (often only appreciable by electron microscopy [153]), the observation may reflect relocalisation of Kupffer cells within the lobule.

### *1.2.b.2 Execution of hepatocyte apoptosis*

There are no ultrastructural or biochemical features to suggest that apoptosis of hepatocytes differs in any unique way from apoptosis of other cell types. Nuclear DNA is cleaved to 30-50 and 200-250kbp fragments by a $Mg^{++}$(but not $Ca^{++}$)-dependent process [158]. However, nucleosome 180bp fragments are often not detectable in apoptotic hepatocytes, therefore "DNA ladders" have low sensitivity as a marker of apoptosis in this cell type [159,160].

Specific recognition and engulfment of apoptotic hepatocytes may be facilitated by altered expression of cell surface carbohydrate receptors. In a rat liver regression model (lead nitrate) hepatocyte apoptosis correlated with increased expression of asialoglycoprotein receptors on hepatocytes and galactose receptors on non-parenchymal cells [157,161]. Endothelial scavenging of apoptotic bodies is increased by IL-1; this may improve the liver's capacity to deal with increased cell destruction in hepatitis or endotoxaemia [162]. Nevertheless, in animal models, extensive hepatocyte apoptosis is followed by increasing serum concentrations of hepatocellular enzymes, indicative of cell lysis [163-165]. In these circumstances saturation of clearance mechanisms has allowed the apoptotic hepatocytes to undergo autolysis, causing leakage of hepatocellular enzymes into extracellular fluid. This probably also accounts for the observation of "necrosis" in tissue sections [163-165]. In a regressing liver model a 20-fold increase in apoptotic rate was estimated as sufficient to saturate clearance mechanisms [164].

### *1.2.b.3 Extracellular regulators of hepatocyte apoptosis*

#### Physiological demands

Apoptosis is the characteristic mechanism by which cells are eliminated from organs during periods of decreased physiological demand. Whether apoptosis is involved in involution of the liver after pregnancy or lactation is not reported; however, in rats reduction of trophic signals by hypophysectomy produced increased hepatocyte apoptosis and liver involution [166]. Nutritional status also regulates liver turnover: caloric restriction increases hepatocyte apoptosis, suppresses proliferation and reduces liver size [152,167]; normal feeding returns the rates of apoptosis and proliferation to normal [152]. The pathways signalling these changes have not been defined, but the observation that calorie restriction also makes hepatocytes resistant to xenobiotic mitogens suggests that diffuse changes in cellular responsiveness accompany any reduction in trophic stimuli [152]. By contrast, the prevalence of apoptosis was shown to be significantly reduced during the first 96 hours after partial hepatectomy [168].

The two responses of atrophy and compensatory proliferation can be produced concurrently, in the model of hepatic portal vein branch ligation: the affected lobe, distal

to the ligated portal vein branch, shows a wave of hepatocyte apoptosis in the first postoperative week, predominantly affecting centrilobular cells, and rapidly shrinks; the other lobes show hepatocyte proliferation in the first few postoperative days [169,170]. Again, the signalling pathways triggering apoptosis are unknown; there is no evidence that hypoxia is a significant factor [171].

In summary, rates of cell death in liver can change to meet fluctuations in physiological demand and are regulated in complementary fashion to proliferation rates.

## Specific signal factors that trigger hepatocyte apoptosis

### *Hepatocyte apoptosis in disease: the role of cytokines*

There is good evidence that apoptosis is the critical mechanism of hepatocyte destruction in immune hepatitis (e.g. the response to virus-infected hepatocytes, autoimmune hepatitis, transplant rejection) [172-175], and also under conditions producing acute liver failure during septic shock [176-179]. Apoptosis is signalled through specific surface receptors, triggered by soluble or cellular ligands. The soluble ligands include cytokines and hormones, whilst cellular ligands include fas ligand. Apoptosis can also be triggered by the perforin-mediated mechanism of cytotoxic T cell killing.

| Apoptotic trigger. |
|---|
| Activin-A |
| Fas ligand |
| Ifnγ |
| Perforin/granzyme |
| $TGF\beta_1$ |
| TNFα |

*Table 4: physiological triggers of hepatocyte apoptosis. For references, see text.*

Many of the key players that can trigger hepatocyte apoptosis, can also regulate hepatocyte proliferation and the synthesis of acute phase proteins that characterise the systemic response to tissue injury or infection. Moreover, similar or overlapping intracellular pathways are usually proposed to mediate these different activities. Thus, the effects of any particular physiological agent are likely to be strongly conditioned by the prevalent microenvironment: some agents may even have opposite actions, depending on concentration and context [180]. TNFα and fas ligand have emerged as dominant triggers of hepatocyte death in liver disease.

### *Tumour necrosis factor-α, interferon-γ, interleukins -1β and -6*

**TNFα** directly triggers hepatocyte apoptosis, signalling through the TNF receptor 1 (TNFR1). The TNF receptors (1 and 2) are normally expressed at low levels on hepatocytes, and expression increases in inflammatory liver disease. TNFR1 can signal very different downstream events in hepatocytes, including proliferation (see earlier), synthesis of acute phase proteins and apoptosis. The pathway to apoptosis involves recruitment of a protein (TRADD) that in turn binds a protein (FADD) which signals directly to the apoptotic protease cascade [181-183] (Figure 5). TNFR1 separately signals activation of Jun kinase, p38 MAP kinase and the transcription factor NFκB (which is inhibitory for apoptosis), demonstrating a divergance between pathways to proliferation and apoptosis [181,182]. There is some evidence that TNFα may also signal engagement of apoptosis through activation of membrane acid sphingomyelinase, to release ceramide. However it is still not certain that ceramide activation is a primary or secondary event [181].

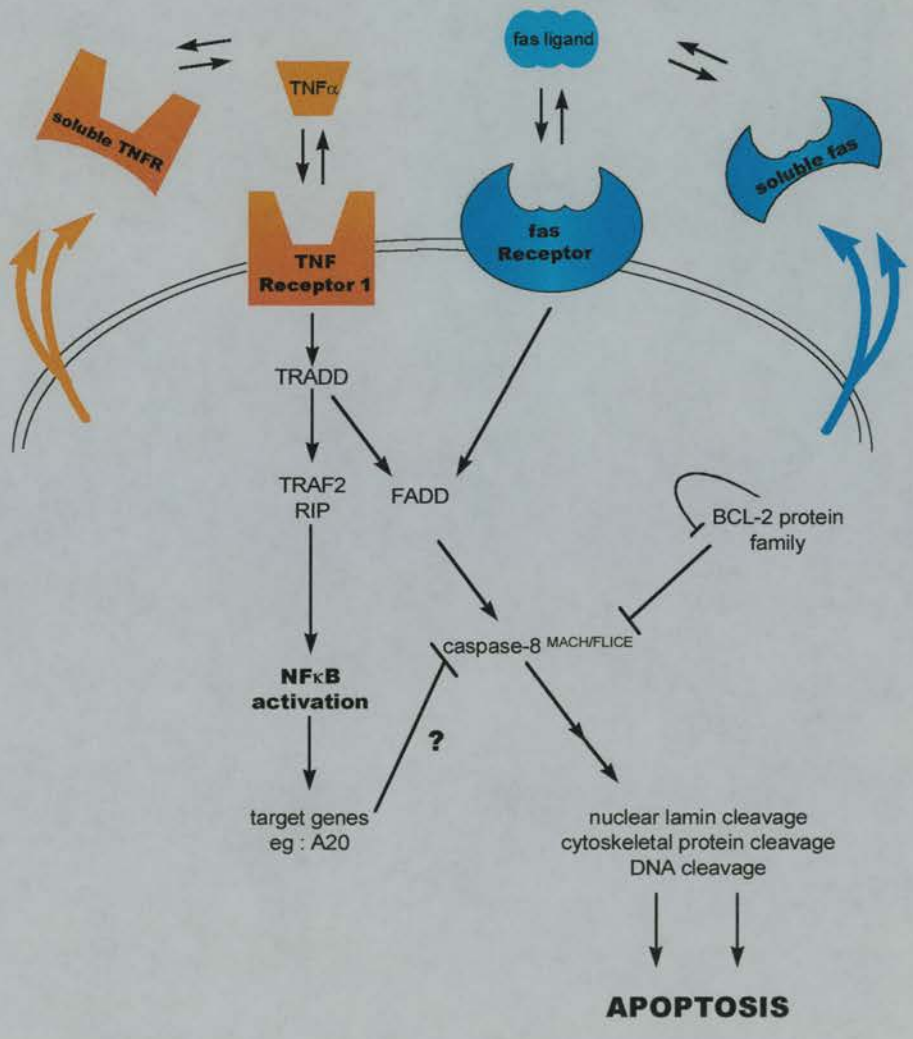



*Figure 5. Fas and TNFRI signal pathways to apoptosis in hepatocytes*

TNF (α or β) is a dominant and final trigger of liver apoptosis in mouse models of **sepsis-induced liver failure** produced by bacterial endotoxin [163,173,176,184]. Mice deficient in the TNF 55kDa receptor are resistant to endotoxin-induced liver failure [184,185]. However, pre-sensitisation of hepatocytes is necessary for TNFα to produce apoptosis: in animal models, hepatocytes are sensitised by pre-exposure to killed bacteria [186] or transcriptional inhibitors [163]; viral infection may also sensitise hepatocytes to TNFα- or Ifnγ-triggered apoptosis [187,188]. In clinical septic shock, ischaemia due to endothelial injury may produce sensitisation [189]. By contrast, **interleukin-1β** (IL-1β) (or inhibitors of translation) inhibits the ability of endotoxin or TNFα to trigger apoptosis [176,190]. IL-1β effects may be mediated through **nitric oxide** synthesis in hepatocytes [191,192], although others have found nitric oxide (which is also induced by TNFα and interferon-γ) to be hepatotoxic [193].

TNFα is also critical to apoptosis in the model of **immune liver injury after T cell activation** by concanavalin A (Con A) or anti-CD3 antibody injection [173,176,194,195]. No presensitisation is necessary in the Con A model of liver injury, but unlike the models of sepsis-associated liver failure, it is not clear whether TNFα or interferon-γ are the final triggers of hepatocyte apoptosis, and both appear to be required [130,195,196]. **IL-6** prevents the liver injury, by reducing TNFα and interferon-γ secretion, but can also act directly on hepatocytes to inhibit apoptosis [195]. Other factors that may be important to modulate hepatocyte responses in the cytokine milieu include HGF, which inhibits interferon-γ cytotoxicity [130]. Fas receptor stimulation (see below) is not necessary in models of TNFα-induced liver failure and apoptosis, since those responses are intact in fas-defective (lpr) mice and hepatocytes cultured from them [197]. By contrast, TNFα was found to suppress rodent hepatocyte apoptosis in primary culture [515].

Taken together, the data show that TNFα can signal different downstream events, whose realisation is context-dependent. In this way the role of TNFα to trigger hepatocyte apoptosis in septicaemia or immune hepatitis is reconciled with its role in promoting hepatocyte proliferation during regeneration.

### *Fas ligand*

Hepatocytes constitutively express the fas receptor (CD95) which, when stimulated in mice, triggers pan-lobular hepatocyte apoptosis within a few hours [165]. By contrast with TNFα, the fas-triggered apoptosis does not require pre-sensitisation *in vivo*. In culture, human hepatocytes are also sensitive to fas-triggered apoptosis, whereas cultured mouse hepatocytes require inhibition of transcription or translation to achieve high levels of fas-triggered apoptosis, suggesting the presence of short-lived inhibitors of the apoptotic pathway [198,199].

Little is known about the regulation of fas expression by hepatocytes. Regulation of fas mRNA splicing may determine the amount of fas expressed on the hepatocyte surface [200], whilst release of a soluble form of fas may reduce cell-surface fas activation, by competion for fas ligand (analagous to the release by hepatocytes of soluble TNF

receptor) was able to prevent endotoxin-induced hepatocyte apoptosis and animal death due to liver failure [186].

The signal transduction steps linking fas stimulation to apoptosis include binding to the receptor "death domain" by an intermediary protein (FADD) that recruits a CED 3-like cysteine protease (caspase 8 [FLICE-MACH]) (Figure 5). This initiates a protease cascade that engages apoptosis [201-203]. FADD also mediates TNFα-triggered apoptosis, and injection of tripeptide inhibitors of caspases reduced both fas- and TNFα-triggered liver apoptosis in mice [183,203]. However, the signals can be differentially regulated: hepatocytes of mice with an SV40 T antigen transgene are resistant to induction of apoptosis by fas stimulation *in vivo* and in primary culture, but retain sensitivity to TNFα-mediated apoptosis [204]. The mechanism of this effect is not known. Fas may trigger more than one pathway to apoptosis in hepatocytes, involving different subclasses of serine proteases [202].

Fas is important for the normal regulation of adult liver size, presumably through apoptosis of redundant hepatocytes: mice with targeted deletion of fas develop considerable liver enlargement after 8 weeks that reaches 160% of normal weight by 16 weeks . The enlargement is due to hyperplasia of liver lobules, and perhaps also hepatocyte hypertrophy or polyploidisation, although only nuclear enlargement is documented [205].

Fas is also likely to be important in the pathogenesis of human liver disease. Observational studies in human chronic hepatitis C infection, hepatitis B-associated cirrhosis and acute (predominantly virus-induced) liver failure show association of hepatocyte destruction with liver infliltration by lymphocytes that express fas ligand [199,206]. Fas expression by hepatocytes was also shown to be upregulated in the areas of lymphocytic infiltration in hepatitis C infection [207]. Most intriguingly, hepatocytes express fas ligand in alcoholic liver disease, suggesting the possibility of autocrine apoptosis (fratricide) as a disease mechanism [199].

Cytotoxic T cells (Tc cells) kill target cells by perforin-granzyme-mediated lysis, or by stimulating target cell fas receptors with fas ligand [208]. Deficiency of perforin, or inhibition of target cell fas stimulation, were each alone sufficient to prevent fulminant hepatocellular injury in similar mouse models of the Tc cell immune response to hepatitis B virus infection [186,209]. Moreover, inhibition of hepatocyte TNF receptor stimulation (by injection of a soluble TNF receptor) also prevented hepatocyte apoptosis and animal death in the same model [186]. Thus multiple mechanisms contribute to immune-mediated hepatocyte apoptosis (TNFα, fas, perforin), and although they are independent, blockade of any one can be sufficient to prevent fulminant hepatic injury, suggesting that at individual cell level there is a threshold level of hepatocyte stimulation/injury for engagement of apoptosis, and that there can be synergy or facilitation between the different signalling mechanisms.

### *TGFβ1*

$TGF\beta_1$ does not produce significant hepatocyte apoptosis in normal rats or mice, when injected at a dose sufficient to suppress regenerative hepatocyte proliferation [151,210]. However, a similar dose increases apoptosis of rat hepatocytes that have been stimulated by xenobiotic mitogen (cyproterone acetate) or culture: in a regressing rat liver model (after cyproterone acetate treatment), $TGF\beta_1$ increased apoptosis throughout the liver lobules, in dose-dependent fashion [164]. Apoptosis increased from within 2–3 hours of treatment up to the final 9 hours timepoint. Apoptosis is more delayed in primary cultures treated with $TGF\beta_1$ — appearing from about 30 hours after treatment, and peaking at about 48 hours [211,212]. This may reflect that the apoptosis is triggered when hepatocytes are in late $G_1$ phase of the cell cycle (see below) [139,213].

These observations have led to the idea that stimulated hepatocytes synthesize inactive $TGF\beta_1$ that when activated can trigger autocrine suicide (fratricide) in sensitised cells. In rats, most apoptotic hepatocytes — but almost no others — were immunopositive for pre-$TGF\beta_1$, and a smaller proportion were also positive for mature $TGF\beta_1$ [214]. Paracrine suicide is also possible, triggered by $TGF\beta_1$ from non-parenchymal cells: cultured myofibroblasts derived from Ito cells secreted $TGF\beta_1$ into culture medium, which was then able to induce apoptosis of hepatocytes [215].

The intracellular events leading to $TGF\beta_1$-induced apoptosis in hepatocytes appear to be closely linked to those producing $G_1$ growth arrest, mediated through effects on the retinoblastoma gene product, pRb and the E2F-1 oncogene [137,139]. The factors determining the threshold between growth arrest and apoptosis are unclear, but results from other cell types suggest that cells become susceptible to apoptosis only during a critical window during late $G_1$ that is after the point at which $TGF\beta_1$ produces growth arrest [213,216]. In hepatocytes, $TGF\beta_1$ increases the proportion of dephosphorylated pRb, which inactivates the E2F-1 oncogene to produce growth arrest and suppress hepatocyte apoptosis [139]. However $TGF\beta_1$ also reduces pRb expression in hepatocytes, which could allow activation of E2F-1; inappropriate E2F-1 activation in $G_1$ produces hepatocyte apoptosis [139]. Thus $TGF\beta_1$ itself, or other unidentified events in $TGF\beta_1$-growth arrested hepatocytes, may lead to E2F-1-triggered apoptosis. The balance between hepatocyte growth arrest and apoptosis can be regulated by extracellular factors: EGF decreases $TGF\beta_1$-induced apoptosis in culture (although HGF does not) [217].

Activity of one or more cysteine proteases (caspases) is necessary for $TGF\beta_1$-induced apoptosis of primary hepatocytes, as shown by use of specific cleavage site inhibitors [218]. DNA cleavage to 300kbp and/or 50kbp fragments occurs in $TGF\beta_1$-induced hepatocyte apoptosis, but there are conflicting reports as to whether further, internucleosomal cleavage occurs [159,160,219-221].

$TGF\beta_1$ produces apoptosis of stimulated rat hepatocytes, but possibly not of mouse hepatocytes [151]. Results from mice bearing a $TGF\beta_1$ transgene that is expressed in hepatocytes are difficult to interpret in the context of regulation of hepatocyte apoptosis,

since the liver microenvironment is so disturbed — increased liver fibrosis, hepatocyte apoptosis and mitosis — as well as numerous inflammatory and fibrotic lesions in other organs [222]. Similarly, the TGFβ1-homozygous-null mouse is not yet sufficiently well investigated to provide data on hepatocyte apoptosis [223-225]. In human liver biopsies, TGFβ1 immunopositivity was demonstrated in hepatocytes of regenerative cirrhotic nodules, and in fulminant hepatitis, but not in hepatocytes of normal liver [226]; its significance for apoptosis is unknown.

### *Activin-A*

Activin-A produces profound centrilobular hepatocyte apoptosis when infused into normal mice and rats at nanomolar concentration. Apoptosis is apparent within 6 hours, and is sufficient to reduce liver weight by 30% within a day [227,228]. The reduction in liver weight continues for about 3 days (to half normal weight), after which the liver becomes refractory to activin-A and starts to regenerate. Indeed by 2 weeks, liver–body weight ratios were significantly greater than normal [227,228]. *In vitro*, activin-A produces a similar increase in apoptosis to TGFβ1, although requiring 10-fold concentration compared with TGFβ1 [227].

By contrast with its effect on normal liver, activin-A administered after partial hepatectomy delays DNA synthesis instead of triggering apoptosis [229]. This may be due to extracellular signals that regulate the hepatocytes' response to activin: in culture, hepatocytes treated with activin-A in the presence of liver mitogens EGF or noradrenaline, undergo growth arrested instead of apoptosis [111]. Physiological inhibitors of activin-A also block its induction of apoptosis (follistatin completely, inhibin partially) in addition to blocking its growth arrest effect [111].

Thus activin-A is a potentially important autocrine regulator of hepatocyte apoptosis, whose effects are dependent on local environment and hepatocyte cell cycle status. Nevertheless, the importance of endogenous activin in the liver remains unknown.

### *Extracellular matrix*

There are empirical descriptions that hepatocyte primary cultures "survive" longer when cultured on specific extracellular matrix proteins than on tissue culture plastic, however, there is no data on rates of apoptosis. Matrix proteins have been shown to influence rates of DNA synthesis in culture [54,55], and this may account for the extended lifespan of the culture. However anoikis (i.e. dependence on specific cell–matrix signals for survival) [516] is likely to be a property of hepatocytes, which survive only briefly as single cells in suspension culture [87]. Moreover, different substrata influence autocrine secretion of TGFβ1 by hepatocytes in culture [230], providing a further indirect mechanism to regulate apoptosis, albeit one of unknown significance.

| Pharmacological triggers of apoptosis. | Reference |
|---|---|
| Cyproterone acetate | (164) |
| Ethanol | (231) |
| uv-irradiation | (232) |
| 2-AAF | (232) |
| Cyclosporine A | (233) |
| Tamoxifen | (234) |
| 1,1,dichloroethylene | (235) |
| Dimethylnitrosamine (DMN) | (236) |
| Heliotrine | (237) |
| Thioacetamide | (238) |
| Acetaminophen | (239) |
| Colchicine, Vinblastine | (240) |
| Cocaine | (241) |
| Menadione, Ionomycin | (242) |
| Microcystin, okadaic acid | (243) |
| Dimethylsulphoxide (DMSO) | (218) |
| Pravastatin | (244) |
| α-amanatin (via TNFα) | (245) |
| Furan | (246) |
| Staurosporine, polymyxin B | (247) |
| Phorbolmyristate acetate | (247) |

*Table 5. Non-physiological agents that trigger hepatocyte apoptosis.*

## Survival factors

The mechanisms by which specific agents can decrease basal or induced hepatocyte apoptosis is poorly understood. The physiological factors are predominantly also mitogens or co-mitogens, whilst the xenobiotic agents were identified through their use as promoters of experimental rat liver carcinogenesis — many also have a weak, transient mitogenic effect on hepatocytes [151].

### *Insulin and insulin-like growth factors (IGFs)*

The decrease in the incidence of heptocyte apoptosis in rats after feeding [152,167], suggests a direct effect of nutrients or anabolic hormones such as insulin. Portal vein ligation rapidly produces apoptosis without evidence of critical ischaemia [169,170], perhaps similarly illustrating a requirement for trophic survival factors. Serum-withdrawal from culture medium produces apoptosis of primary hepatocytes [247], indicating that soluble factors are necessary for hepatocyte survival. Empirically, certain hormones, particularly insulin and EGF, are described to increase culture longevity, apparently independently of proliferation [87]. However, a direct effect on the incidence of apoptosis has not been tested. Overexpression of receptors for EGF or insulin in hepatoma cell lines, and of an

activated HGF receptor in hepatocytes *in vivo*, has been reported to inhibit apoptosis [248,249].

The insulin-like growth factors (IGF1, IGF2) can substitute for serum to reduce apoptosis in hepatocyte cell lines expressing a N-myc-transgene [250,251], and may also inhibit $TGF\beta_1$-induced apoptosis [252]. The specific receptor(s) involved is unclear, since insulin and IGF1 can activate each others' receptors, as can IGF1 and IGF2. Hepatocytes constitutively express insulin and IGF2 receptors, however the IGF1 receptor is expressed by adult hepatocytes only during regeneration [253].

### *Other survival factors*

Several other physiological agents are known to inhibit hepatocyte apoptosis. They are discussed in other sections of this introduction and are listed in Table 6. Pharmacological agents shown to inhibit hepatocyte apoptosis are listed in Table 7.

| Physiological survival factor (in primary culture) | Apoptotic stimulus | Reference |
|---|---|---|
| EGF/TGFα | TGFβ, activin-A | (111,217) |
| Follistatin, inhibin | activin-A | (227) |
| Fructose (ketohexoses) | Bile salts | (254) |
| Glucagon | "spontaneous" apoptosis | (255) |
| HGF, activated c-met | γIfn | (130,249) |
| L-carnitine | "spontaneous" | (256) |
| Noradrenaline | activin-A | (111) |

*Table 6. physiological agents that inhibit hepatocyte apoptosis in primary culture.*

| Pharmacological survival factor | Reference |
|---|---|
| α-chlorocyclohexane | (257) |
| Caspase inhibitors (Z-VAD.fmk; AcYVAD.cmk) | (183,203) |
| Dichloroacetic acid | (258) |
| Heparin | (259) |
| Nafenopin | (260) |
| Phenobarbitone | (232,257) |
| 2,3,7,8 tetrachlorodibenzo-p-dioxin | (232,261) |

*Table 7. pharmacological agents that inhibit hepatocyte apoptosis in vivo or primary culture.*

### *1.2.b.4 Intracellular regulators of hepatocyte apoptosis*

Signal transduction pathways that trigger hepatocyte apoptosis after surface receptor stimulation have already been discussed for TGFβ, TNFα and fas. This section considers more general aspects of intracellular regulation of hepatocyte apoptosis, not necessarily tied to a particular pathway.

Apoptosis of hepatocytes is energy dependent [262]. However, studies investigating the requirement for protein or RNA synthesis have produced contradictory results [163,243,262,263]. Given that suppression of these activities is invariably incomplete and the pharmacological agents used to determine these requirements can themselves produce apoptosis, the data are difficult to interpret. Moreover, the state of the hepatocytes themselves and the system under test are factors, as illustrated by the easy triggering of hepatocyte apoptosis by fas stimulation *in vivo*, but the requirement for inhibition of RNA synthesis to get the same effect in primary culture. Taken together, the data show that there are a variety of susceptible and resistant states for hepatocyte apoptosis, which are changeable, and determined by patterns of gene expression.

#### Protein phosphorylation

Phosphorylation-dephosphorylation reactions have many functions in intracellular signalling. The use of pharmacological inhibitors to dissect these complex and delicate cascades is hampered by the low specificity of the inhibitors, at both the biochemical and the subcellular compartmental level [264]. Thus the phosphatase inhibitors okadaic acid (serine/threonine phosphatase inhibitor) or microcystin, produce rapid hepatocyte apoptosis [243], whilst the protein kinase inhibitors staurosporine or polymyxin, or protein kinase stimulation by phorbol myristate acetate, also produce hepatocyte apoptosis

[218,247]. Collectively, these data are difficult to interpret beyond showing that sufficiently severe disruption of normal intracellular signal pathways can (still) trigger hepatocyte apoptosis.

## Oncogenes

The finding in other cell types that forced oncogene expression triggers apoptosis, unless there is co-stimulation by survival factors, has been extended to hepatocyte cell lines forced to overexpress an *N-myc* transgene [250]. Thus hepatocytes share with other cell types fundamental protective mechanisms that prevent autonomous oncogene activation. There is also increased hepatocyte apoptosis in double transgenic TGFα/*c-myc* mice, although the precise basis for the increase is less clear [265]. However, forced overexpression experiments may reveal important protective mechanisms relevant to tumour suppression, but may not represent a normal signalling pathway to apoptosis. Indeed, *c-myc* has not been shown to regulate hepatocyte apoptosis triggered by physiological signals.

*c-jun* (but not *c-fos*) has been suggested to regulate hepatocyte apoptosis in an ischaemia-reperfusion model, based on its temporal and spatial expression patterns in the injured liver [266]. However, it is difficult to separate causal from casual associations relating to the tissue response to injury. An association of *c-fos* induction with TGFβ-induced apotosis has been reported [217], but again the correlation may not reflect cause.

## Intracellular proteases

Caspase activation is a prevalent and necessary phase preceeding hepatocyte apoptosis triggered by a variety of different signals, many of which have already been discussed, and which include $TGF\beta_1$ [218], fas antibody [183,202,203,267], TNFα [182,183], and also re-oxygenation of hypoxic hepatocytes [268].

Serine protease activity may be required for hepatocyte apoptosis after bile acid exposure [269], and also for hepatocyte chromatin cleavage to 50kbp fragments [270]. Serine proteases may also be important in TNFα-induced apoptosis, although this has not been shown for hepatocytes [271,272].

## Inhibitory regulators of apoptosis

### *NFκB*

NFκB activation can determine whether a cell is stimulated or dies in response to certain agents, of which TNFα is the best understood. TNFα activates NFκB which induces genes that suppress apoptosis, in addition to functions such as induction of antiviral proteins. Target genes of NFκB that suppress apoptosis include the zinc finger protein A20 and possibly manganese superoxide dismutase [273]. If NFκB is deficient or not activated, the cells instead undergo apoptosis. This mechanism is important for survival of macrophages, fibroblasts, and hepatocytes — mice deficient in the $p65^{rela}$ subunit of

NFκB die in utero with massive liver apoptosis, due to lack of inducible, NF-κB-regulated pathways [274].

Thus, activation of NFκB has to be prevented or its effects blocked, for stimuli such as TNFα to trigger apoptosis. This may explain why stimuli such as TNFα (or fas activation in cultures) do not trigger hepatocyte apoptosis unless RNA or protein synthesis are inhibited (see earlier), blocking the protective actions of NFκB [163]. More authentic stimuli that sensitise hepatocytes to killing by TNFα include hepatitis B virus infection [187]. Viral infection of hepatocytes subverts cellular protein and RNA synthesis, perhaps preventing NFκB from acting. Thus there is selective, cytokine-triggered killing of virus-infected hepatocytes, whilst adjacent non-infected cells respond normally with NFκB-induced expression of antiviral genes [273]. Other cytokines, such as IL-1β, activate NFκB and so may raise the level of protection against apoptosis induced by TNFα, although this has not been formally tested in hepatocytes [130,163].

Thus NFκB is part of a pathway that induces anti-apoptotic genes and that can be manipulated to alter the balance between cytokine-mediated stimulation and cell killing. Intriguingly, ionising radiation can activate NFκB, suggesting that regulable autoprotective mechanisms might also be induced after DNA damage [123,273].

### *bcl-2 family*

The bcl-2 family of proteins are believed to directly influence the activation of apoptotic caspases [275]. Most data on members of the bcl-2 family in liver are observational or of no determined physiological significance, although BAG-1 may be important. BAG-1 is a bcl-2–binding protein that cooperates with bcl-2 to inhibit apoptosis [276]. The HGF receptor binds BAG-1, and overexpression of BAG-1 in liver progenitor cells protects against apoptosis [277], suggesting a mechanism for the survival factor effects signalled through the HGF receptor [130,249]. However, bcl-2 or BAG-1 were not able to suppress apoptosis of hepatoma cell lines, produced by expression of a Wilms tumour 1 (WT-1) splice variant [248].

Transgenic bcl-2 overexpression in hepatocytes inhibits fas-triggered apoptosis [278,279], but how this occurs, and whether the observation has physiological significance, are unknown, given that adult hepatocytes do not normally express bcl-2 and have not been shown to induce it [254,280-284]. bcl-2 and the related antiapoptotic protein A1 are expressed at low levels in human foetal and mouse embryonic hepatocytes, respectively, suggesting differential expression of this protein family in developing and adult liver [285,286].

Several other members of the bcl-2 family have been identified in adult hepatocytes, including bax, $bcl_{XL}$ $bcl_{XS}$ and other uncharacterised Bax and Bcl-2 cross-reactive proteins — no significance has been attached to these observations [284,286,287]. bax protein levels were shown to increase after partial hepatectomy, whilst bcl-2 and $bcl_{XL}$ protein levels did not [286].

***Hepatitis viral proteins***

Hepatitis C virus (HCV) core protein inhibits apoptosis induced by DNA damage or *c-myc* overexpression in genital tract cell lines [288], however its effect on hepatocyte apoptosis has not been reported. The HCV NS3 protease inhibits actinomycin D–triggered apoptosis of NIH3T3 fibroblasts, by a mechanism that was suggested to be p53-dependent and $p21^{WAF1}$-independent, however again this has not been evaluated in hepatocytes [289].

### *1.2.b.5 Ploidy and apoptosis*

The possibility that hepatocyte ploidy populations have differential susceptibility to apoptosis has received little attention. This could be of interest since diploid cells differ from polyploid cells in responsiveness to mitogens and may be more susceptible than polyploid cells to carcinogenesis (see earlier). In primary hepatocytes, apoptosis produced by inhibition of *Rb* expression led to proportionately greater loss of diploid compared with tetraploid cells (41% vs 11% reduction), suggesting that diploid hepatocytes are more likely than polyploid cells to undergo apoptosis [139]. Increased liver polyploidy produced by administration of a xenobiotic mitogen (lead nitrate) did not reverse after withdrawal of the mitogen, despite considerable apoptosis during regression, further suggesting that polyploid cells are not more sensitive than diploid to undergo apoptosis [4].

## *1.3 p53 in the liver*

### 1.3.a Normal liver

Little is known about how p53 functions in normal liver. In adult liver, p53 is virtually undetectable and levels of the p53-responsive gene, $p21^{WAF1}$, are low, together suggesting that p53 has no constitutive role, for example in the maintenance of replicative quiescence [7,37]. This is confirmed by observations that mice made germline-deficient in p53 have normal, quiescent liver [290-293]. Indeed, the transcription factor C/EBPα is an important determinant of quiescence in many cell types, including liver, and does not require p53 [29,147].

After partial hepatectomy, p53 mRNA and protein concentrations increase transiently from 12–15 hours, during late $G_1$, and return to basal level by 18–24 hours [37]. No significance has been attached to this change, which has been largely ignored in subsequent papers on the regulation of liver regeneration. Tsukada reported that hepatocytes from p53-deficient mice did not differ from wild type in growth characteristics in primary culture; however, no data was presented [294]. $p21^{WAF1}$ expression after partial hepatectomy is normally independent of p53; however p53-dependent induction of p21 can occur, for example, if mice are protein-deprived before partial hepatectomy — presumably, this is a stress response [7].

The role of p53 in hepatocyte apoptosis is also unknown; Columbano found that cycloheximide treatment that was sufficient to cause hepatocyte apoptosis, also increased hepatocyte p53 mRNA levels. However, there was no increase of p53 expression during apoptosis associated with liver regression after mitogen-withdrawal [295]. Thus there is no direct evidence that p53 is involved in physiologically-triggered liver cell death.

## 1.3.b Liver after DNA damage

Hepatocytes increase p53 concentration after exposure to agents that damage DNA (Table 8), and mice deficient in nucleotide excision-repair show extensive hepatocyte p53 immunopositivity, together suggesting a role for p53 in hepatocyte responses to DNA damage, similar to other tissues [296]. By contrast with other genotoxins and its effects on some other tissues, γ-irradiation does not increase p53 protein or mRNA concentration in hepatocytes [283,297,298]. However, hepatocytes do sense γ-irradiation because there is induction of the GADD45 gene and the DNA repair enzyme $O^6$-alkylguanyl transferase [299,300].

The only biological response shown to be regulated by p53 in hepatocytes is the recently-demonstrated mitogen-resistant $G_1$/S arrest after DNA damage (uv-b irradiation, diethylnitrosamine, mitomycin C) or actinomycin D [301,302]. The growth arrest can be reversed by antisense p53, demonstrating p53-dependence. The association of increased p53 protein or mRNA concentrations with increased hepatocyte apoptosis after uv-c, 2-acetylaminofluorene or portal vein ligation, implied that hepatocytes show p53-dependent apoptosis, but casual association was not excluded [169,232]. The association of p53 protein increase and induction of apoptosis by prostaglandin was shown to be casual using hepatocellular carcinoma cell lines with and without functional p53 [303].

The identification of a pathway linking p53 to DNA excision-repair proteins in fibroblasts [304], and observations of p53-dependent excision-repair in different cell types [305-312] (Prost S., Bellamy C.O.C., Clarke A.R., Wyllie A.H., Harrison D.J., submitted), suggest the possibility that p53 could regulate DNA repair in hepatocytes. However, no studies have addressed this issue directly; the induction by γ-irradiation of the DNA repair enzyme $O^6$-alkylguanyl transferase is p53-dependent; however, since the enzyme does not repair DNA strand breaks (the DNA lesion of γ-irradiation), the data is only suggestive of a role for p53 in hepatocyte DNA repair [300]. The frequency of spontaneous mutations in liver was not increased in mice heterozygous or null for p53, compared with wild type (assessed on the basis of inactivating mutations in a *lac* transgene) [313].

| Stimulus. | Detection. | Study. | Reference. |
|---|---|---|---|
| Partial hepatectomy | protein, mRNA | In vivo, rats | (37) |
| Carbon tetrachloride | mRNA | In vivo, rats | (57) |
| Portal vein ligation | mRNA | In vivo, rats | (169) |
| SV40T transgene | protein | In vivo, mice | (314) |
| ERCC-1 deficiency | protein | In vivo, mice | (296) |
| uv-b irradiation<br>uv-c irradiation | protein | Culture, rats | (301) (232) |
| Diethylnitrosamine injury | protein | In vivo, rivulus<br>Culture, rats | (315)<br>(302) |
| Mitomycin C | protein | Culture, rats | (301) |
| 2-acetylaminofluorene | protein | Culture, rats | (232) |
| Cadmium | mRNA | In vivo, mice | (316) |
| Copper | mRNA | In vivo, rats | (317) |
| Actinomycin D | protein | Culture, rats | (301) |
| Peroxisome proliferator | mRNA | In vivo, rats | (318) |

*Table 8. Stimuli that increase p53 protein/mRNA levels in primary hepatocytes.*

## 1.3.c p53 in hepatocarcinogenesis

Liver carcinoma is the fourth commonest cause of cancer death world-wide [319]. Mutations in the p53 tumour suppressor gene, usually with loss of the residual wild type allele, are frequent in human hepatocellular carcinomas (HCC) and correlate positively with increasing histological grade of carcinoma and early recurrence, suggesting that defective p53 function is of pathogenic significance and confers increased clinical aggression [320-322]. Studies of intratumour heterogeneity have suggested that p53 mutation and allele loss are late events in human hepatocellular carcinoma, mediating tumour progression but occurring after carcinogenesis [323,324]. However, several lines of evidence indicate that p53 dysfunction can precede hepatocarcinogenesis and have a pathogenic role. First, abnormal accumulation of p53 protein has been observed in hepatocytes of patients with cirrhosis and liver cell dysplasia, high risk lesions for carcinoma development [325-327]. Second, exposure to Aflatoxin B1, a dietary mutagen and a cofactor in human hepatocarcinogenesis, is associated with a characteristic point mutation in p53 [328]. This mutation has been demonstrated in non-neoplastic hepatocytes, particularly in areas of high aflatoxin exposure where hepatocellular carcinoma is prevalent [329]. Third, dysfunctional p53 is probably more common in hepatocellular carcinoma than appreciated from standard genetic screens of "hotspot" regions, because of both mutation distribution [330,331] and functional inactivation without mutation [332-334]. Functional inactivation of p53 is best characterised for chronic hepatitis B infection.

Hepatitis B virus is the major aetiological agent of human hepatocellular carcinoma; the Hepatitis B X protein heterodimerises with and inactivates p53 *in vitro* and *in vivo*, and in transgenic mice, this is temporally and spatially associated with the development of hepatocellular carcinoma from preneoplastic lesions, providing strong evidence that p53 dysfunction is relevant to hepatocarcinogenesis [335-337].

## p53 mutation profile in human hepatocellular carcinoma

The prevalence and spectrum of p53 mutations in hepatocellular carcinoma is strongly influenced by geographic location. Dietary exposure to Aflatoxin B1 is associated with a characteristic G-T transversion mutation in p53 (Ser249) [338,339] that can be identified in over half of hepatocellular carcinomas in areas of high exposure risk, such as Qidong (China) and Mozambique, but less than 1/25 hepatocellular carcinoma in low exposure areas [340]. Individual susceptibility to the aflatoxin-induced Ser249 mutation correlates postitively with particular mutant alleles of hepatic detoxifying enzymes [341]. hepatocellular carcinoma p53 mutations have been categorised into geographic groups: G to T transversions (including Ser249) predominate in the sub-Saharan Africa and Southeast Asia continent, whereas the majority of p53 mutations in hepatocellular carcinomas from Europe/North America are G to A transitions. By contrast, the mutation spectrum in hepatocellular carcinomas from Southeast Asia islands is a mixture of base substitutions, deletions and insertions [342].

In regions of little Aflatoxin B1 exposure, p53 mutations are still prevalent; however, about one quarter of the mutations lie outside the most commonly evaluated exons 5-8, and most of those (>70%) generated stop codons or frameshifts that would probably be missed by immunohistochemical screening [330]. Therefore, the true prevalence of p53 mutation in hepatocellular carcinoma is difficult to estimate. Nevertheless, there is considerable geographic variability, for example, in Japan (19% [343], 32% [344], or 65% when multiple nodules were evaluated [321]); in the United States (45% [345]); in Taiwan (33% [346]); Germany (15% [347], 10/22 [348]); France (16% [349]); Great Britain (11% [350], 9% [351]); Alaskan natives (0/7 [352], 0/13 [353]); Australia (0/15 [354]).

In summary, p53 mutation is prevalent in hepatocellular carcinomas; the geographic heterogeneity of mutation prevalence and mutation profile may reflect patterns of exposure to different risk factors for hepatocellular carcinoma.

## Consequences of p53 mutation

A few studies have evaluated the functional consequences of p53 mutation in hepatocyte cell lines. The Ser249 p53 mutation is the prevalent, but not exclusive, mutation produced by Aflatoxin B1in human hepatocytes, and its common identification in hepatocellular carcinoma presumably reflects a selective advantage for affected cells [355]. Human p53ser249 is transactivation-deficient, and in human hepatocellular carcinoma cells showed the strongest dominant negative inhibition of wild type p53 transactivation activity compared with a panel of p53 mutants [356,357]. p53ser249 may also have novel

gain-of-function properties, since it enhanced survival (colony formation) and mitotic activity of the p53-deficient human hepatocarcinoma-derived cell line, Hep3B (HBV-positive). However, p53ser249 did not increase the weak tumorigenicity of Hep3B — postulated to be because TGFβ-induced apoptosis was retained [356].

A murine p53 mutant (Ser246), equivalent to the human Ser249 mutant, was transfected into a hepatocyte cell line and shown to reduce serum-dependence for growth, and to increase colony formation and cellular pleomorphism. However, the mutant did not transform the cell line [358]. Thus the human and murine equivalent mutations appear to have similar properties in hepatocyte-derived cells. Interestingly, the phenotype of p53ser246 was specific to hepatocytes — not seen in transfected murine fibroblasts — emphasising the limitations of modelling genetic lesions in irrelevant cell types. Moreover, the phenotype was also mutant-specific — not seen using a p53val135 mutant that is well described in other cell systems as a temperature-sensitive, dominant-transforming oncogene [359]).

Taken together, the data suggest there are p53 mutant-specific effects, on hepatocyte-specific growth factor pathways, perhaps acting through altered transcriptional activation. The data suggest that p53 dysfunction can augment clonal growth in carcinoma progression, perhaps through manipulating sensitivity to growth factors, and leave open the possibility of a similar contribution to carcinogenesis.

p53 dysfunction is not essential for hepatocarcinogenesis: human hepatocellular carcinoma-derived cell lines, HepG2 and 2215, have wild type p53 that appears to be functionally intact, as assessed by basal transactivation of a reporter gene, increased p53 and $p21^{WAF1}$ protein concentrations after exposure to DNA-damaging agents (uv, doxorubicin), and inhibition of DNA synthesis [360]. However, by contrast with the effects of γ-irradiation on normal rodent hepatocytes, γ-irradiation increased p53 immunopositivity in the HepG2 cells and a murine hepatocellular carcinoma cell line [360,361]. It is not clear if this difference is due to a malignancy-associated change in p53 regulation, or has a more trivial, methodological explanation.

## Liver carcinogenesis in genetically modified animals

Germline p53-deficiency in mice, either heterozygous or homozygous, did not predispose to spontaneous or carcinogen-induced hepatocarcinogenesis (γ-irradiation, diethylnitrosamine, dimethylnitrosamine), at least not before the mice succumbed to lymphomas or sarcomas [292,293,362,363]. Moreover the number and size of phenotypically altered foci, and the relative proportions of hepatocellular carcinoma and hepatocellular adenomas were not significantly different between wild type and p53-heterozygous mice exposed to a single dose of diethylnitrosamine at 12 days [293]. In a different model, mice bearing a mutant p53 transgene that dominantly inactivates wild type function and that is specifically expressed in hepatocytes (and choroid plexus), also lived a normal lifespan without liver disease [364]. Thus there is no evidence that p53 dysfunction alone in otherwise normal liver influences hepatocarcinogenesis. This suggests that the

development of hepatocellular carcinoma in HBX-transgenic mice, discussed above, involves properties of HBX additional to inactivation of p53. The idea is supported by the increased susceptibility of HBX-transgenic mice to diethylnitrosamine-induced hepatocellular carcinoma, not shown in simple p53 deficiency [365].

The SV40 T antigen binds and inactivates p53, as well as affecting other proteins, particularly members of the pRb family. Mice bearing an SV40 T antigen transgene that is expressed in hepatocytes, show abnormal hepatocyte proliferation, dysplasia, and develop phenotypically-altered foci and hepatocellular carcinoma from within a few weeks of birth [314,366-368]. The incidence of hepatocellular carcinoma is upto 100%, depending on background strain. However, comparison with p53-deficient mice suggests that transgene effects on gene products such as the pRb family are critical to hepatocarcinogenesis.

A few animal models of hepatocarcinogenesis recapitulate the disease sequence of chronic hepatocellular destruction, inflammation and compensatory regeneration that preceeds human hepatocarcinogenesis. However, p53 function is not usually evaluated. Mice carrying a hepatitis surface antigen transgene, whose overexpression in hepatocytes leads to chronic hepatocyte destruction, regeneration and eventually hepatocellular carcinoma, did not show p53 mutation in the carcinomas [369]. However, neither was any abnormality detected in a large number of other oncogenes and oncosuppressor genes that were evaluated. This suggests the model may have special features that replace the selective advantage of oncogene or oncosuppressor mutation. Mice with a *c-myc* transgene, or a *TGFα* transgene, expressed in hepatocytes, and double transgenic *c-myc/TGFα* mice show accelerated hepatocarcinogenesis. However, no p53 mutations were found in hepatocellular carcinomas developing in these mice [370].

In summary, where it has been evaluated, p53 mutation has not been identified in transgenic mouse models of hepatocarcinogenesis, in the hepatocellular carcinomas that develop. The HBX model of functional inactivation remains the best evidence for a contribution of p53 dysfunction to hepatocarcinogenesis in these models.

### Hepadnaviral models of hepatocarcinogenesis

Woodchucks and ground squirrels infected with the species hepatitis virus and/or exposed to aflatoxin B1, did not show p53 mutation in the hepatocellular carcinomas that developed [371].

### Chemical hepatocarcinogenesis

Genetic background is a critical determinant of rodent susceptibility to hepatocarcinogenesis, and studies are frequently performed in strains known to be susceptible, and in the more susceptible gender. Whether this strategy could reduce any selective advantage from p53 mutation is not known. Most studies have failed to identify p53 mutations in chemically-induced hepatocellular carcinomas from mice [372-375] or rats [376]. However, Smith reported immunohistochemical evidence for abnormal p53 in a high

proportion of murine hepatocellular carcinomas induced by choline-deficiency [377] or diethylnitrosamine [377], whilst Lilleberg found abnormal restriction fragments suggesting mutant p53 in hepatocellular carcinoma from rats treated with Aflatoxin B1 [378]. Aflatoxin B1 treatment of non-human primates, mice, rats, or ducks does not produce an analagous p53 mutation to that in humans, although it is an extremely potent animal hepatocarcinogen; however, other p53 mutations have been reported in animal, Aflatoxin B1–induced hepatocellular carcinomas [328,378-380]. The reason for the species difference is uncertain, but in humans, the Ser249 mutation has been linked to specific mutant alleles of detoxifying enzymes, which may not occur in other species [341].

By contrast with animal hepatocellular carcinoma *in vivo*, p53 mutations were rapidly acquired when cells from the carcinomas were cultured, indicating a specific selective advantage under culture conditions, which is clearly not relevant, or suppressed, *in vivo* [374].

## 1.4 Chemical carcinogenesis in the liver

The field of hepatocarcinogenesis is large, and the discussion here will be limited to issues pertinent to the experimental data that will be presented on diethylnitrosamine (DEN) carcinogenesis.

Liver is commonly used to study the stepwise development of cancer. Strategies of chemical carcinogenesis involve a carcinogen (a DNA-damaging agent such as diethylnitrosamine), which may be combined with a regenerative stimulus such as partial hepatectomy, and/or long term administration of a tumour promoter (e.g. phenobarbitone). Intriguingly, hyperplasia induced by direct mitogens cannot substitute for the regenerative stimulus of partial hepatectomy or $CCl_4$, to support initiation of carcinogenesis by carcinogens [381]. This remains unexplained [64]. Carcinogenesis protocols are many and varied, but can be categorised according to the possible target population, into protocols producing oval cell proliferation (e.g. 5-acetylaminofluorene with choline deficient diet) and those in which oval cell proliferation is not a feature, but development of carcinoma is preceeded by phenotypically altered foci and nodules (e.g. diethylnitrosamine with partial hepatectomy).

### 1.4.a Phenotypically altered foci

A variety of convenient phenotypic changes that occur after carcinogen treatment and before development of malignancy, are used to identify putative mutated cells and their progeny (so-called "altered foci"). The relation between altered foci and subsequently developing carcinomas is uncertain, although there is a positive correlation. Only a tiny proportion of foci (0.04-0.1% [382]) may progress to neoplasms, and there is no evidence that they represent an obligate, homogeneous or irreversibly-altered population in the progression towards cancer [383,384]. Indeed, different phenotypic markers identify overlapping, but not congruent, subsets of cells [385]. There is some evidence that foci are

clonal, but this may not be universal [386]. Nevertheless, studies of altered foci have revealed interesting changes that will now be discussed.

In early carcinogenesis, altered foci increase in size (cell number) only very slowly, if at all [151], perhaps at comparable rates to the microclones contributing to normal fractal replenishment of liver [14,15], although a direct comparison has yet to be reported. However, there is a critical alteration in the hepatocytes constituting the foci, such that their average lifespan is dramatically curtailed, accompanied by a manyfold (5-10x) increase in cell turnover (proliferation and apoptosis) [151]. Foci are thus regions of altered population kinetics in which numerous short-lived hepatocytes are generated to fill a relatively stable volume of liver. One feature of a shorter-lived cell population is that its size changes much more rapidly for similar relative changes in apoptosis or proliferation rates. Indeed, calorie restriction sufficient to eliminate 20% of normal liver cells by reducing DNA synthesis and increasing apoptosis, reduced the size and number of foci by a total volume of 85% in the same period, and halved the incidence of carcinomas after return to normal feeding - suggesting that many initiated cells had been eliminated [152]. Conversely, pharmacological inhibition of hepatocyte apoptosis by phenobarbitone (or other tumour promoters such as nafenopin) rapidly increases the size of altered foci [257,387]; the effect is reversed on withdrawal of the tumour promoter. Although such tumour promoters also stimulate cell proliferation, the effect is transient and not sufficient to account for the promoting effect, which correlates better with the inhibition of apoptosis [151,384].

Compared with altered foci, the rates of proliferation and apoptosis in neoplastic nodules and in hepatocellular carcinoma are progressively increased [151,388]; whether this could in part reflect a subset of newly generated hepatocytes that are very short-lived, or a more generalised reduction in the lifespan of lesional hepatocytes, is unknown.

Genetic marking studies have shown that the sporadic, fractal pattern of hepatocyte proliferation becomes disturbed after carcinogen treatment (5-acetylaminofluorene with choline deficient diet, or diethylnitrosamine with partial hepatectomy): proliferation becomes concentrated in fewer sites that consequently produce larger cell clusters [389]. In part this may be due to carcinogen-induced damage causing cycle arrest of many cells. The clusters could not be linked to phenotypically altered foci. The data are preliminary, but it is tempting to speculate that the subsequent development of nodular liver disease is an extension of this process.

## 1.4.b Diethylnitrosamine hepatocarcinogenesis

Diethylnitrosamine (DEN) is an alkylating hepatocarcinogen that produces a variety of DNA adducts, of which $O^4$ethyldeoxythymidine is the most slowly removed from hepatocyte DNA, and is probably the most significant [390]. High doses of DEN (200mg/kg) produce liver necrosis; lower doses (1-20mg/kg) are not appreciably

cytotoxic, but a single neonatal exposure is sufficient to produce hepatocellular carcinomas (see

Table 9). The morphology and kinetics of DEN-induced liver lesions are well described in mice [391,392]. Mathematical modelling indicated that at least 2 critical time-dependent events were required for the development of atypical foci, and 4 for carcinomas. The probabilities of initiation of foci and carcinomas differed by more than 3 orders of magnitude, suggesting qualitative differences in the initiating event required, and so foci cannot necessarily be considered as early biological steps in carcinoma development.

| | Dose of DEN (single exposure at 15 days): | |
|---|---|---|
| | 0.625mg/kg | 5mg/kg |
| Atypical foci | 26 | 12 |
| Hyperplastic nodules | 46 | 21 |
| Hepatocellular adenomas | 55 | 30 |
| Hepatocellular carcinomas | 65 | 46 |

*Table 9. Time (weeks) to 50% incidence of liver lesions in male B6C3F mice, after a single intraperitoneal dose of DEN at 15 days. (adapted from [391,392])*

Specific genetic lesions can modulate DEN-induced hepatocarcinogenesis: a retroviral vector that expressed activated *Ki-ras*, produced increased proliferation of infected hepatocytes, and accelerated hepatocarcinogenesis [393]. By contrast, TGFα-deficient mice exposed to DEN failed to develop the large liver lesions that were produced in wild type mice, suggesting a role for TGFα in promotion [104]. DEN accelerated liver carcinogenesis in HBX-transgenic mice, illustrating cooperativity between chemical and virus-associated carcinogenesis [372].

### *1.4.b.1 p53 and diethylnitrosamine hepatocarcinogenesis*

After exposure to DEN, hepatocytes show unscheduled DNA synthesis, indicating that excision repair is stimulated [394]. Specific adducts are also removed by $O^6$ alkyl transferase activity. However, the repair is imperfect: mutation frequency in a reporter transgene was still increased in "normal" mouse liver, one year after a dose of DEN [395]. The DNA damage caused by DEN stimulates a p53 response: cultured hepatocytes from rats exposed to DEN showed p53-dependent $G_1$/S cell cycle arrest when exposed again to DEN. However, the p53 response was much diminished in cells that were presumed to be from atypical foci (immunopositive for glutathione S transferase P), showing that cells in foci respond abnormally to further genetic injury [302]. Goodwin reported abnormal, persistent p53 immunopositivity in occasional hepatocytes 3 weeks after a necrogenic dose of DEN; this could reflect a response to persistent damage [315].

Despite the evidence for a p53 response to the DNA damage that DEN produces in hepatocytes, p53 mutation has not been found in mouse hepatocellular carcinomas produced by single-dose DEN treatment; moreover — as discussed earlier — p53-deficiency did not accelerate hepatocarcinogenesis after single dose DEN treatment at 15 days [293,372,373,375]. Smith combined DEN treatment with a methyl-deficient diet in rats, and demonstrated mutant p53 atypical foci that developed — but restricted to the larger foci. This suggests either that p53-mutant foci grew larger than others, or that p53 mutation occurs late in the clonal expansion of initiated cells [396].

In summary, DEN produces a p53 response in normal hepatocytes, but although this response may be muted in atypical foci, there is no evidence that p53-deficiency is sufficient to facilitate carcinogenesis.

# 2. Aims

The hypothesis underlying this thesis is that p53-deficiency in hepatocytes alters the regulation of hepatocyte turnover in ways that might be relevant for liver carcinogenesis. By comparison of wild type with p53-deficient hepatocytes — where possible *in vivo* and *in vitro* — the experiments described sought to identify whether and how p53 regulates hepatocyte turnover and responses to DNA damage.

Specific aims were:

1. To establish and evaluate a system that allowed primary culture of mouse hepatocytes in defined conditions.
2. To study the consequences of p53 deficiency for hepatocyte proliferation, ploidy and apoptosis.
3. To establish the involvement of p53 in the responses of hepatocytes to DNA damage, and the particular downstream responses regulated through p53 in this cell type.
4. To compare the consequences for wild type and p53-deficient livers, of exposure to a liver carcinogen.

# 3. Materials and methods

## *3.1 Primary hepatocyte culture*

### 3.1.a Gene-targeted mice

Generation of the homozygous p53-deficient mice used in these experiments has been described [397]. Mouse E14 embryonic stem cells (derived from strain 129/Ola) bearing a targeted deletion of exons 2 through 6 of the p53 gene were injected into blastocysts to generate germline chimaeras, which were bred to homozygosity. The targeting construct consisted of a fragment from within intron 1 of the p53 gene, ligated to a *pgk-neo* cassette and a 5kb fragment that incorporated exons 7–11 of the p53 gene. The *neo* cassette contained numerous STOP codons in all 3 reading frames to prevent downstream transcription of the truncated gene.
To establish mouse p53 genotypes, DNA was prepared from tail biopsies and selectively amplified by polymerase chain reaction, using a primer for intron 7 (common to both genotypes), and primers specific for exon 6 (wild type) and the *neo* construct.

Mice used in the experiments described here were outbred on a mixed background, segregating for 129, Ola and Balb C. For each experiment the mice were age and sex-matched, and were littermates if possible. p53 genotype was rechecked on tail biopsy after killing. Unless otherwise stated, hepatocytes were cultured from adult male mice of 6–10 weeks age. The mice were housed in plastic cages in a room with a 12 hour day-night cycle and controlled temperature and humidity. They were given a standard diet and water *ad libitum*.

### 3.1.b Isolation of murine hepatocytes

#### *3.1.b.1 Two step retrograde perfusion of adult mouse liver*

The perfusion apparatus was sterilised and cleaned before and after use by running through 70% absolute ethanol/tissue culture grade water followed by tissue culture grade water. All reagents and equipment in contact with the hepatocytes were cleaned to tissue culture grade standard and sterilised. Perfusions were carried out under still air conditions and once hepatocytes were isolated all subsequent handling was performed in a sterile laminar flow tissue culture hood (class 2) using sterile technique.

Young adult mice weighing 20–25g were anaesthetised with pentabarbitone (300mg/kg i.p.). Once deeply anaesthetised (deep reflexes lost), the mouse was prepared for surgery and the skin sterilised with alcohol. The abdominal cavity and thoracic contents were displayed by a midline incision and reflection of peritoneum and the anterior thoracic cage. The hepatic portal vein was identified and a loose ligature placed around the

thoracic inferior vena cava (Figure 6). The inferior vena cava was cannulated by inserting a polyethylene cannula (0.4/0.8 or 0.5/1.0 mm i.d./o.d.) through an incision in the right atrium, and the cannula was secured in position by the ligature. The hepatic portal vein was transected. Oxygenated *perfusion medium* (Appendix) at 37°C was pumped through the cannula (8.5mls/minute) for at least 5 minutes. The liver clears immediately as blood is flushed into the portal vein by incoming perfusate. The perfusate was then changed to oxygenated *digestion medium* (Appendix) and perfusion continued for a further 10–15 minutes, until fissures form spontaneously or with mild pressure on the liver surface. During this time the liver typically enlarges to almost twice normal size, and any structural detail visible through the capsule is lost. The perfusion was then terminated; the digested liver, still held intact by its capsule, was excised and the gall bladder discarded.

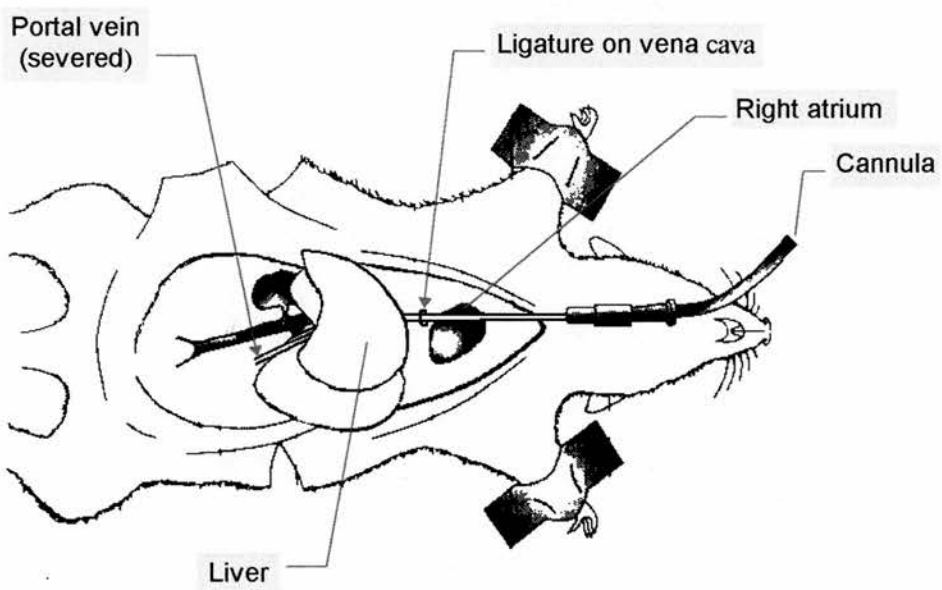



*Figure 6.* ***Retrograde perfusion of mouse liver.***

The liver was placed into a sterile plastic tissue culture dish and the capsule snipped open with sterile scissors. Hepatocytes were then gently but thoroughly mechanically dissaggregated from the fibrous stroma using a "brushing" action from hilum to periphery, along the branching strands of fibrous supporting tissue. This produces a thick, homogeneous cell "soup", consisting of isolated hepatocytes, small clumps of hepatocytes, the intact fibrous stroma and some non-parenchymal cells. Depending on the type and batch of collagenase used and the quality of the digestion, it was sometimes necessary to continue the digestion for a further 10–15minutes in fresh *digestion medium* at 37°C in a conical flask within an orbital incubator set at 90–100rpm.

### *3.1.b.2 Purification and removal of damaged cells from the hepatocyte isolate*

Ice cold *plating medium* (Appendix) was then added to the cell suspension. This was gently aspirated up and down a wide-bore pipette (2mm orifice) 10–15 times to further break up small clumps, and then filtered through a 250μm mesh into an ice cold beaker. The suspension was made up to 50ml with ice-cold *plating medium* and centrifuged (50g for 5 minutes, at 4°C). The supernatant, containing debris and non-parenchymal cells, was discarded. The cell pellet — more than 90% hepatocytes on morphology — was then further purified and damaged cells removed by centrigugation through Percoll:

#### Isopyknic centrifugation in Percoll

The protocol was adapted from that of Kreamer et al [398] for rat hepatocytes, and exploits the observation that damaged cells will float and murine hepatocytes will pellet, in a dense ($\rho$ = 1.055 $gcm^{-3}$), isotonic, Percoll gradient.

The hepatocyte pellet was resuspended in 6ml ice-cold *plating medium* and 8ml cold Percoll stock solution (Appendix) and spun at 50g for 10 minutes at 4°C. After discarding the layer of floating cells (dead cells, non-hepatocytes and debris) and the remainder of the supernatant the cell pellet (99% hepatocytes as assessed by morphology under phase contrast illumination) was resuspended in ice-cold *plating medium* to 5mls, for estimation of viability and yield.

### *3.1.b.3 Assessment of hepatocyte viability and yield*

The trypan blue exclusion method was used to estimate hepatocyte viability. Cells with damaged membranes admit the dye and are easily differentiated from non-staining cells by light microscopy. Trypan blue has a greater affinity for serum protein than cellular protein, so the assays were performed in serum-free solutions.

Immediately after gentle resuspension (hepatocytes settle very rapidly in suspension) a 20μl aliquot of hepatocyte suspension was added to a mixture of 40μl of 0.5% Trypan blue in isotonic saline (prefiltered at 0.2μm) with an equal volume of sterile PBS at pH 7.4. One drop of the well mixed suspension was allowed to flow by capillary action into a tightly coverslipped improved Neubauer haemocytometer (chamber depth 0.1cm), filling but not overflowing the measuring compartment. The cell number in five 1$mm^2$ blocks was then counted and trypan blue negative cells expressed as a proportion of total to give an estimate of hepatocyte viability. The viable hepatocyte yield was calculated from the number of trypan blue negative cells in the known volume assessed ($5x10^{-4}$ $cm^3$), allowing for the sample dilution (5-fold) and original volume (5ml).

## 3.1.c Primary hepatocyte monlayer culture

### *3.1.c.1 Plating of freshly isolated hepatocytes*

Freshly isolated, purified hepatocytes in suspension were diluted as necessary with *plating medium* and plated out at the required density (usually 0.2-0.4$x10^5/cm^2$) on

coated tissue culture plastic (either Lab Tek Permanox chamber slides or Nunc culture ware). Culture surfaces were normally precoated with fibronectin, although in some experiments rat tail collagen was evaluated (Appendix). Before attachment hepatocytes were evenly distributed over the culture surface by gently agitating the dish from side to side then backwards and forewards. This was important to avoid otherwise inevitable aggregation of hepatocytes at the center of the culture wells. Cultures were then left at 37°C for 40–60 minutes to allow hepatocytes to attach, incubating either in air or 5% $CO_2$/95% air depending on the plating medium formulation.

#### *3.1.c.2 Routine culture maintenance*

After the attachment period, *plating medium* and unattached cells were aspirated and replaced by fresh modified Chee's culture medium (Appendix). Cultures were incubated in a humid atmosphere of 5% $CO_2$/air at 37°C and the medium changed the next day and at least every 2 days afterwards. The medium was kept <2mm deep to maintain optimum oxygen tension (60mm Hg) [399].

## *3.2 Cell biology and in vivo studies*

### 3.2.a Drugs

#### *3.2.a.1 1,4 bis 2-(3,5dichloropyridyloxybenzene) (TCPOBOP)*

In order to evaluate proliferative (hyperplastic) responses of hepatocytes *in vivo*, mice were exposed to a chemical mitogen that had been previously used in our laboratories to produce liver hyperplasia, 1,4 bis 2-(3,5dichloropyridyloxybenzene) (TCPOBOP, a gift from Dr G. Smith, Dundee) [400]. Male mice aged between 29 and 33 days were given a single intraperitoneal (i.p.) injection of 3mg/kg TCPOBOP, suspended in corn oil. Control animals received corn oil only. For assessment of DNA synthesis BrdU labelling agent (Amersham, 0.1ml/10g body weight) was administered as a single i.p. bolus 18 hours before the animals were killed by cervical dislocation. Liver and body weights were recorded, and liver tissue was processed for DNA analysis by flow cytometry (section 3.2.g). The remainder was divided and fixed in buffered 10% formalin for routine histological analysis and in methanol for BrdU immunohistochemistry (section 3.2.f.2).

#### *3.2.a.2 Induction of liver regeneration by carbon tetrachloride*

Mice received an intraperitoneal injection of 0.5ml/kg $CCl_4$ (approximately 100μmol), mixed 5% v/v in corn oil, or corn oil alone (negative controls). This was administered at a fixed time of day (midday) in order to control for any confounding circadian effects on liver regeneration [401]. Two hours prior to killing, mice received an i.p. bolus of 3mg/ml BrdU labelling agent (Amersham, 0.1ml/10g body weight). The mice were then killed at various timepoints by cervical dislocation. Liver and body weights were recorded. The liver was divided and fixed in buffered formalin for routine histological analysis and in methanol for BrdU immunohistochemistry (section 3.2.f.2).

### *3.2.a.3 Exposure of mice to the hepatocarcinogen diethylnitrosamine*

Mice were given a single intraperitoneal bolus of diethylnitrosamine (DEN) (10 or 50mg/kg, diluted 1/1000 in corn oil) at twelve days of age. Ten weeks after treatment the mice were killed by cervical dislocation and necropsied. In addition to any lesions, the liver, one lung, one kidney, one submandibular gland, the pancreas, thymus and spleen were excised and fixed in buffered 10% formalin. The colorectum and the distal 15cm of small intestine were excised and flushed with water prior to mounting *en face* and fixing overnight in methacarn (4:2:1 methanol:chloroform:acetic acid). The fixed bowel lengths were then wound into a "swiss roll" which was subsequently paraffin embedded edge on and sectioned, producing a continuous spiral of bowel from proximal to distal ends on a single section. Lesion type and number were recorded by simultaneous microscopic analysis of 5μm haematoxylin and eosin-stained sections by two observers (myself and Dr AR Clarke) at a double headed microscope. Assessments were made without knowledge of the genotype or treatment.

Livers were scored for the prescence and number of phenotypically-altered foci and nodular lesions (hyperplastic, adenomatous or carcinomas), according to established criteria [391]. They were also scored for the presence of diffuse cytological changes that were present in hepatocytes throughout the liver. These changes were termed diffuse atypia. Diffuse atypia was defined as increased variability in the sizes of hepatocyte nuclei (many of which appeared enlarged), with easily identified irregular nuclear contours in some hepatocytes. No attempt was made to discriminate different degrees of severity of diffuse atypia, although there was variation between livers scored as positive.

## 3.2.b Cytokines

Aliquots of stock solutions of each cytokine were stored at -20°C until required.

### *3.2.b.1 Transforming growth factor $\beta_1$ (TGF$\beta_1$)*

A stock solution of 1μg/ml (40nM) transforming growth factor $\beta_1$ from human platelets (Sigma) with 50μg/ml human serum albumin was prepared in 4mM HCl containing 1mg/ml bovine serum albumin. This was diluted in culture medium to the desired concentration. Control cells were treated similarly but without TGF-$\beta_1$.

### *3.2.b.2 Insulin*

A stock solution of 300μM Bovine pancreatic insulin (Life Technologies) in culture medium was diluted to 100nM. Control cells did not receive this supplement.

### *3.2.b.3 Dexamethasone*

A stock solution of 100μM dexamethasone (Sigma) was prepared by 27-fold dilution in culture medium of a 1mg/ml solution of dexamethasone in absolute ethanol. This was added to culture medium to a final concentration of 30nM. Control cells were similarly treated but without dexamethasone.

### *3.2.b.4 Epidermal growth factor (EGF)*

A stock solution of 25μg/ml EGF from mouse submaxillary glands (Sigma) was prepared in PBS containing 0.5% bovine serum albumin and added to culture medium to a final concentration of 25ng/ml. Control cells were treated similarly with medium containing an equivalent volume of bovine serum albumin in PBS.

## 3.2.c Irradiation

Mice and cultures were γ-irradiated by a $Cs^{137}$ source at 0.33 Gy/minute. Unirradiated controls were otherwise transported and handled identically. uv-c irradiation (254nm) was administered to cultures after complete removal of culture medium, using a Spectrolinker XL-1500 (Spectronics Corporation) preset to the required flux ($J/m^2$).

## 3.2.d Evaluation of apoptosis

### *3.2.d.1 Electron microscopy*

Culture medium was removed and hepatocyte monolayers fixed *in situ* by ice cold 3% glutaraldehyde in 0.1M sodium cacodylate. After detachment by scraping the cell pellets were prepared for transmission electron microscopy by standard methods. These entailed postfixation by cacodylate buffered 1% osmium tetroxide, dehydration through graded alcohols and impregnation by grades of araldite, which was then polymerised at 56°C. 50nm sections were cut and stained by uranyl acetate and lead citrate, ready for viewing on a Phillips CM12 Transmission Electron Microscope. An additional 1μm section was cut and stained by toluidine blue for light microscopic examination.

### *3.2.d.2 Fluorescence microscopy*

Monolayers of hepatocytes on Permanox chamber slides (Lab Tek) were fixed in ice cold 90% ethanol/10% formalin for at least 1 hour. The cells were then exposed to 0.2M acetic acid for 1 minute before staining by acridine orange (10μg/ml in PBS). Excess stain was rinsed off in water before coverslipping and viewing under uv light.

### *3.2.d.3 Light microscopy*

Cultured monolayers fixed at 4°C overnight by Boum's solution (Appendix) were Feulgen-stained by exposure to 5M HCl for 40 minutes, rinsing in tap water, incubating in Schiff solution (Appendix) for 1 hour, followed by three 5–10 minute washes in tap water. Cells were counterstained by 1% aqueous light green, air dried, mounted in cedarwood oil (Sigma) and coverslipped.

## 3.2.e Recognition of apoptosis

The morphology of apoptosis was easily recognised by light microscopy in fixed, Feulgen-stained hepatocyte monolayers on chamber slides, and was confirmed by electron microscopy (Figure 7).

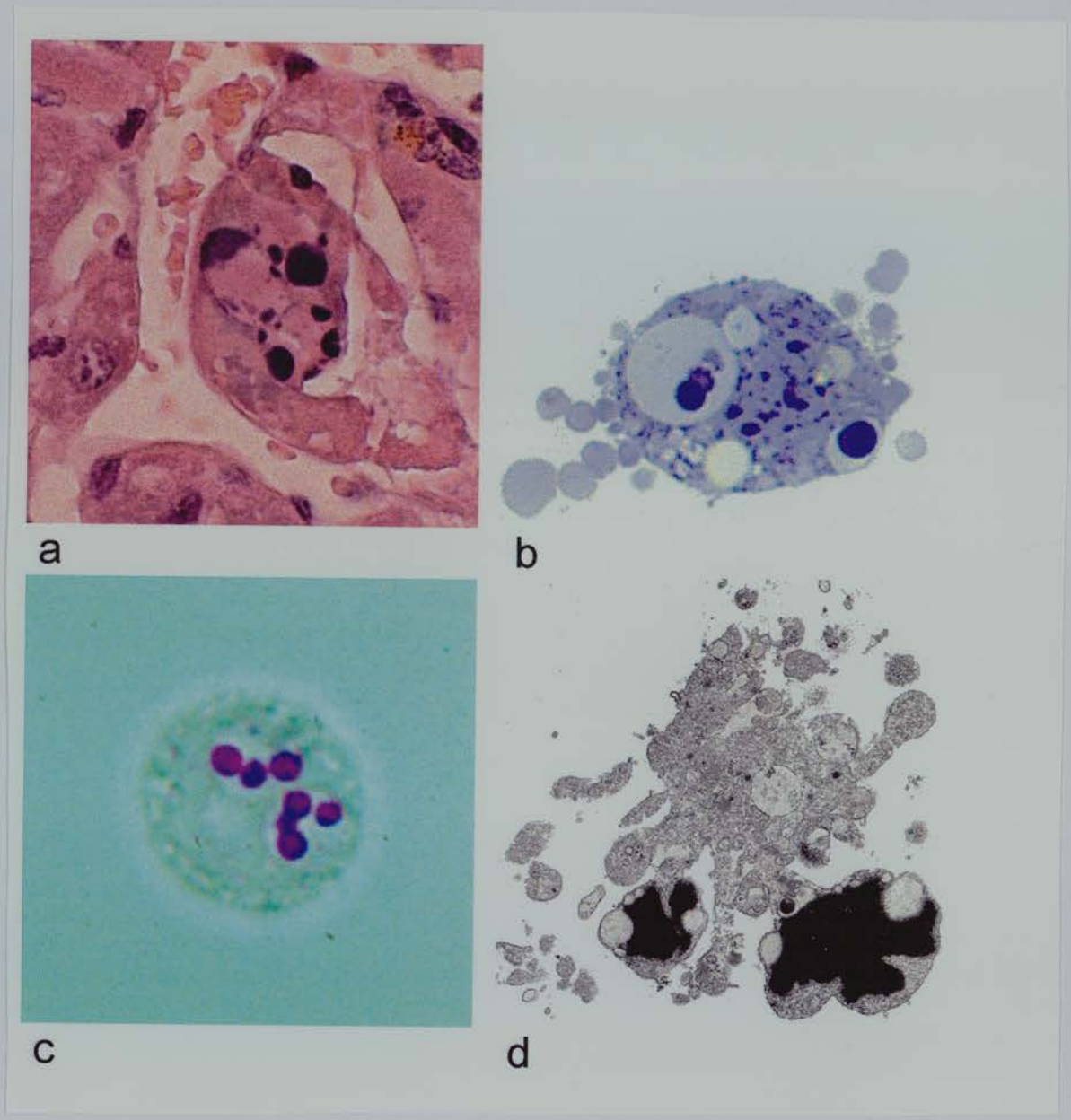



*Figure 7. Morphology of hepatocyte apoptosis, showing nuclear fragmentation and cytoplasmic condensation with blebbing*
*a, apoptotic hepatocyte in mouse liver (paraffin section, haematoxylin and eosin, original magnification x600);*
*b, apoptotic hepatocyte in primary culture (toluidine blue 200nm plastic section, original magnification x400);*
*c, apoptotic hepatocyte in primary culture (Feulgen stain, original magnification x400);*
*d, apoptotic hepatocyte in primary culture (transmission electron micrograph).*

The use of chamber slides gave improved optical resolution at high magnification when compared with conventional tissue cultureware. Boum's fixative was selected from a variety of formalin, formalin/alcohol and alcoholic fixative mixtures as giving the best nuclear morphology with a crisp nuclear membrane. Feulgen staining provided slightly crisper, more detailed nuclear morphology than acridine orange staining, avoiding problems of cytoplasmic autofluorescence and RNA fluorescence that lent a slight haziness to hepatocytes. Moreover, the problems of rapidly-fading fluorescence were eliminated and counting by light microscopy was less fatiguing than by fluorescence in a dark room.

In order to compare the relative sensitivity to detect apoptosis using these stains, acridine orange-stained monolayers were restained by the Feulgen method, and the results of counting similar areas of the culture slide compared (Figure 8). The results showed that compared with acridine orange, Feulgen staining had a similar sensitivity to detect apoptosis. The figure also shows that counts of the same culture slide were consistent in their evaluation of apoptotic index (Figure 8a,b).

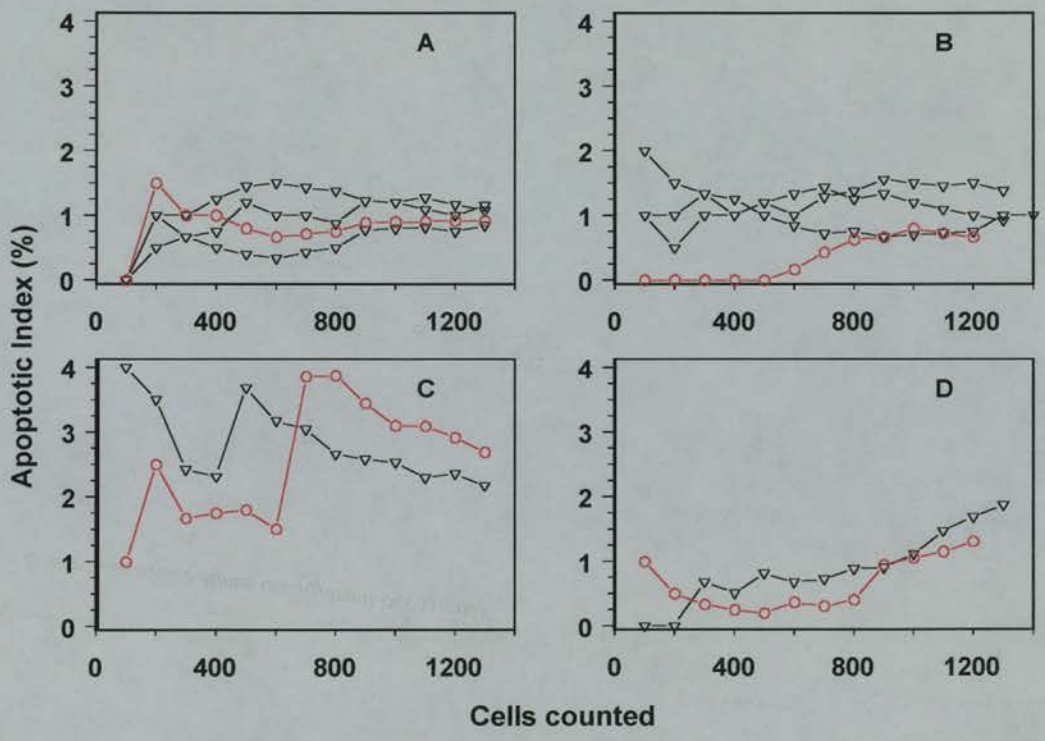



*Figure 8: Running means for apoptotic index in culture, and comparison of acridine orange with Feulgen staining. Cultures of hepatocytes derived from the same mouse and plated onto 4 culture slides (A-D) were stained with acridine orange and counted for apoptosis, then Feulgen-stained and recounted. Slides A and B were fibronectin-coated; slides C and D uncoated.*
*The variability in apoptosis counts between slides C and D was a feature of cultures on uncoated slides, where heterogeneity of cell survival over the culture surface was increased compared with coated slides (i.e. dispersed aggregates of dying cells, better survival toward the edges of the chamber slides), so requiring higher counts to achieve running means (the mean apoptotic indices of C and D are converging only by 1200–1300 cells counted).Fibronectin-coated slides were used for all experiments described in Results.*
***Key**: Red lines show counts with acridine orange staining; black lines depict counts after Feulgen staining (slides A and B were counted 3 times on different occasions).*

## 3.2.f Immunocytochemistry

### *3.2.f.1 p53 protein*

The two primary antibodies used to label p53 protein were pAb421 (Oncogene Science), a mouse monoclonal $IgG_{2a}$ that binds an epitope near the C-terminus, and CM5 rabbit polyclonal antiserum, that recognises seven distinct epitopes distributed throughout the p53 protein [402].

p53 immunohistochemistry was performed on formalin-fixed low temperature paraffin sections on polylysine coated slides after steam exposure in a pressure cooker for 10 minutes, according to the recommended protocol supplied with the CM5 antiserum (Novocastra, UK). Hepatocyte cultures on chamber slides were fixed in ice cold acetone/methanol (1:1 v/v) for 10 minutes and stored dry at -80°C. Positive nuclei were visualised by an avidin-biotin peroxidase labelling procedure, summarised below:

After rehydration, the slides were exposed to 1% hydrogen peroxide for 10 minutes, washed in PBS, then incubated for 10 minutes in 20% serum, 0.5% Tween 20 in PBS. Endogenous biotin was blocked using a kit (Vector laboratories), according to the manufacturer's instructions. Paraffin sections were incubated in CM5 for 1 hour at room temperature, whilst chamber slides were incubated with primary antibody in a humid chamber overnight at 4°C. pAb421 was diluted 1/1000 in PBS containing 5% rabbit serum and 0.5% Tween 20. CM5 was diluted 1/1500 for labelling cultured cells and 1/300 for paraffin sections. Negative controls omitted the primary antibody. After washing in PBS, the appropriate secondary antibody was applied for 30 minutes, either biotinolated rabbit anti-mouse $F(ab')_2$ (Dako) at 1/400 dilution or biotinylated sheep anti-rabbit (Dako) at 1/500 dilution. Avidin-biotin horseradish peroxidase complex (Dako) was used as the final labelling step and the chromogen was Liquid DAB (Diaminobenzidene) with DAB enhancer (Biogenex), each applied according to the manufacturers' instructions. Slides were then counterstained, mounted and coverslipped.

### *3.2.f.2 5-Bromo-2'-deoxyuridine (BrdU)*

Cultures plated onto Lab Tek Permanox chamber slides were exposed to medium containing 40μM BrdU for 3 hours, after which they were fixed overnight at 4°C in 70% ethanol/PBS. Cells that had incorporated BrdU into DNA were labelled by an indirect peroxidase immunocytochemistry technique, as follows:

Chamber slides were exposed to 1% $H_2O_2$ in distilled water for ten minutes, then rinsed in PBS and incubated in 5M HCl for 1 hour, followed by three 5-minute washes in PBS to restore neutrality. Cells were then incubated in 20% normal rabbit serum with 0.5% Tween 20 in PBS for 10 minutes. This was blotted off and cells incubated for 1 hour in purified monoclonal rat anti-BrdU $IgG_{2a}$ (Sera Lab) diluted 1/100 in 5% normal rabbit serum/Tween 20/PBS. Negative control slides omitted the primary antibody. After two five minute washes in PBS, the slides were incubated for 30 minutes in affinity isolated

horseradish peroxidase-conjugated rabbit anti-rat IgG (Sigma) that had been adsorbed in normal mouse serum and was diluted 1/50 in Tween 20/PBS. Excess antibody was then removed by three 5 minute washes in PBS. Positively stained nuclei were visualised by Liquid DAB Chromogen with DAB enhancer (Biogenex), used according to the manufacturer's instructions. Slides were counterstained with haematoxylin and light green, air dried, mounted in cedarwood oil and coverslipped.

Immunohistochemistry was performed on methanol-fixed low temperature paraffin sections preincubated in 5M HCl for 1 hour, using a standard avidin-biotin peroxidase procedure (section3.2.f. 1). The primary antibody was the rat anti-BrdU monoclonal (Sera Lab) and the secondary a biotin-labelled goat anti-rat immunoglobulin (Dako). Negative controls omitted the primary antibody.

For evaluation of BrdU positivity in experiments utilising TCPOBOP (see section3.2.a.1) 700 hepatocytes (sufficient to achieve a stable running mean — see Appendix) were counted from at least seven randomly selected fields and the results expressed as a percentage (BrdU labelling index). BrdU positivity in experiments utilising γ-irradiation or $CCl_4$ was expressed as positive cells per 20 randomly chosen high power (x400) fields (running mean achieved at 14 high power fields, Appendix).

### *3.2.f.3 Immunofluorescence*

Indirect immunofluorescence was performed on hepatocyte cultures using polyclonal antisera to murine albumin (rabbit IgG, Nordic Immunological Labs Ltd), murine fibrinogen (goat IgG, Nordic Immunological Labs Ltd) and a purified mouse monoclonal $IgG_{2a}$ antibody to β-galactosidase (Oncogene Science).

Hepatocyte cultures on chamber slides were fixed in ice cold acetone/methanol (1:1 v/v) for 5 minutes and stored dry at -80°C until use. After rehydration in PBS indirect immunofluorescence was performed by incubation in primary antibody for 30 minutes followed by three PBS washes and incubation in the appropriate fluorescein isothionate-conjugated (FITC) secondary antibody for 30 minutes. After further washes in PBS, aqueous mounting and coverslipping, slides were viewed under uv illumination from a mercury lamp using a dichroic filter appropriate for FITC (green) fluorescence.

## 3.2.g Flow cytometric evaluation of nuclear ploidy

Fresh liver tissue for DNA analysis was collected, dissaggregated and nuclei stained by propidium iodide as described by Vindelov [403,404]. This method produces clean, single nuclei essentially devoid of residual cytoplasm, as confirmed by examination of wet preparations and cytospins. Ten thousand nuclei from each sample were analysed for DNA content by a Coulter EPICS CS flow cytometer, measuring fluorescence emitted by each nucleus at 610nm (red fluorescence) in 488nm Argon laser light. The gate was set to red fluorescence and the results of each analysis were viewed as a histogram of recorded red fluorescent events. The total red fluorescence emitted by each nucleus is

proportional to the amount of bound propidium iodide. Since propidium iodide binds stochiometrically to DNA the integral red fluorescence recorded by the flow cytometer for each nucleus is proportional to DNA content. The histograms of red fluorescence are therefore histograms of nuclear DNA content.

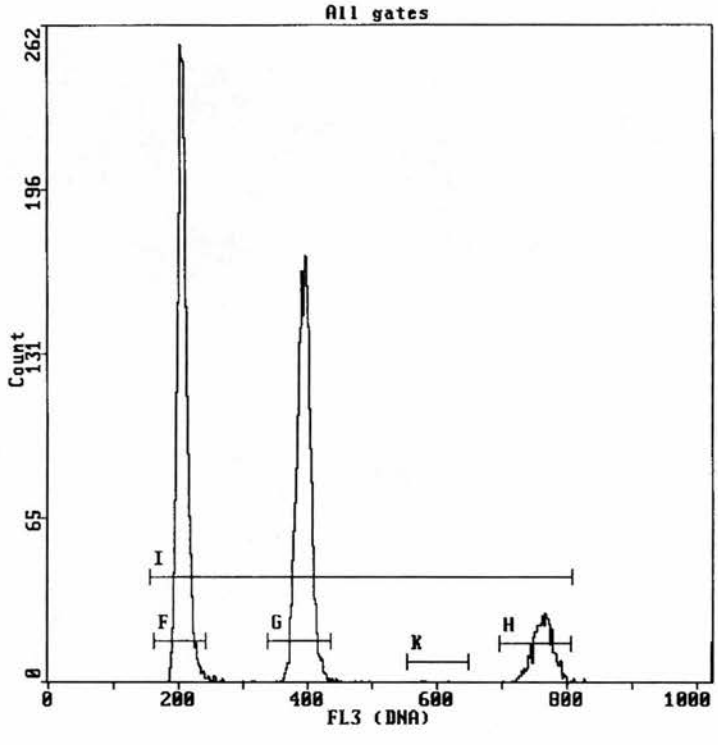



*Figure 9. Histogram of linear red fluorescence for propidium iodide-stained fresh hepatocyte nuclei. Peaks F, G, H represent DNA contents of 2n, 4n and 8n respectively.*

## 3.2.h Transient transfection of primary hepatocytes

Under appropriate conditions, eukaryotic cells can take up exogenous DNA, and a portion of this DNA becomes localised in the nucleus. This phenomenon has been widely exploited to obtain both transient and stable expression of various genes. A wide variety of methods have been developed to facilitate this process, of which one of the most efficient and reproducible in mammalian cells utilises liposomes containing cationic and neutral lipids [FelgnerPL1987].

The mechanism by which cation-liposomes mediate transfection of DNA into animal cells is incompletely understood. Negatively charged phosphate groups of DNA associate with the positively charged surface of the liposome, forming a lipid-DNA complex. Residual positive charge on the liposome causes it to associate with cell surfaces, following which there is evidence for both endocytosis of the whole complex by the cell and for fusion of the liposome-DNA complex with the plasma membrane, releasing the DNA directly into the cytoplasm [405,406][FelgnerPL1987]. Several synthetic cationic lipid formulations are available of which, for primary hepatocytes, Lipofectin has been reported to be the most

effective, or at least equally effective when compared with other lipids [405-407]. The requirement for transfection to occur under serum-free conditions was not a problem in the present culture system.

### *3.2.h.1 Plasmids transfected*

pCMVβ (Clontech) is a mammalian expression vector designed for the constitutive expression of β-galactosidase from the *Escherichia coli lacZ* gene in mammalian cells, under the control of the human cytomegalovirus immediate early promoter/enhancer. This promoter was selected since it has been shown to give greater expression of reporter genes in lipofected primary hepatocytes when compared with several other promoters [408].

pRGCΔFosLacZ is a reporter plasmid for transcriptionally active wild type p53. It is based upon the pBSK plasmid into which has been cloned oligonucleotides containing two copies of a p53 RGC binding site, a transcriptionally inactive mutant murine *fos* promoter, the *lacZ* gene (open reading frame), and the small t intron with the polyadenylation signals of SV40 [409]. Wild type p53 binds strongly to the RGC elements and stimulates expression of β-galactosidase. pRGCΔFosLacZ and a negative control plasmid pΔFosLacZ, which is identical except lacking the RGC fragment necessary for p53 binding, were the kind gift of Professor S.H. Friend, (Dept. of Paediatrics, Harvard Medical School, USA).

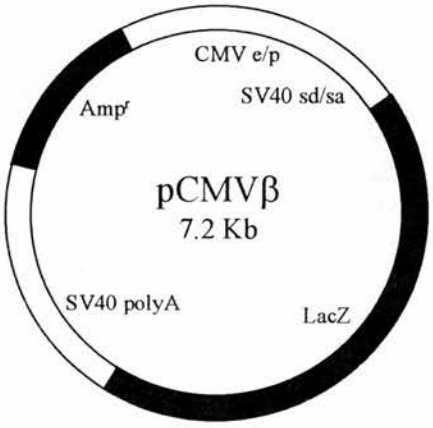



*Figure 10.* ***pCMVβ plasmid****. key: CMV e/p, cytomegalovirus immediate early promoter enhancer; SV40 sd/sa, SV40 splice donor/splice acceptor; LacZ, β-galactosidase gene; SV40 polyA, SV40 polyadenylation signal; Amp$^r$, Ampicillin resistance gene.*

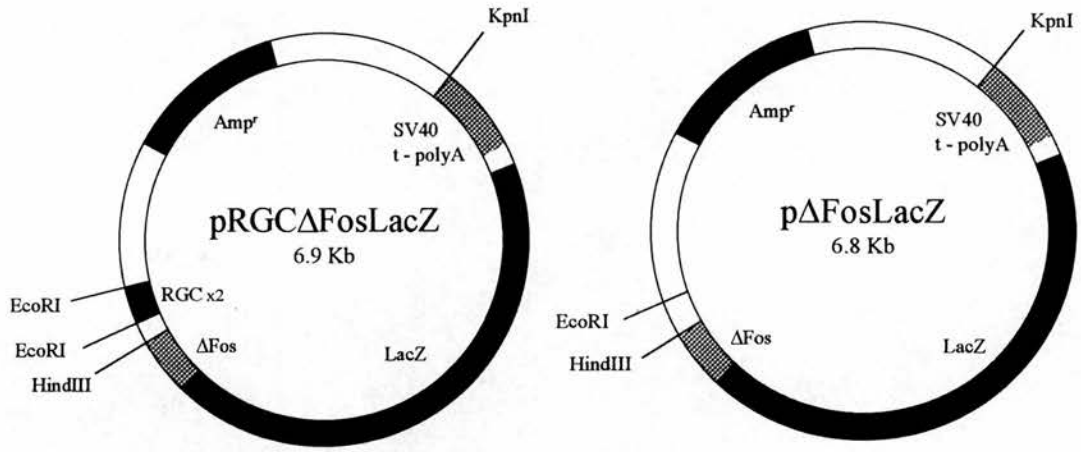



*Figure 11.* ***p53 reporter plasmid and control plasmid.*** *Key: SV40 t-polyA, small t intron and polyadenylation signals of SV40; LacZ, β-galactosidase; ΔFos, mutant murine Fos promoter; RGC, ribosome gene cluster p53 binding site; Amp$^r$, Ampicillin resistance gene.*

## Plasmid preparation

Plasmid DNA (approximately 10ng in not more than 1μl TE) was introduced into competent Escherichia coli (e.g. 40μl strain DH5α) by incubation on ice for 20 minutes followed by heat shock at 42°C for 40 seconds and chilling on ice for 2 minutes. The bacteria were then incubated for 1 hour at 37°C with 160μl of SOC medium (Appendix) in an orbital incubator at 220 rpm in order to allow expression of antibiotic resistance by the transformed bacteria. Using a sterile glass spreader the organisms were then inoculated onto LB agar plates with appropriate antibiotics (Appendix) and cultured at 37°C for 16–20 hours. Several single colonies were then isolated and individually cultured in 20mls LB medium with the appropriate antibiotic in the orbital incubator at 220 rpm and 37°C until cloudy (approximately 3 hours). Aliquots of each culture were stored at -80°C in 50% glycerol, whilst the remainder was pelleted by centrifugation at 6500 rpm for 5 minutes. Plasmid DNA was prepared from each of these mini-cultures, using the QIAprep Spin Plasmid Kit (Qiagen). These mini-preparations of plasmid DNA were done to allow verification of plasmid identity by restriction enzyme digestion and gel electrophoresis, before larger scale preparation of the plasmid. The large scale preparation was done with QIA Tip-100 or Tip-2500 kits (Qiagen) from a 100ml or a 500ml bacterial culture (up to 16 hours incubation in antibiotic-supplemented LB medium). In each case the manufacturers' instructions were followed. Essentially, the Qiagen kits utilise alkaline lysis of bacteria with denaturation of proteins, plasmid and chromosomal DNA, followed by separation of soluble, renatured plasmid DNA from complexed chromosomal DNA, protein and detergent. The plasmid is then highly purified by selective binding to a resin, followed by washing and subsequent elution. Purified plasmid from these procedures was desalted and concentrated by precipitation in isopropanol, centrifugation and washing of the pellet in ice-cold 70% ethanol. The air-

dried plasmid pellet was resuspended in 1ml tissue culture grade PBS. Plasmids were stored in this form at -80°C.

### Verification of plasmid identity

Purified plasmid DNA (>1μg) from the mini-preparations was digested in sterile microfuge (Eppendorf) tubes containing a mixture of the appropriate restriction endonuclease (Promega or Boehringer Mannheim) with the recommended buffer diluted in DDW. Digestion was usually carried out at 37°C for at least 1 hour. Enough enzyme was used to ensure complete digestion (1 unit of enzyme completely digests 1μg of plasmid DNA in 1 hour at 37°C). The volume of enzyme in the final reaction mixture was adjusted to keep glycerol from the stock enzyme solution less than 5% v/v, in order to prevent interference by the glycerol with enzymatic activity.

In order to characterise the prepared plasmids, plasmid DNA and the products of restriction enzyme digestion were separated on agarose gels, along with a reference kilobase ladder molecular weight marker (Life Technologies). Gel electrophoresis separates DNA fragments of different sizes and conformation (supercoiled, nicked circular or linear) because of their different migration rates through the gel toward the anode in an electric field. Ethidium bromide in the gel intercalates into double stranded DNA, allowing visualisation under uv light of fluorescent bands corresponding to migrated DNA fragments of various sizes. Preparation of 1% agarose gels in TBE and electrophoresis followed the protocols of Sambrook et al [410]. Samples were mixed with $\frac{1}{6}$ volume of gel loading buffer (Appendix) and loaded into the wells of a gel submerged in TBE. Electrophoresis was continued until the bromophenol blue marker had migrated across 80–90% of the gel. Plasmid fragment sizes were estimated by reference to the kilobase ladder marker.

The concentration of DNA in plasmid preparations was estimated by measuring the optical density (OD) at $\lambda$=260nm of a 1:100 diluted sample, using a Genequant II spectrophotometer (unit $OD_{260}$=50μg DNA/ml). $OD_{260}/OD_{280}$ ratios were used as a measure of sample DNA purity.

### *3.2.h.2 Cationic liposome-mediated transfection*

Lipofectin Reagent (Life Technologies) was used as the vehicle for transfection of DNA into hepatocytes. Lipofectin is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in water.

Primary hepatocytes were plated into fibronectin-coated 24 or 12 well tissue culture dishes at a density of 0.2–0.3 x $10^5/cm^2$ and transfection was generally performed after 48 hours culture. The monolayers were fed with fresh, serum-free modified Chee's culture medium a few hours prior to transfection. The quantities of reagents given here have been optimised for a single 24 well chamber (1.90$cm^2$), and were scaled up appropriately for

larger well sizes. Lipofectin reagent (6µl of 1mg/ml) was diluted into 100µl serum-free, antibiotic-free culture medium and incubated at room temperature for 45 minutes. This suspension was then mixed gently with 100µl of serum-free, antibiotic-free medium containing purified plasmid DNA (1µg) and incubated for a further 45 minutes at room temperature. The mixture was then made up to 300µl with serum and antibiotic-free medium, and layed over hepatocytes in place of the existing culture medium. The cultures were incubated at 37°C in a humid 5% $CO_2$/95% air atmosphere for 6 hours, whereupon the DNA-containing medium was replaced with 500µl standard culture medium.

#### *3.2.h.3 Effects of irradiation on p53 reporter plasmid expression*

Primary hepatocytes were cultured for at least 40 hours on fibronectin-coated 12 well culture dishes and then transfected with pCMVβ or pRGCΔFosLacZ or pΔFosLacZ plasmids, according to the protocol described in section 1. 24-48 hours later the cultures received either uv-c (0 or 10J/$m^2$) or γ-irradiation (0 or 15Gy) as described in section 3.2.c. The culture medium was changed after a further 48 hours if the cells were not yet harvested for estimation of β-galactosidase activity (section 3.2.i.3).

## 3.2.i Cytochemical staining

#### *3.2.i.1 Tetrazolium dye assay of culture growth and survival*

Microculture tetrazolium assays depend upon the reduction of exogenous tetrazolium salts to coloured formazans by mitochondrial succinate dehydrogenase in living cells. The present protocol is based on a modification by Carmichael [411] to the assay originally described by Mossmann [412], utilising 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide (MTT).

Hepatocytes cultured in Permanox chamber slides or 24 well tissue culture plates were incubated under normal culture conditions for 3 hours in culture medium containing 1mg/ml filtered MTT (from a 10mg/ml stock solution in medium). After this time the medium was discarded and cultures washed with PBS. The PBS was aspirated and the culture wells allowed to dry. DMSO was then added (100µl/1.9$cm^2$ well) and mixed briefly to ensure that all the formazan dye was solubilised. Aliquots were then transferred to a 96 well ELISA plate and the absorbance read at 490nm (negative control omitting MTT) by an ELISA plate reader (MR 5000, Dynatech).

#### *3.2.i.2 In situ staining of transfected hepatocytes for β-galactosidase activity*

After removal of culture medium and rinsing with PBS, cells were fixed by 0.05% glutaraldehyde in PBS for 5 minutes at 4°C. The fixative was discarded and cells washed three times in PBS, leaving the second wash on for at least 10 minutes. Monolayers were then incubated at 37°C in 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution (Appendix) overnight, using sufficient volume to cover the cells. Positive cells were easily visualised under light microscopy by their strong, blue colour (Figure 12).

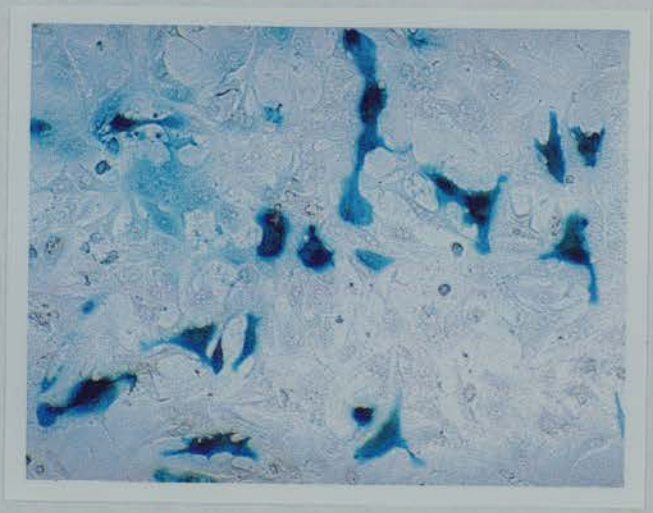


*Figure 12 X-gal staining of pCMVβ-transfected hepatocyte primary cultures.*

### *3.2.i.3 Quantitation of β-galactosidase activity by colorimetric assay*

β-galactosidase activity in transfected hepatocytes was quantified using the Beta-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega), following the manufacturer's instructions. Firstly, cell extracts were prepared by rinsing the monolayers with PBS and then incubating in Reporter Lysis Buffer, appropriately diluted in water, using sufficient to just cover the cells (eg: 100µl for one chamber of a 24 well plate). After 15 minutes incubation at room temperature the dishes were scraped clean, the resultant lysate vortexed, centrifuged in a microfuge at 4°C for 2 minutes (13,000 rpm), and the supernatant stored at -80°C until use.

The cell extracts were then assayed for β-galactosidase activity by measuring the amount of yellow hydrolysis product (o-nitrophenol) generated from ONPG substrate (o-nitrophenyl-β-D-galactopyranoside). Untransfected hepatocytes were used as a negative control for the assay. A 30µl aliquot of each cell extract was incubated with an equal volume of 2x Assay buffer, containing the ONPG substrate, in a 96 well plate for 30 minutes to 3 hours, until a yellow colour was appreciable. A standard curve for β-galactosidase activity was also prepared over a range of 0–5.0 milliunits, according to the manufacturer's instructions, using the β-galactosidase supplied (1unit/µl; one unit of enzyme hydrolyzes 1µmol ONPG per minute at pH7.5 and 37°C.) Standard curve assays were incubated in parallel with and for the same length of time as the test sample extracts. The reaction was then stopped by mixing in 90µl 1M $Na_2CO_3$ and the absorbance at 420nm read by an ELISA plate reader (MR 5000, Dynatech).

To account for variability in the number of cells recovered from each well for estimation of β-galactosidase activity, the protein concentration of each cell extract was estimated. A known aliquot of each cell extract (usually 20µl, but this was altered if necessary to

keep the final absorbance within the linear range of the standard curve) was diluted into 800µl distilled water, mixed with 200µl of Protein Assay Dye Reagent (Biorad) and incubated at room temperature for 10–60 minutes. The negative control used Lysis Buffer. A standard curve was constructed using a bovine serum albumin 2mg/ml standard solution (Biorad), diluted to final concentrations of 1–8µg/ml and incubated in parallel with the test samples. The absorbance was then read at 595nm.

# 4. Optimisation of hepatocyte isolation and culture

Primary hepatocyte culture offers an oppurtunity to complement and extend observations made in vivo, by combining the use of authentic hepatocytes (i.e. not cell lines) with the ability to regulate and control the hepatocytes' environment. For a fully-defined culture environment, it is necessary to use serum-free culture medium. This chapter describes the setting up and evaluation of a method for isolation and monolayer culture of primary mouse hepatocytes.

## *4.1 Hepatocyte isolation*

### 4.1.a Background discussion

Production of a single cell suspension from liver requires that intercellular junctions be disrupted and cell-to-matrix adhesion abolished. Current techniques for the isolation of viable hepatocytes are based on original studies on rats. Early attempts using mechanical disruption generally produced non-viable cells in low yield and have been superceded by protease enzyme-based isolation methods, of which collagenase has remained the most widely used enzyme since its introduction by Howard in 1967 [413]. Howard reported the isolation of intact rat hepatocytes representing 3-5% of the original liver using a collagenase/hyaluronidase solution injected into hepatic sinusoids, followed by incubation of thin liver slices in further enzyme solution. This was the first unequivocal demonstration that the preparation of suspensions of intact hepatic parenchymal cells was possible.

In order to obtain better exposure of the liver connective tissue to enzyme and better survival of hepatocytes during digestion, Berry and Freind perfused the liver through the hepatic portal vein with oxygenated, balanced saline solution containing collagenase/hyaluronidase [414]. This gave hepatocyte yields representing 30-50% of the original liver. Adequate separation of parenchymal cells from each other requires exposure to a $Ca^{++}$-free medium in order to irreversibly disrupt intercellular calcium-dependent junctions, but the collagenase activity necessary to digest the extracellular hepatic matrix is calcium-dependent. Therefore Seglen devised the **two-step perfusion**, whereby perfusion of the liver with a $Ca^{++}$-free medium is followed by perfusion with a calcium-enriched collagenase-containing medium [415]. Although numerous minor refinements have since been introduced [416], the collagenase perfusion technique has remained essentially as described by Seglen. It is now common practice to obtain yields of rat hepatocytes between 40-50% of the theoretical maximum yield, and with trypan blue exclusion routinely exceeding 95%.

### *4.1.a.1 Murine hepatocyte isolation*

Only Klaunig has published a systematic comparison of different techniques for the isolation of mouse hepatocytes [417]. Other authors have reported particular variations of the one-step method of Berry and Friend or the two-step method of Seglen, applied to mouse hepatocyte isolation [418-425]. Both hepatic portal and retrograde (i.e. abdominal or thoracic inferior vena caval) perfusion techniques have been tested, with no consistently preferred technique emerging. Klaunig favoured portal perfusion, however this gave lower yields than retrograde techniques unless intermittent pressure was allowed to build up within the liver during perfusion by occluding the perfusate outflow, a procedure considered by Berry to be damaging to the cells [87]. The greatest yields were reported by Renton, using a retrograde technique similar to that described here [418]. However there is difficulty in comparing different authors' results, because of frequent failure to report yields and viabilities and uncertainties about nutritional state, weights, gender and strains of mice used. In general, yields are proportionately lower than for rat livers and many groups report trypan blue exclusion of only 80-90%, a level unacceptable for rat hepatocyte isolation and which suggests greater fragility of mouse compared with rat hepatocytes [87]. Furthermore, incomplete dissagregation causing cell clumping seems to be a more frequent problem for murine hepatocytes, even using batches of collagenase known to be satisfactory for use on rat livers (R. Schulte-Hermann, communication).

### *4.1.a.2 Changes to hepatocytes during isolation*

Although hepatocyte responses are conveniently studied in primary culture, phenotypic changes may affect the interpretation or relevance of results for hepatocytes *in vivo*. It is therefore important to consider the effects on hepatocytes of isolation and culture. This section reviews the changes to hepatocytes during isolation, before discussing the chosen method of isolation; phenotypic changes in culture are reviewed in the section on monolayer culture.

The original report of one-step liver perfusion, by Berry and Friend, described the ultrastructural changes that occurred in the rat liver, and others have added little to that report. Klaunig has since described the ultrastructure of mouse liver during 2-step perfusion, which is similar to the changes described by Berry and Friend [417]. Perfusion with $Ca^{++}$-free solution has little effect on hepatic architecture: sinusoids swell and there is partial denudation of sinusoid lining cells. Hepatocytes themselves show only swelling of endoplasmic reticulum and partial separation at areas of interdigitation, although junctional complexes remain intact. However perfusion with collagenase solution causes gross architectural disruption: hepatocyte plates break down as hepatocytes separate into single cells or small aggregates, held together by the tight junctions around bile canaliculi. Desmosomes are cleaved, forming hemidesmosomes which are endocytosed. Isolated cells separate by physical shearing of tight and gap junctions or by tearing off blebs of cytoplasm from their neighbor around the junctions. The resulting defects in membrane integrity appear to reseal quickly, and excess water entering the cell is probably exported

via the Golgi apparatus. However, there is substantial loss of cellular potassium during perfusion (up to 60% of total [414]). With the loss of intercellular contacts, the hepatocyte cytoskeleton disperses, however this change is reversed in monolayer culture when cell junctions are reestablished and much of the polarity of the native cell can return [426].

Foy has recently added to these morphological assessments by magnetic resonance imaging of rat liver during perfusion *ex vivo* to measure intrahepatic flow, pH and ATP levels [427]. Hepatic perfusion with $Ca^{++}$-free solution was homogeneous and did not significantly reduce intracellular pH or ATP levels. By contrast, perfusion with collagenase solution led to dramatically disrupted intrahepatic flow within 3 minutes (multiple unperfused foci), associated with profound decreases in intrahepatic ATP concentration (undetectable by 6 minutes), and pH (nadir 6.79). These effects were not a direct effect of collagenase on hepatocytes, but were probably related to local ischaemia due to poor local perfusion. Thus, despite minimal intracellular ultrastructural injury, hepatocyte isolation inflicts considerable, albeit brief, metabolic stress to the cells. This is apparently tolerated quite well: the ability to synthesize glucose from lactate is retained to 50% of the level *in situ* [414]. The gluconeogenic assay is a searching test of metabolic integrity, since the anabolic process involves both mitochondrial and cytoplasmic compartments [428].

Most of these changes appear to be temporary if the stress is not prolonged. However an important consequence of isolation is shift of hepatocytes from replicative quiescence ($G_0$) into $G_1$ phase of the cell cycle. *In vivo*, adult hepatocytes are largely unresponsive to mitogens and become competent only after a "priming" stimulus that causes them to progress into $G_1$. Such stimuli include partial hepatectomy, necrosis, metabolic stress or any disruption of extracellular matrix or cell-cell contacts. Even sublethal concentrations of collagenase, infused through the hepatic portal vein *in vivo*, are sufficient as a priming stimulus for mitogen responsiveness [49], and it has been demonstrated that isolation into culture of primary hepatocytes causes expression of immediate early genes *fos*, *jun* and *myc* that mark entry to $G_1$ [51]. Therefore primary cultured hepatocytes are a system representative of a stimulated liver ("primed for regeneration") rather than quiescant liver.

## 4.1.b Surgical method for hepatocyte isolation

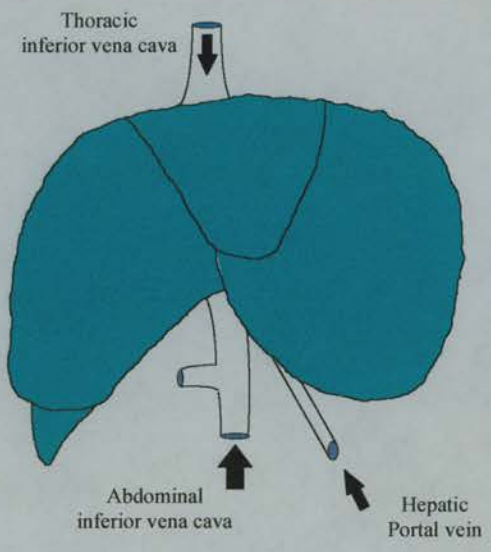



*Figure 13: Routes for liver perfusion*

The present technique of retrograde 2-step perfusion under terminal anaesthesia, was based on the studies cited above. It was also possible to perform perfusion on mice that were killed by cervical dislocation, 30-60 seconds after injection of heparin (Multiparin, 100 units) into the tail vein. Anterograde (portal venous) and retrograde (vena caval) routes of perfusion (Figure 13) were tested with this protocol and each yielded viable hepatocytes (Table 10). However, despite heparinisation, intrahepatic venous or sinusoid thrombosis was a recurring problem that resulted in poor perfusion of a particular segment or of the peripheral liver. Occasionally the entire liver did not perfuse. Furthermore, hepatocyte clumps (5–20 hepatocytes in aggregates, often including dead cells) were relatively more frequent in the final isolate, reducing the quality of the preparation. Therefore this approach was abandoned in favour of perfusion under terminal anaesthesia, in which circulation continued almost until perfusion was started, thus preventing intrahepatic stasis and thrombosis. Retrograde perfusion through the inferior vena cava and hepatic veins was selected as the route of choice, primarily because of relative speed and ease compared with portal vein cannulation.

| | Number of mice | Viable hepatocyte yield (millions) | | | Trypan blue exclusion (mean %) |
|---|---|---|---|---|---|
| | | Mean | Median | Upper quartile | |
| **Heparinised, killed:** | | | | | |
| retrograde perfusion | 23 | 19 | 14 | 23 | 90 |
| portal perfusion | 6 | 20 | 17 | 27 | 89 |
| **Anaesthetised:** | | | | | |
| Collagenase a | 16 | 13 | 17 | 14 | 88 |
| Collagenase I | 40 | 26 | 27 | 33 | 93 |
| Collagenase IV | 18 | 30 | 25 | 37 | 93 |

*Table 10: Hepatocyte yields and viabilities for different perfusion protocols*

## 4.1.c Choice of collagenase

The type and batch of collagenase are major factors affecting the quality and yield of isolated hepatocyte preparations [415]. There is unpredictable batch-to-batch variability that is due to uncertainty about which components of "crude" collagenase are essential for satisfactory cell separation; different ratios of various non-collagenase protease activities (clostripain, tryptic, caseinase etc.) are present in "crude" collagenase, removal of which can substantially decrease the quality of cell separation achieved. These preclude definitive statements about the suitability of a particular collagenase for perfusion without prior testing on the particular species of interest. For mouse liver perfusion, Boerhinger collagenase h gave relatively poor yields and was also not recommended by others (personal communication). Collagenase a (Boerhinger) has proved satisfactory for mouse perfusion by others (personal communication) but gave dissapointing yields with mice used here (Table 10). Better results were obtained using Sigma Collagenase I and IV. These were similar in terms of viable hepatocyte yields produced (Table 10), however collagenase IV generally gave fewer hepatocyte aggregates. The inclusion of DNAse I in the digestion medium was also effective to reduce the prevalence of aggregates. Continued incubation in digestion medium *ex vivo* for 10–15 minutes, as suggested by Seglen [415] was found to be useful if the initial perfusion digestion was not optimal, or after collagenase I perfusions — to decrease aggregation without compromising cell viability.

## 4.1.d Perfusate composition

The value of continuously oxygenating the perfusate was not specifically evaluated and was reported by Klaunig to be unnecessary [417]. However of a series of otherwise comparable isolations utilising collagenase I the average viable hepatocyte yield with

means over 99% of freshly plated cells in monolayers were identified as hepatocytes. Immunofluorescence cytoplasmic positivity for fibrinogen and albumin was also demonstrated as further confirmation of cell phenotype.

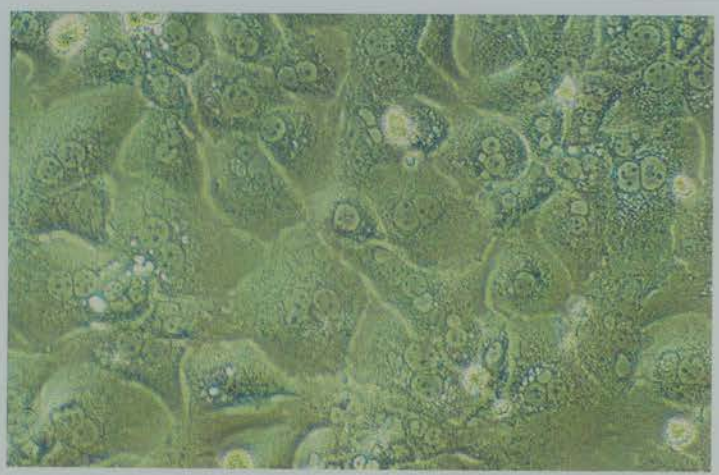


*Figure 14 phase contrast micrograph of hepatocytes in primary culture*

### 4.1.g Precision of cell counting

It is standard practice to evaluate the yield of hepatocytes using a haemocytometer, however there are potential sources of error. The occurrence of cell doublets and aggregates in the hepatocyte isolate causes departure of the sample from the Poisson distribution, resulting in underestimation of the true count (overdistribution error). The number of cells counted also affects precision: the particular dilution of cells used here (see Methods, section 3.1.b.3) was chosen so that approximately 500 cells would be counted (representing a yield of 25 million); this gives a reasonably precise estimate without being impractical [431]. If smaller numbers of cells were counted (250–300), repeated counts from the same hepatocyte isolate were found to differ more than expected from random fluctuation (e.g. 295, 322, 259, 260; $p=0.02$, $\chi^2$ test) and therefore to provide unreliable estimates of the true yield. In practice, the average of 2 independent fillings of the haemocytometer was used to estimate yield.

## *4.2 Monolayer culture*

Maintenance of a differentiated phenotype in cultured hepatocytes is obviously important for data to remain relevant to hepatocytes *in vivo*. This section discusses some of the

phenotypic changes that can occur in cultured hepatocytes and factors that affect those changes. The results of attempts to optimise culture conditions for primary hepatocytes will then be presented. In particular, serum-free culture was desirable — firstly, because 5–10% serum in culture medium does not promote differentiated function; secondly, to allow fully-defined conditions of supplementation with cytokines to be tested; and thirdly to prevent growth of contaminant cell populations.

## 4.2.a Background discussion

Hepatocytes can be plated onto tissue culture plastic and maintained for a few days in any of several "off the shelf" simple culture media, such as Leibowitz L15 or Dulbecco's modified Eagle's medium, supplemented with 5–10% serum. The major problems associated with this "simple" monolayer culture of primary hepatocytes are shortened cell survival time, including detachment from monolayers after 4–5 days, and alterations in gene expression and differentiated function that rapidly depart from the *in vivo* state. Such alterations include decreases in gluconeogenic enzymes, cytochromes P-450, NADPH cytochrome P-450 reductase, cytochrome $b_5$, and associated drug metabolising activities; reduced synthesis and secretion of albumin and other serum proteins, shifts in isoenzyme expression patterns away from those typical of hepatocytes and increased expression of proteins found in foetal liver (e.g. α-fetoprotein and γ-glutamyl transpeptidase). There is also increased expression of genes for some cytoskeletal proteins such as actin and tubulin [reviewed by [432,433]]. The changes do not necessarily indicate "dedifferentiation" of the hepatocytes; several liver-specific enzyme activities are hormone-dependent and can be restored with the appropriate hormone supplement. However fundamental changes in gene expression do occur in simple cultures that reflect greatly diminished transcription of the relevant genes [434]. Variables known to influence these changes include cell density, matrix attachment and composition, culture medium composition, heterologous cell interactions and the source animal species. An exhaustive investigation of these factors was not possible, however the effects of varying substratum and medium composition were tested and will first be briefly discussed.

### *4.2.a.1 Cell substratum*

Use of culture-ware coated with adsorbed matrix protein greatly enhances cell attachment, particularly in serum-free media, and also promotes DNA synthesis, depending on the particular matrix protein [54,55]. Matrix films probably have little additional effect on preservation of differentiated function, although use of collagen gel "sandwiches" with hepatocytes intercalated has been reported to greatly improve survival and albumin secretion [435]. Complex biomatrices such as "Matrigel" also greatly improve functional preservation, but contain many undefined components in addition to the cocktail of known cytokines (e.g.TGFβ) and matrix proteins (e.g.laminin). Until recently it was thought that such biomatrices were essential for preservation of phenotype *in vitro*, however several reports now indicate that highly supplemented culture media are capable of preserving many hepatocyte functions at levels comparable to those *in vivo*, with only

simple substrata such as collagen-coated tissue culture plastic [436]. This is particularly true for primate hepatocytes [437].

### *4.2.a.2 Culture medium*

Medium formulation is a major determinant of both culture survival and maintenance of hepatocyte phenotype. The literature on the subject is large, confusing and almost entirely devoted to rat hepatocytes, however it is clear that most commercially available media, while containing many of the basic requirements for cell survival, are inadequate to fully support the complex requirements of hepatocytes. Serum-free media are desirable, since serum is undefined, contains factors that do not aid maintenance of differentiated function [432], and promotes growth of non-parenchymal cells. High initial concentrations of amino acids (5–10 fold greater than in serum) are required to offset rapid utilisation and maintain protein synthesis [438,439]; DNA synthesis also depends strongly on proline content [440]. Of carbohydrates, lactate and pyruvate are better substrates than glucose unless it is at very high concentration (>20mM). Selenite, transferrin, and linoleic acid are commonly added to hepatocyte culture media, in addition to other trace elements not present in simple media, whose individual contribution to culture quality is uncertain. Indeed such assessments are often difficult to make or even irrelevant in isolation, given the complex interaction by which a hormone/factor with a positive effect on one function may have no or a negative effect on others, and that interactions between hormones are not predictable sums of their individual actions [441,442].

Glucocorticoids have the most marked effect of hormones on preservation of function and of hepatocyte viability and are universally added to media. Two-dimensional gel analyses have shown that synthesis of approximately 80 proteins changes in the first 20 hours of rat hepatocyte culture [443] and that dexamethasone at 30–100nM is sufficient to reverse or retard these changes (with induction or repression of a further group), and preserve albumin synthesis and morphology [444]. Insulin enhances cell attachment, survival and anabolic functions — effects that are maximal at 100nM [439]. Epidermal growth factor may also preserve hepatocyte morphology and albumin synthesis in addition to its mitogenic effects [441]. The beneficial effects on hepatocyte function of other hormones such as glucagon and tri-iodothyronone, are less well documented [442,445].

Non-physiological supplements have also been reported to benefit hepatocyte preservation or function, although how exactly is not usually clear. DMSO (2%) is best characterised and causes initial detachment of a proportion of cells but in the remainder preserves several liver specific functions and hepatocyte morphology for several weeks, in a glucocorticoid and insulin dependent manner [444]. However these effects are largely due to abnormal mRNA stability rather than restoration of gene expression levels. Phenobarbital and nafenopin extend culture lifespans, and phenobarbital promotes long term retention of albumin secretion (10–20% initial values) and cytochrome P-450b activity [436,446]. Nicotinamide may benefit cultures [447]. Butyrate (5mM) prolongs

hepatocyte survival and preserves phenotype and morphology, perhaps by modulating histone acetylation [448,449].

**In summary**, there are few published systematic evaluations of the contributions of individual medium components to maintenance of hepatocyte phenotype; those available are performed on rat hepatocytes. However, defined complex media are now formulated that extend survival and substantially preserve at least some hepatocyte-specific functions and characteristics in simple monolayer culture.

## 4.2.b The MTT assay.

The MTT assay was chosen here as a convenient means to compare the effects of different substrata and medium formulations on primary hepatocyte culture. For a given medium formulation, formazan dye production by hepatocytes was proportional to number plated (Pearson's linear correlation coefficient, $r = 0.997$; $p<0.0001$), although falling off at higher plating densities as the culture surface became saturated and plating efficiency dropped (Figure 15) . However it was not possible to use this method to accurately track cell number over time because hepatocyte metabolic activity decreases with time in culture, reducing the MTT-metabolising capacity of individual cells [87,450]. Thus the assay results are a product of both cell number and preservation of hepatocyte metabolic capacity in culture. However, as it is desirable to maintain both of these factors the assay was considered a useful if not completely specific index for culture optimisation. One advantage of the assay was that direct observation of monolayers after incubation with MTT, in which viable cells contained dark formazan crystals, permitted validation of culture viability on a cell-by-cell basis, in addition to measurements of total formazan production by the colorimetric assay.

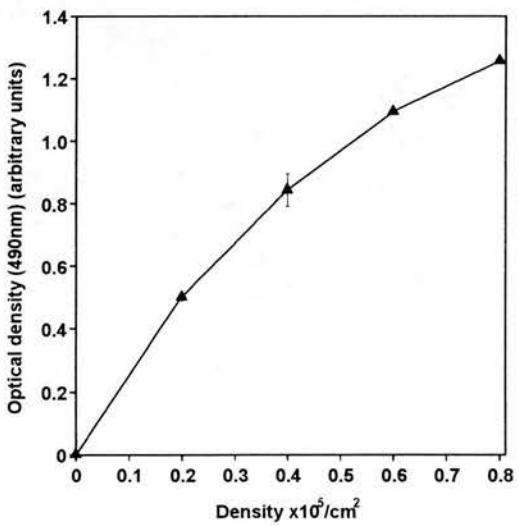



*Figure 15:Correlation of hepatocyte plating density with MTT assay. Cells were plated on fibronectin-coated permanox wells (0.78cm$^2$) for 1 hour before beginning the MTT assay.*

## 4.2.c Substratum composition.

Permanox has been reported to give better preservation of at least some hepatocyte enzyme activities (cytochrome P450) [436,451], compared with other tissue culture plastic, and was used in most of the experiments recorded here. Precoating culture surfaces with fibronectin or collagen I considerably reduced hepatocyte detachment after 4–5 days of culture. Fibronectin has also been reported to promote hepatocyte DNA synthesis [54] and to retard expression of γ-glutamyl transpeptidase, a foetal hepatocyte enzyme [55].

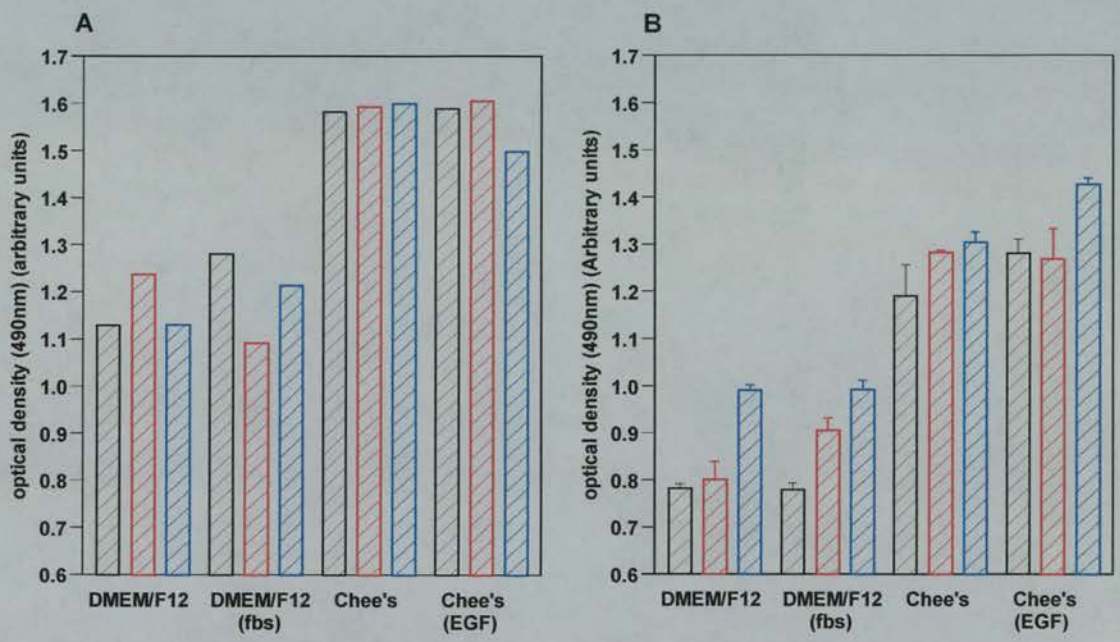



*Figure 16: Effects of different culture substrata and media on hepatocyte culture, as assessed by MTT assay. Hepatocytes were plated ($0.6x10^5/cm^2$) as described in methods, then changed after 45 minutes to the indicated medium.*
*(a) Freshly plated hepatocytes; (b) After 2 days culture.*
***Key**: black, uncoated permanox,; red, collagen coated ($20\mu g/cm^2$); blue, fibronectin coated ($20\mu g/cm^2$).*
*All media were supplemented with insulin (300nM) and dexamethasone (100nM). Fbs indicates 1% serum supplement, EGF indicates 25ng/ml EGF supplement. Triplicate observations.*

Fibronectin or collagen I coating of permanox did not affect MTT assay values for freshly plated hepatocytes (Figure 16a), however by 48 hours of culture there was a tendency for fibronectin-coated surfaces to give superior MTT results compared with either collagen I or uncoated permanox, irrespective of the culture medium formulation (Figure 16b). Rates of apoptosis were also slightly lower for cultures maintained on fibronectin-coated permanox, compared with uncoated tissue culture plastic, suggesting a direct action of specific extracellular matrix binding as a survival factor (Figure 17).

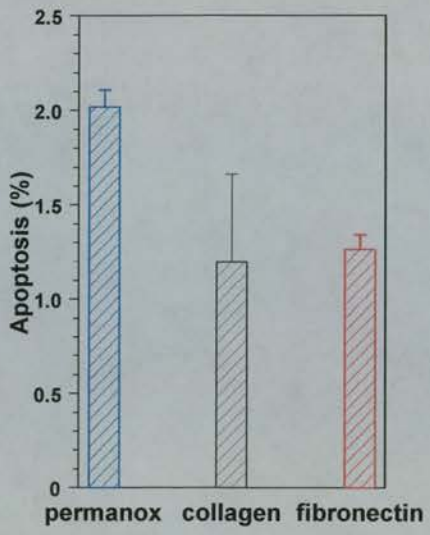



*Figure 17: Effect of culture substratum on hepatocyte apoptosis after 4 days culture in DMEM/F12, with dexamethasone (100nM), insulin (300nM) and EGF (25ng/ml). Collagen and fibronectin were coated at 20μg/cm$^2$.Cells were plated at 0.3x10$^5$/cm$^2$. At least 1000 cells counted per observation; n=2 (permanox), 3 (collagen), 5 (fibronectin).Values expressed as mean + standard error of the mean.*

## 4.2.d Culture medium formulation

Three media were evaluated on mouse hepatocyte cultures, a 1:1 mixture of Ham's F12 with Dulbecco-modified Eagle's minimal essential medium (DMEM/F12), Hepatozyme SFM (Gibco) and a custom modification of Chee's medium (Appendix). DMEM/F12 is a readily available culture medium that has been reported to be more effective than other commonly available formulations for murine hepatocyte culture and growth [452]. Hepatozyme SFM is a recently marketed complex medium designed for serum-free culture of hepatocytes with increased preservation of liver-specific functions. Chee's medium is a substantially modified form of Eagle's Minimal Essential Medium, with a particularly high amino acid content and which contains a novel combination of glucose with other carbohydrates. It has been shown to allow prolonged hepatocyte survival with excellent preservation of several differentiated functions [436,444,453].

The medium formulation had an immediate effect upon MTT-metabolising activity of freshly plated hepatocytes, with Chee's medium giving values over 35% greater than those with DMEM/F12, independent of supplementation with serum or epidermal growth factor (Figure 16a). At this stage there was no difference in the viable cell numbers, as assessed by trypan blue exclusion and counts of MTT-metabolising cells. Therefore this effect was due to increased metabolising activity by the plated cells.

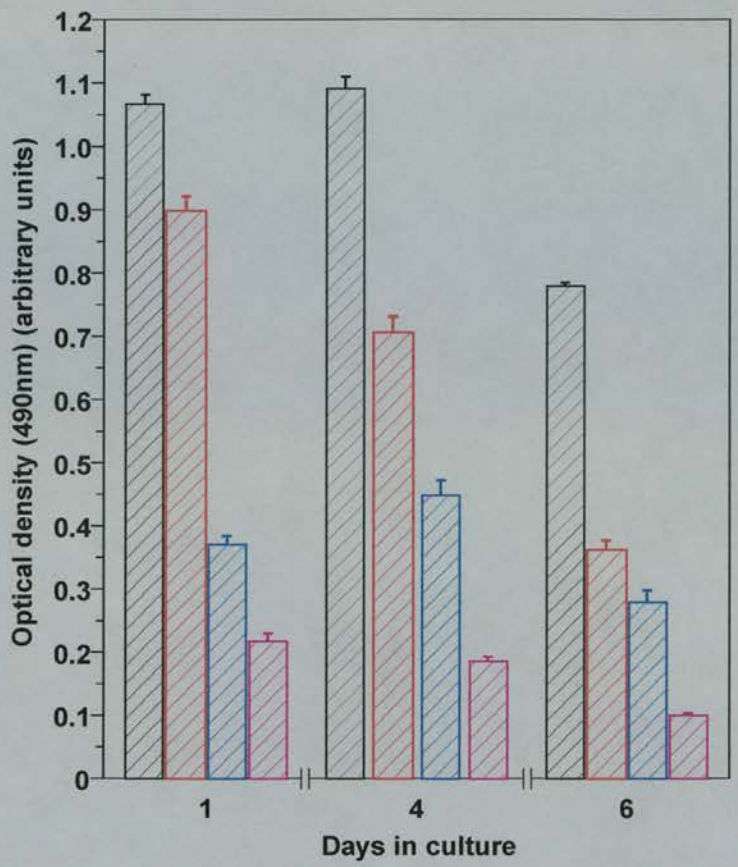



*Figure 18: Effect of medium formulation on primary hepatocyte culture, assessed by MTT assay.*
*Cells were plated ($0.3x10^5/cm^2$) on fibronectin-coated plastic and changed to test media after 1 hour. MTT assays were performed at the indicated times after plating. All media were supplemented with dexamethasone (100nM) and insulin (1.5μM).*
***Key to media:** Black bars, Chee's + 2% serum; red bars, Chee's; Blue bars, Hepatozyme SFM; Purple bars, DMEM/F12. Six replicates per observation (values expressed as mean + standard error).*

Comparison of all 3 media over the first 6 days of primary culture (Figure 18) showed that the two complex media, Hepatozyme SFM and modified Chee's, were superior to DMEM/F12, however modified Chee's medium preserved MTT values best. Direct observation of cultures confirmed that the differences were due at least in part to greater final cell numbers in Chee's medium (Figure 19).

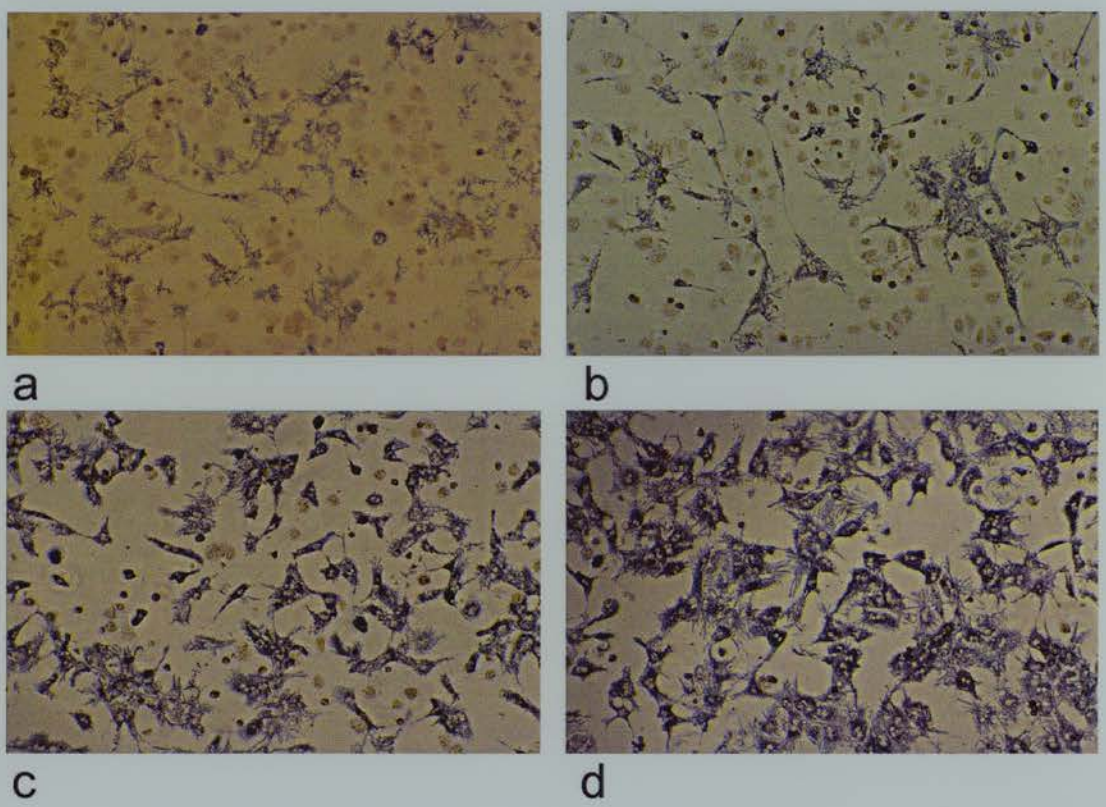



*Figure 19 Phase contrast micrography of 4-day-old hepatocyte cultures after MTT exposure. The viable cells are replete with dark crystals in the cytoplasm. Hepatocytes had been cultured in different media, as follows:* ***a,*** *DMEM/F12* ***b,*** *Hepatozyme SFM;* ***c,*** *Chee's;* ***d,*** *Chee's + 2% serum.*

### *4.2.d.1 Apoptosis in different culture media.*

The observations just described suggested the possibility that rates of hepatocyte cell death differed in the different culture media. Therefore comparative rates of apoptosis in the different media were evaluated. The results in Figure 20 show lower apoptotic indices in Chee's compared with F12/DMEM medium, most evident at the latest timepoint. Moreover, although serum-free Chee's medium initially provoked a prolonged increase in apoptotic indices compared with serum-supplemented medium, these reduced to levels comparable with serum-supplemented Chee's. Inspection at the 6 day timepoint showed a greater degree of confluence in Chee's (both serum and serum-free) compared with the F12/DMEM-cultured wells. Thus, the culture medium formulation is relevant to achieving low levels of hepatocyte apoptosis under serum-free conditions, and different rates of apoptosis between media are responsible at least in part for the differences observed in MTT values and culture longetivities.

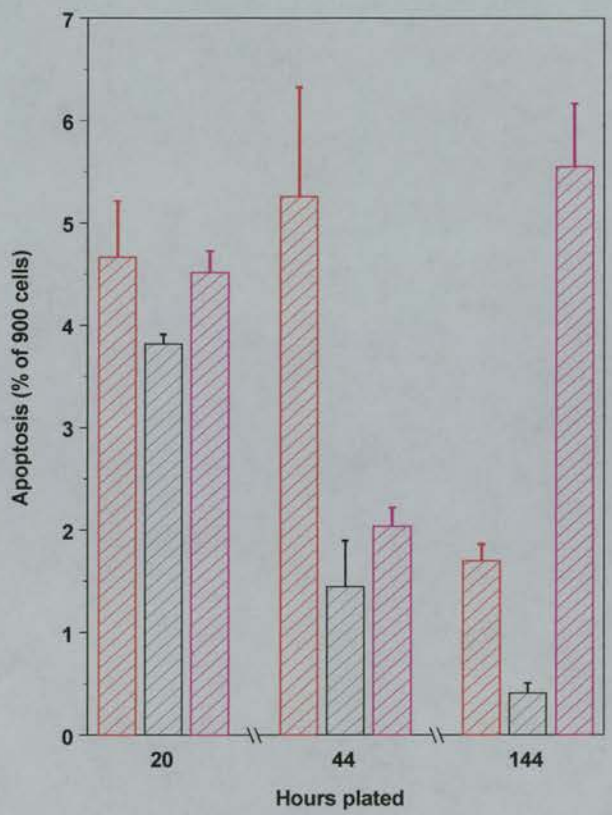



*Figure 20: apoptotic indices in different media. After plating and attachment (1hr), primary hepatocytes were cultured in either Chee's medium or DMEM/F12 for up to six days, before fixing and feulgen-staining for assessment of apoptosis, as described in methods. Both media were similarly supplemented with ITS, 100nM dex and 1% serum, and comparison was also made with serum-free Chee's medium.*
***Key**: red bars, Chee's medium (serum free), black bars, Chee's medium (1% serum), purple bars, DMEM/F12 (1% serum).*

## 4.2.e Culture medium osmolality

Normal rat plasma osmolality is approximately 290mOsol/kg, and most mammalian cell culture media are within the range 280–310mOsmol/kg. However, when formulating a new culture medium (SM1) for primary rat hepatocytes, containing high concentrations of amino acids, Schwarze found that the best preservation of cultures occurred when the medium osmolality was increased to 405mOsmol/kg [438]. This unusually high osmolality requirement was thought to be because facilitated transport of amino acids across the hepatocyte plasma membrane made the large amino acid component of the medium osmotically "transparent", necessitating an increased concentration of NaCl in the medium to compensate.

Murine serum osmolality is normally about 310mosmol/kg, significantly greater than that of humans or rats, and like medium SM1, Chee's medium contains very high concentrations of amino acids. Therefore the effect of increasing the osmolality of modified Chee's medium was tested by adding NaCl to medium of known osmolality and incubating hepatocytes for 48 hours (Figure 21). Allowing that NaCl does not ionise completely so the contribution of extra NaCl to osmolality is slightly less than the combined molar concentrations of $Na^+$ and $Cl^+$ (as assumed in the figure), and that evaporation over the 48 hours increases osmolality, the results indicate that optimal value for hepatocyte culture is about 400mOsmol/kg, with a sharp fall in culture viablility at greater osmolalities. Thus the findings of Schwarze for defined medium SM1, also apply to mouse hepatocytes cultured in Chee's medium; moreover the mouse hepatocytes are shown to tolerate a wide range of medium osmolality.

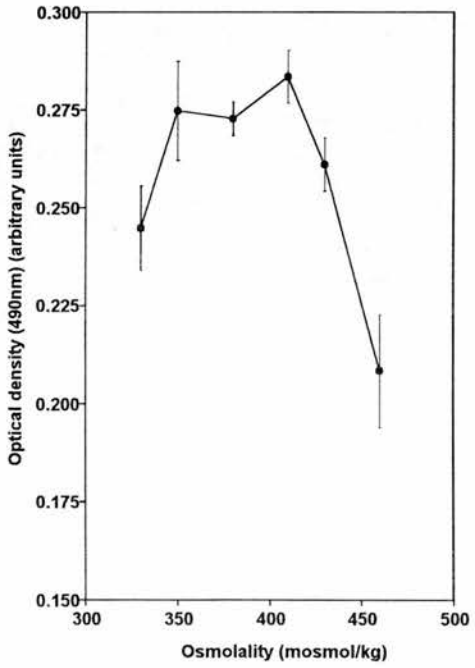



*Figure 21: Effect of medium osmolality on hepatocyte culture, assessed by MTT assay.*

### 4.2.f Elimination of non-parenchymal cells

Although the cell isolates were highly purified hepatocytes, contaminant growth of non-hepatocyte populations was initially a concern for the p53-deficient animals, given the spontaneous immortalisation of p53-null mouse embryo fibroblasts *in vitro* [454]. Therefore, Chee's medium was made hepatocyte-selective by substituting 0.4mM ornithine for arginine. Hepatocytes contain ornithine decarboxylase, which allows them to synthesize arginine de novo from ornithine, whereas arginine is an essential amino acid for the other liver cell populations [438]. Dexamethasone (25nM) was included in the medium for its beneficial effects maintaining hepatocyte phenotype, but it also suppresses fibroblastic growth. Under serum-free conditions no survival or outgrowth of fibroblastic cells was seen, whereas with even 1–2% serum rare clones of morphologically distinct, small, fibroblastic cells could be detected after 7–8 days culture of p53-null cells.

## *4.3 Summary*

A method of primary murine hepatocyte isolation and serum-free culture has been established, with attention to factors that promote culture purity and viability. Chee's medium has been reported to promote maintenance of differentiated function in cultured rat hepatocytes and for mouse hepatocytes the present modification — with increased osmolality, increased buffering capacity and ornithine substituted for arginine — is also shown to give superior culture preservation, compared with the other media formulations tested that have been recommended for hepatocyte culture. The use of serum-free conditions allows fully defined conditions of supplementation with cytokines to be tested, and prevents growth of any non-parenchymal cells.

# 5. Results.

## 5.1 Hepatocyte proliferation

### 5.1.a p53-deficient hepatocytes are more likely than wild type to proliferate in culture

Before isolation for culture from quiescent adult liver, virtually all hepatocytes are in the $G_0$ phase [43]. They are thus effectively synchronised before receiving the proliferative stimulus of isolation and primary culture. In culture, both wild type and p53-null hepatocytes showed a rise in BrdU positivity from near zero at plating to a peak at 72–96 hours in culture, but the BrdU positive fraction was consistently greater in the null cells (Figure 22) ($p<0.0001$). Thus more null hepatocytes were cycling, but with a similar kinetics of entry from $G_0$ to S phase as wild type. This finding was independent of the presence of mitogens (EGF or serum) or co-mitogen (insulin) in the culture medium (Figure 22).

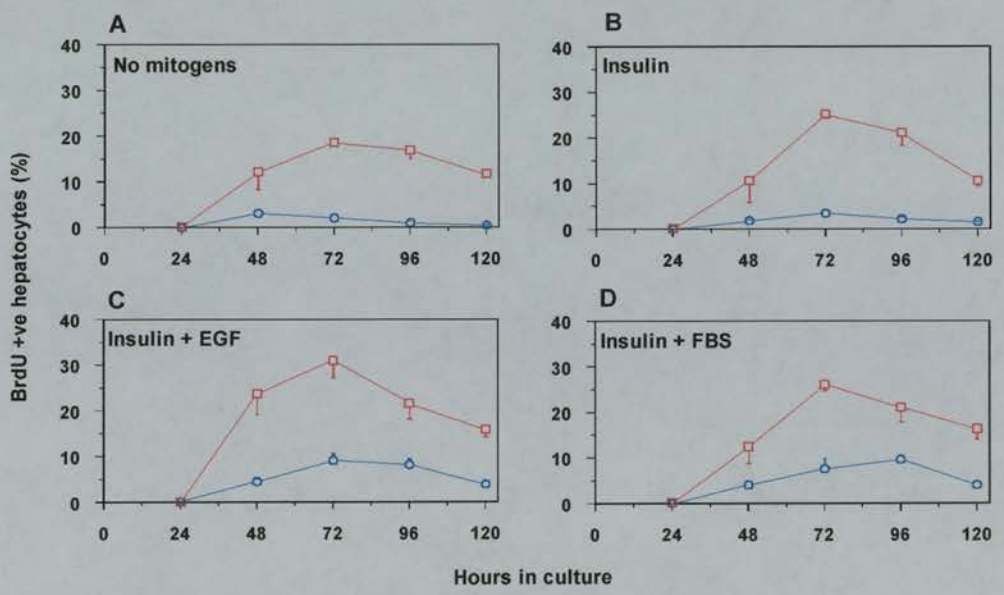



*Figure 22. Effect of p53 genotype on hepatocyte DNA synthesis in primary culture.*
*BrdU incorporation into hepatocytes was measured at the indicated times after isolation and plating. Cells were cultured in Chee's medium with 30nM dexamethasone. Four degrees of continuous mitogen supplementation were evaluated: (a) no mitogen; (b) 300nM Insulin; (c) 300nM Insulin + 25ng/ml EGF, (d) 300nM Insulin + 1% FBS. Results expressed as mean ± standard error of the mean for duplicate cultures from 3 mice of each genotype (i.e. n=6)*
***Key**: wild type ○; p53 homozygous null □.*

| Treatment | Wild type | p53-deficient |
|---|---|---|
| No mitogen | 16 (100%) | 127 (100%) |
| Insulin (300nM) | 20 (125%) | 145 (115%) |
| Insulin + 25ng/ml EGF | 55 (346%) | 199 (157%) |
| Insulin + 1% FBS | 53 (336%) | 161 (127%) |

*Table 12. Area under curve, caluculated for the proliferation curves in Figure 22 (arbitrary units).*

## 5.1.b p53-deficient hepatocytes are less sensitive to cytokine growth regulation

Mitogens (EGF, serum) in culture medium much increased the BrdU labelling indices of wild type hepatocytes (Table 12). By contrast, the already high BrdU indices of p53-null hepatocytes were less influenced by these factors (Table 12). This suggests that p53-null cells were more likely than wild type to proliferate but were relatively unresponsive to further stimulation by cytokines.

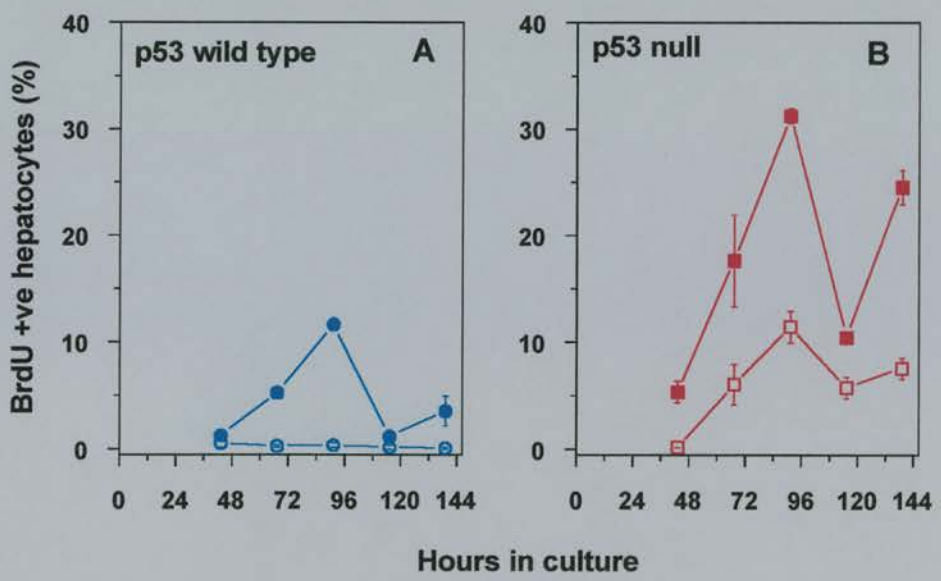



*Figure 23. Inhibition of hepatocyte DNA synthesis by TGF$\beta_1$*
*BrdU incorporation into cultured hepatocytes was measured at the indicated times after isolation and plating. Cells were cultured in Chee's medium with 100nM dexamethasone, 1.5$\mu$M insulin, 25ng/ml EGF, to which 160pM TGF$\beta_1$ was added from 20 hours. Negative controls omitted TGF$\beta_1$. Results expressed as mean $\pm$ standard error of the mean.*
*(a) wild type (b) p53 homozygous null. **Key**: open symbols, TGF$\beta_1$-treated; closed symbols, negative controls.*

| Treatment | Wild type | p53-null |
|---|---|---|
| Control | 52 (100%) | 179 (100%) |
| $TGF\beta_1$ (160pM) | 6 (12%) | 68 (38%) |

*Table 13. Area under curve, calculated for the proliferation curves in Figure 23 (arbitrary units).*

Responsiveness to cytokine inhibition of proliferation was also decreased in the p53-null hepatocytes. When incubated in high concentrations of $TGF\beta_1$ — sufficient to abrogate wild type hepatocyte proliferation — p53-null hepatocyte proliferation was only reduced to the level of untreated wild type controls (Figure 23, Table 13).

In addition to inhibition of proliferation, $TGF\beta_1$ produces apoptosis of rat and human hepatocytes; therefore the effects on apoptosis of mouse hepatocytes were tested. However, when primary wild type murine hepatocytes were cultured with $TGF\beta_1$ for up to 48 hours, there was no induction of apoptosis over 3 logs of $TGF\beta_1$ concentration (10–1000pM) (Figure 24). Therefore $TGF\beta_1$ does not readily induce mouse hepatocyte apoptosis *in vitro* — an observation also made by others [151] — and this cannot account for the differences between genotypes in the ability of $TGF\beta_1$ to inhibit proliferation.

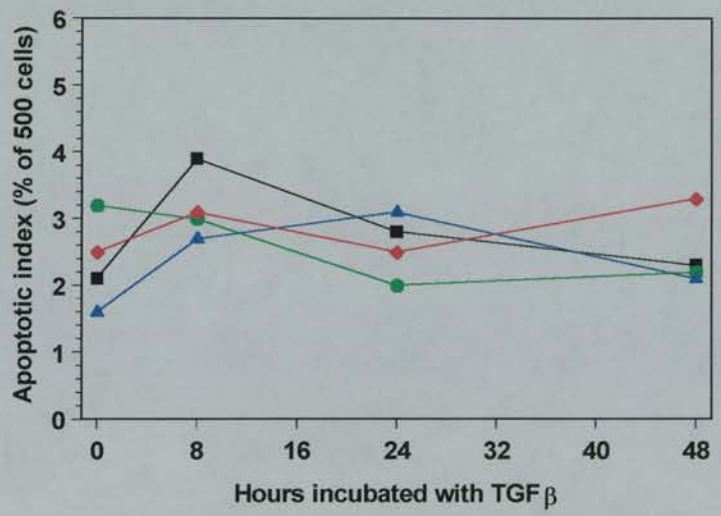



*Figure 24: Apoptosis after treatment of wild type hepatocytes with $TGF\beta_1$.*
*$TGF\beta_1$ at the indicated concentrations was added to culture medium from 24 hours after hepatocyte isolation. Apoptosis was counted on feulgen-stained cultures at the indicated time points. Cells were cultured in serum-free Chee's medium supplemented with 30nM dexamethasone, 100nM insulin. Results are the mean of duplicate observations for each point.*
***Key**: Black, negative control; Green, 10pM TGFβ; Blue, 100pM TGFβ; Red, 1000pM TGFβ.*

## 5.1.c p53-deficient livers have an enhanced proliferative response to hyperplastic and regenerative stimuli

To test whether the p53-dependent differences in proliferative activity also occur *in vivo*, liver regeneration was induced by administering a necrogenic dose of carbon tetrachloride. Necrosis was fully developed by 24 hours after drug administration and affected 35–40 % of the hepatocyte parenchyma, as determined by image morphometry. Necrosis was followed by a sharply defined wave of DNA synthesis in residual hepatocytes, as assessed by pulse labeling with BrdU, peaking 60 hours after administration of $CCl_4$. As shown in Figure 25 and Figure 26, regenerating p53-null livers had a synchronous but significantly greater peak of DNA synthetic activity when compared with wild type ($p<0.05$), in agreement with the findings in culture. Liver:body weight ratios after $CCl_4$ did not show differences between genotypes (Figure 27), however the usefulness of this measurement to assess changes in liver mass was reduced by inflammatory infiltration, oedema and the continued presence and contribution to weight assessments of the necrotic tissue in the liver.

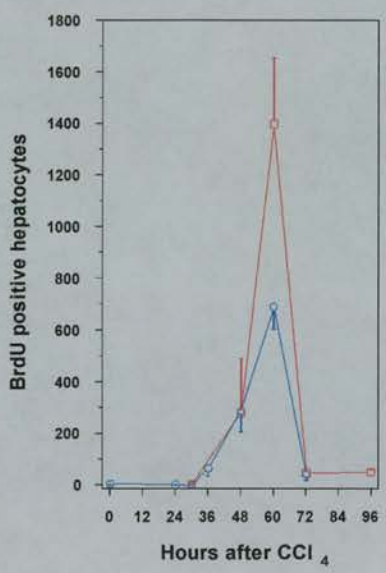
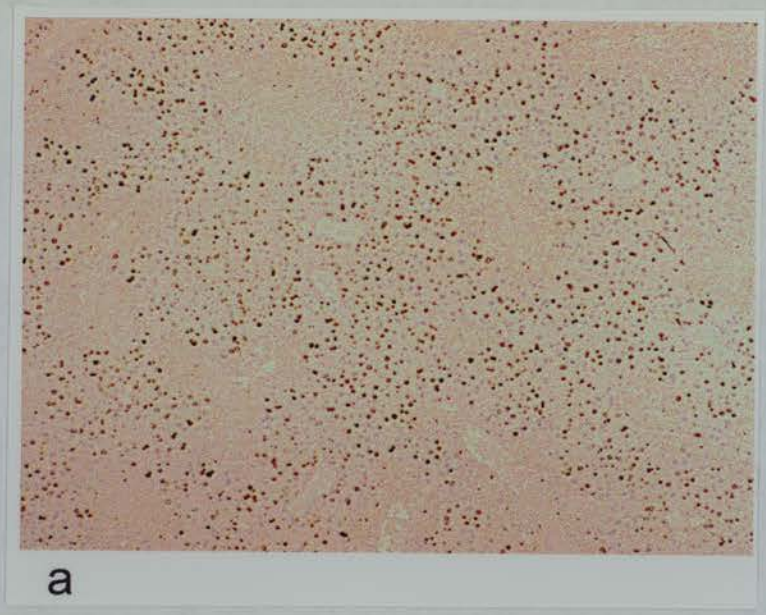



*Figure 25. Liver regeneration by wild type and p53-deficient mice after administration of $CCl_4$.*
*(a) BrdU incorporation into hepatocytes was evaluated at the indicated times after $CCl_4$ injury. Results are expressed as mean ± standard error of the mean (3–6 mice per observation).*
*Key: p53 wild type ○; p53 homozygous null □.*



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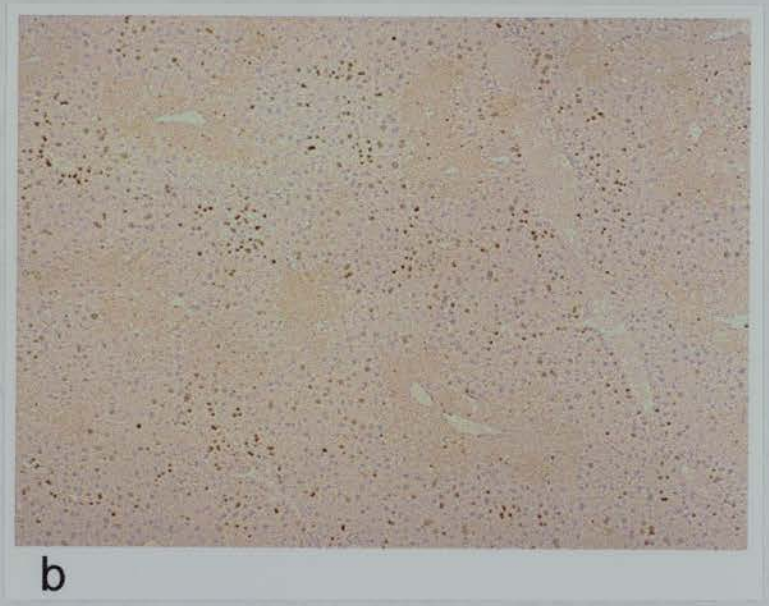

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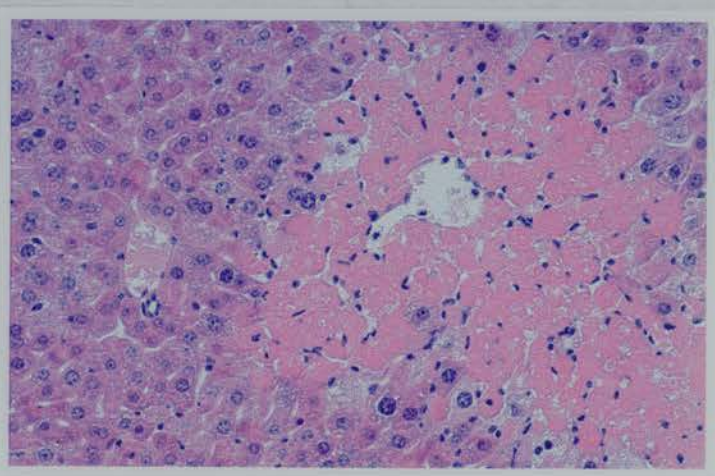


c

*Figure 26: Pulse BrdU labelling of livers, 60 hours after $CCl_4$ injury, showing increased BrdU immunopositivity (darkly stained nuclei) in p53 null liver (a), compared with wild type (b). Original magnification x100. (c) shows the pattern of hepatocyte necrosis 24 hours after administration of $CCl_4$— affecting acinar zone 3 hepatocytes (indicated to the right of picture, around the central vein)(haematoxylin & eosin stained tissue section).This is a wild type animal; p53-null mice showed no differences in the character, timing or extent of necrosis produced after $CCl_4$.*

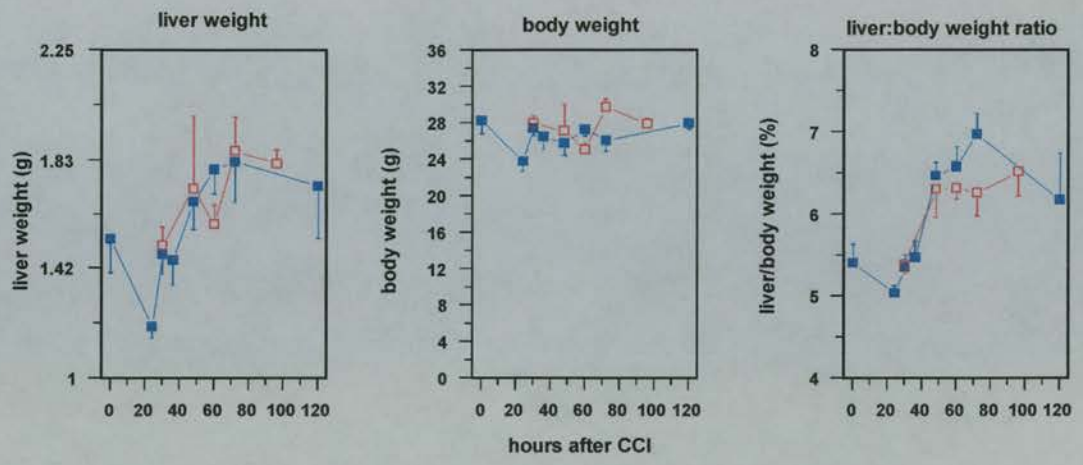



*Figure 27 Changes in liver and body weights of wild type and p53-deficient mice following $CCl_4$ treatment at time zero. Results expressed as mean ± standard error of the mean of observations on 2–9 mice per point.*
***Key:** blue, wild type; red, p53 null.*

Next the hyperplastic responses of livers to a xenobiotic mitogen were compared between wild type and p53-deficient mice. Such agents are well described as tumour promoters in experimental hepatocarcinogenesis. A single dose of a non-genotoxic mitogen, 1,4 bis 2-(3,5 dichloropyridyloxybenzene) (TCPOBOP), 3mg/kg i.p. [400], induced a surge of DNA synthesis and mitotic activity within hepatocytes by 3 days which appeared to be randomly distributed within the hepatic acini. This was accompanied by an increase in relative liver mass (liver:body weight index) which was maintained at 23 days despite the return of BrdU incorporation and mitotic indices to baseline levels (Table 14). Both the mitogen-induced surge in DNA synthesis and the ultimate increase in liver:body weight ratio were greater in p53 null livers compared with wild type, showing an amplified response of p53-deficient hepatocytes to the TCPOBOP (Table 14). No dysplasia or foci of persistent proliferation were apparent in any treated livers.

| | | 3 days after treatment | | 23 days after treatment | |
|---|---|---|---|---|---|
| | | Corn oil | TCPOBOP | Corn oil | TCPOBOP |
| BrdU (% +ve of 700 cells) | wild type | 0.32 (0.13) | 8.25 (0.13) | 0.23 (0.19) | 0.07 (0.04) |
| | p53 null | 0.57 (0.24) | 14.70 (2.91)* | 0.13 (0.06) | 0.04 (0.04) |
| Liver/body weight (%) | wild type | 5.51 (0.18) | 8.82 (0.51) | 5.52 (0.12) | 9.80 (0.43) |
| | p53 null | 5.41 (0.40) | 8.61 (0.49) | 5.65 (0.37) | 11.17 (0.70) |

* significantly greater than wild type (p=0.04, Mann Whitney test)
n=3–5 mice per observation.
Values expressed as mean (standard error of mean)

*Table 14. Effect of p53 genotype on hepatic responses to an exogenous mitogen (TCPOBOP).*

Flow cytometric analyses of nuclear ploidy showed that the proportion of diploid nuclei increased after TCPOBOP treatment, but this did not differ significantly between p53 genotypes (Figure 28). There was no aneuploidy.

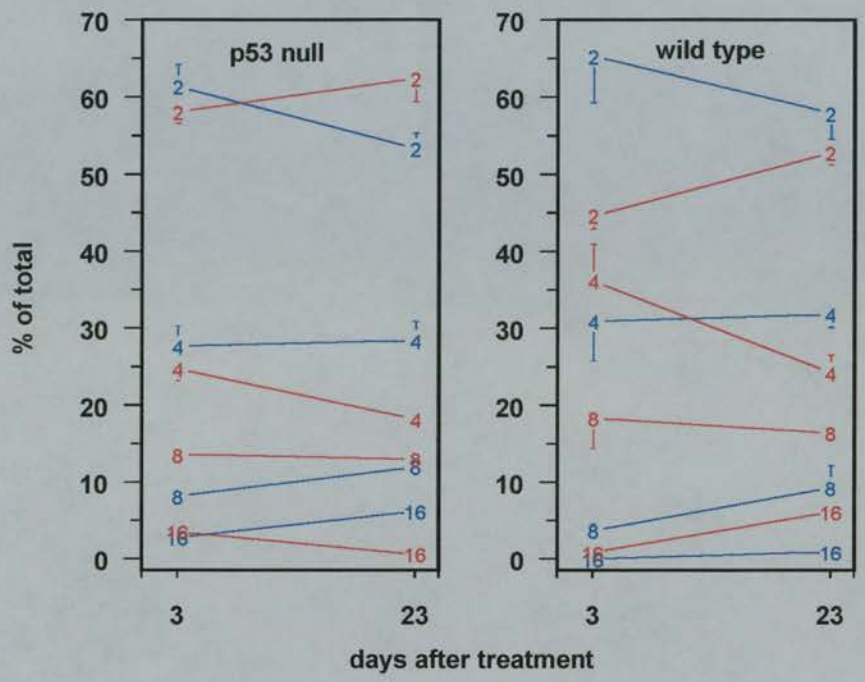



*Figure 28. Hepatocyte nuclear ploidy after single dose TCPOBOP treatment of wild type and p53 deficient mice. Results are the mean ± standard error of the mean of observations on 3-5 mice per time-point.*
***Key:** red, TCPOBOP-treated; blue, negative controls. The symbol numbers indicate the ploidy population represented (i.e. 2n, 4n, 8n,16n).*

## 5.1.d Polyploidisation in the aging liver is p53-independent

Since the results showed differences between wild type and p53-null mice in proliferative characteristics, it was of interest to examine the effect of p53-deficiency on hepatocyte nuclear ploidy. Hepatocyte nuclear DNA content was evaluated by flow cytometry in livers from one hundred and thirty mice aged between 2–137 days (42 wild type, 50 p53-heterozygous and 38 p53-null). At necropsy all livers appeared grossly normal. Twenty six mice were female. The results for males and females have been combined (Figure 29) as preliminary analysis showed no significant differences in ploidy according to gender. In neonatal mice, hepatocytes are diploid, having a 2n $G_0$ DNA content. At this stage, any 4n cells are $G_2$ proliferating hepatocytes or cycling residual extramedullary haemopoietic cells. Shortly after weaning and continuing through adult life, there is a progressive increase in the relative proportion of polyploid hepatocytes and, concomitantly, increasing degrees of polyploidisation. However, it is evident from Figure 29 that p53 genotype does not influence the timing, trend or degree of hepatocyte polyploidisation under these basal conditions. Aneuploidy was not detected in any sample.

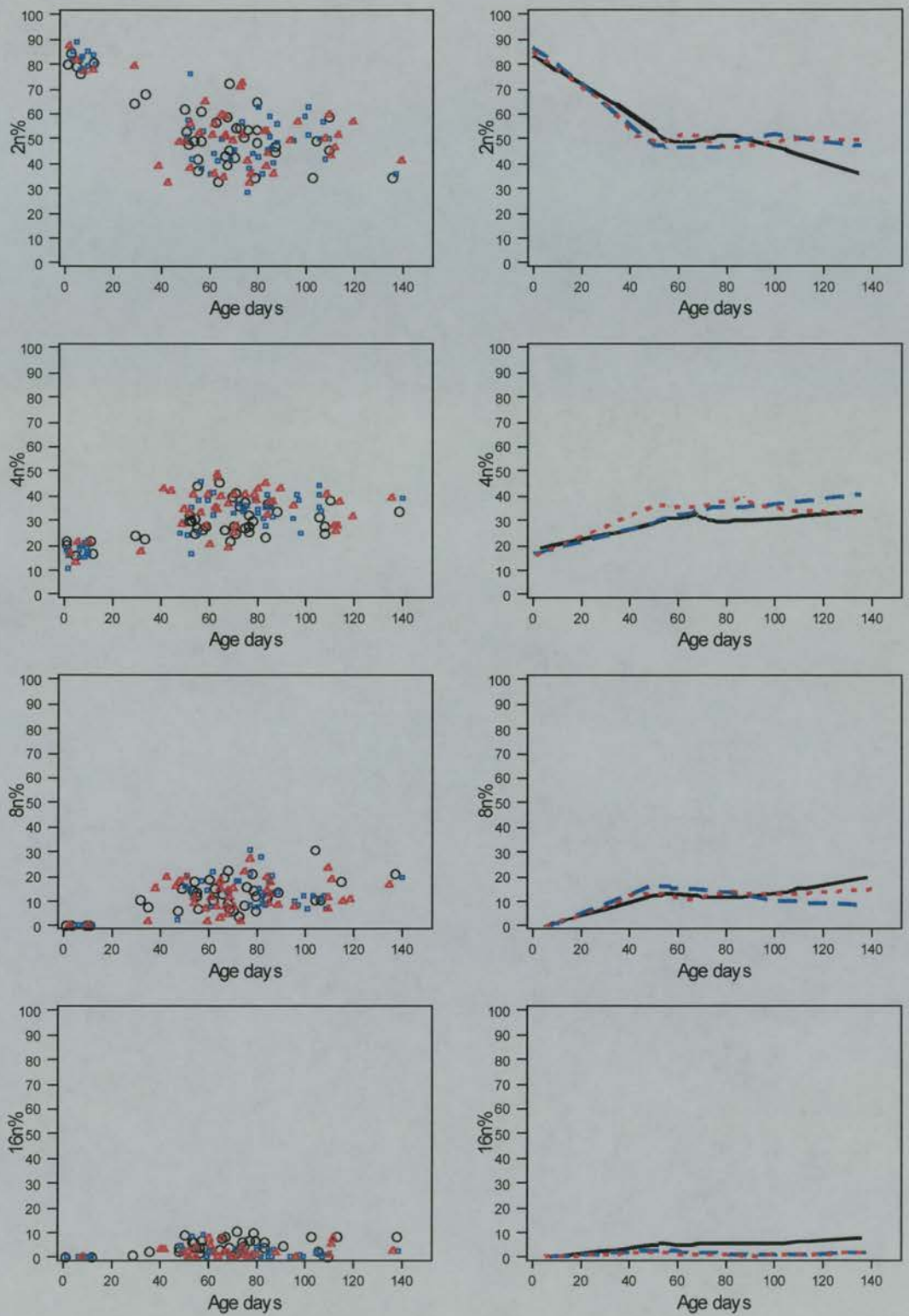



*Figure 29. Effects of p53 genotype on postnatal hepatocyte polyploidisation.*
*The relative proportions of diploid (2n%), tetraploid (4n%), octaploid (8n%) and hexadecaploid (16n) nuclei at different ages (range 2-137 days) are depicted as scatterplots (left column) and as Lowess plots (right column).*
***Key**: - - - -, Δ wild type; – – –, □ p53-heterozygous; ——, ○ p53-homozygous-null.*

## 5.2 Hepatocyte apoptosis

### 5.2.a p53 regulates hepatocyte dependence on survival factors

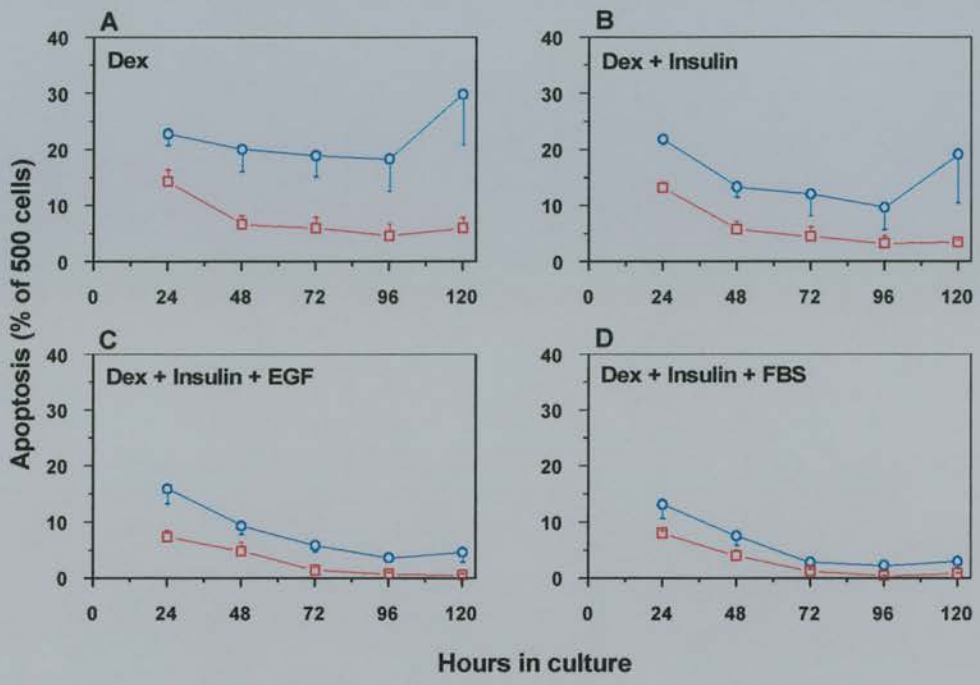



*Figure 30: Hepatocyte apoptosis in culture, assessed at the indicated times after plating. Cells were cultured in Chee's medium with 30nM dexamethasone. Four degrees of supplementation were evaluated: (a) none; (b) 300nM Insulin; (c) 300nM Insulin + 25ng/ml EGF, (d) 300nM Insulin + 1% FBS. Results are the mean ± standard error of the mean for duplicate cultures from 3 mice of each genotype (i.e. n=6).*
***Key**: p53 wild type ○; p53 homozygous null □.*

| Treatment | Wild type | p53-null |
|---|---|---|
| Dex | 195 (100%) | 64 (100%) |
| Dex + insulin | 129 (66%) | 49 (77%) |
| Dex + insulin + EGF | 69 (35%) | 25 (39%) |
| Dex + insulin + FBS | 49 (25%) | 24 (38%) |

*Table 15. Area under curve, calculated for the apoptosis curves in Figure 30 (arbitrary units).*

Insulin, or insulin with EGF or serum, reduced apoptosis of wild type hepatocytes in culture ($p<0.01$). These factors are therefore survival factors for mouse hepatocytes. When apoptosis of wild type and p53-deficient hepatocytes in culture was compared, p53-deficient hepatocytes had lower apoptotic indices than wild type, under a variety of culture conditions (Figure 30a–d). The lower apoptotic indices were apparent before BrdU uptake began, so were not directly related to either DNA synthesis or cell

proliferation. The survival benefit conferred by p53 deficiency was greatest when trophic support in the culture medium was least (compare Figure 30a,b with c,d). p53 deficiency therefore reduces dependency on survival factors, but confers little survival advantage when these factors are not limiting and wild type apoptotic rates are low anyway.

## 5.3 Hepatocyte responses to DNA damage

p53 function is important in many other cell types for normal responses to DNA damage. To evaluate the role of p53 in hepatocyte responses to DNA damage, two genotoxic stimuli were used: γ-irradiation (which causes DNA strand breaks and base damage) and uv-c irradiation (254nm; which predominantly produces photodimers) [455].

### 5.3.a p53 in DNA-damaged hepatocytes

p53 protein responses to the genotoxic injuries were evaluated in 2 ways. First, by immunocytochemistry for p53 protein; second, by a transfected p53 reporter plasmid in cultured hepatocytes — to assess transcription activation by p53.

#### *5.3.a.1 uv-c injury (10J/m$^2$) produces p53 protein and transactivation responses*

uv-c irradiation (10J/m$^2$) of hepatocyte cultures produced nuclear p53 protein accumulation in most hepatocytes, as assessed by immunocytochemistry, and the response peaked at around 24 hours after irradiation (Figure 31, Figure 32).
p53 immunopositivity of unirradiated, control cultures slowly increased with time in culture. This may reflect a "stress response" to the culture environment and the acquisition of undefined cellular injuries.

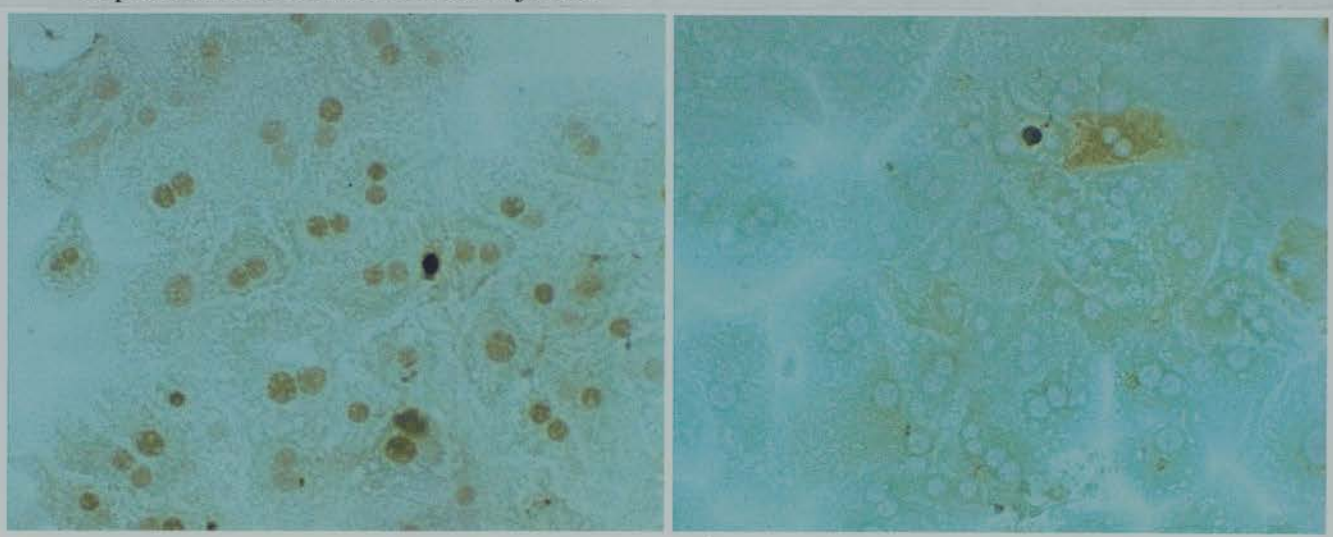



*Figure 31: (a) immunocytochemistry for p53 protein, performed on hepatocyte monolayers fixed 24 hours after 10J/m$^2$ uv irradiation. There is strong nuclear immunopositivity (brown staining) in most of the irradiated hepatocytes. The primary antibody used was pAB421 (see methods section 3.2.f.1). (b) is the negative control. Cells were cultured in Chee's medium with dexamethasone 30nM, insulin 300nM.*

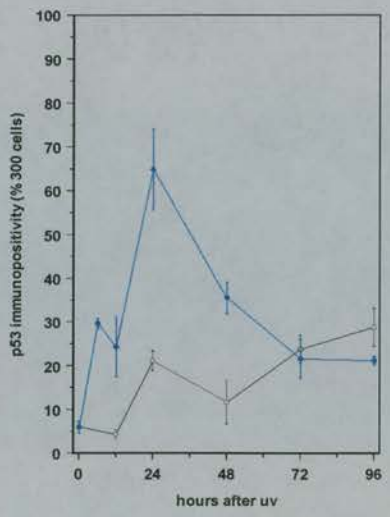



*Figure 32. p53 immunopositivity (pAb 421) of hepatocyte cultures after uv-c injury (10J/m$^2$). Cells were cultured for 24 hours before irradiation. Results are the mean ± standard error of the mean for duplicate cultures from 2 mice.* ***Key****: ○ open symbols unirradiated, ● solid symbols irradiated.*

**Transfection experiments**. Plasmids were introduced into primary hepatocytes by lipofection using cationic liposomes. Optimum transfection efficiency was determined by standard means of varying the mass of DNA transfected, the lipid:DNA ratio, and the duration of transfection. There was no evidence of toxicity with the protocol used and transfection efficiency was consistently between 15 and 20% of cells — as determined on cultures transfected with the constitutive *lacZ*-expression plasmid pCMVβ, by estimating the proportion of stained, i.e. *LacZ*-expressing, hepatocytes after incubation with 1mg/ml X-Gal, 1mM $MgCl_2$, 10mM $Fe_3(CN)_6$, 10mM $Fe_4(CN)_6$ in phosphate buffered saline (Figure 12). The Xgal assay is relatively insensitive and therefore the transfection efficiency indicated by this method is a minimum figure. The assay did serve however to demonstrate the repeatability of the transfection method.

The optimised lipofection protocol differed somewhat from those suggested for primary rat hepatocytes by previous authors [406,407,456], although overall transfection efficiencies (15-20%) were a little better (eg 5-10% for Ponder et al, 10% for Jarnegin). In particular, whilst optimum lipid:DNA ratios (6:1) and transfection times (6 hours) were comparable, the optimal amount of DNA (1μg/1.9cm$^2$ plate) utilised in each transfection was considerably greater, and the reason for this is not clear. Watanabe has reported that with mouse primary hepatocytes very high (circa 15:1) Lipofectin:DNA ratios and extended lipid/DNA preincubation times yielded greater transfection efficiencies (upto 45%) [405], however this was not the case in the present system.


*Figure 33. β-galactosidase activity in primary hepatocytes transfected with pCMVβ plasmid that constitutively expresses* LacZ. *n=4*

Firstly, the kinetics of plasmid expression after transfection were examined in hepatocyte cultures transfected with the constitutively-expressing pCMVβ plasmid. In these cultures βgalactosidase activity was detectable by the ONPG colorimetric assay within 12 hours, and showed relatively stable levels from 24–48 hours, slightly decreasing by 72 hours (Figure 33). Therefore, subsequent experiments on transfected cells were performed between 24 and 48 hours after transfection.

Wild type hepatocytes transfected with the **p53-reporter plasmid** (pRGCΔFosLacZ) or its control plasmid (pΔFosLacZ) showed no significant β-galactosidase activity, equivalent to untransfected controls (Figure 34). However after treatment with **10J/m$^2$ uv-c** there was specific induction of β-galactosidase expression from the p53 reporter plasmid, peaking at about 14 hours after irradiation and subsequently decreasing to control levels by 24 hours (Figure 34a). Activity of the negative control plasmid remained at baseline (Figure 34b). Parallel experiments on pCMV-transfected hepatocytes showed that the same dose of uv-c irradiation produced a 37% decrease in the (constitutively expressed) β-galactosidase activity (Figure 34b). This decrease was probably due to non-specific transcriptional suppression by uv, a well documented effect [457] that makes the observation of increased p53-reporter gene expression after uv injury even more significant.

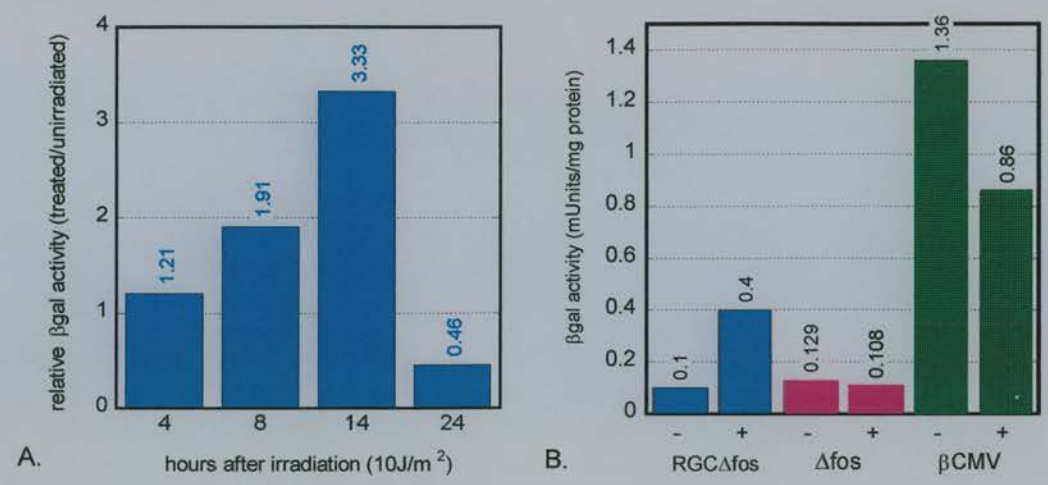



*Figure 34. p53 reporter plasmid β-galactosidase expression following uv-c treatment of transfected primary hepatocytes.*
*(a) p53 reporter activity in hepatocytes following 10J/m² uv-c. β-galactosidase activity is expressed relative to untreated transfected hepatocytes. The activity at 14 hours is significantly greater than control values (p=0.04).*
*(b) Effect of uv-c treatment on β-galactosidase expression from the p53 reporter plasmid (RGCΔFosLacZ), the negative control plasmid (ΔFosLacZ) and the constitutive pCMVβ plasmid. Results are shown 14 hours after uv-c (indicated by +), untreated controls are indicated by -. Activity of the treated RGCΔFosLacZ reporter is significantly greater than the control (p=0.04); the treated pCMVβ plasmid is significantly lower than control (p=0.02)*
*(a) and (b) are result of independent experiments, each involving duplicate observations at each time point.*

### *5.3.a.2 No p53 response to γ-irradiation injury (15Gy)*

By contrast to uv injury, γ-irradiation produced no significant change in expression from any plasmid, with the p53-reporter and its control remaining at basal levels from 2 to 24 hours after irradiation (Figure 35a), and constitutive pCMVβ expression equivalent to unirradiated control transfectants (Figure 35b). Furthermore, p53 protein did not accumulate to levels detectable by CM5 or pAb421 immunocytochemistry in wild type hepatocytes irradiated either in vivo, in accord with previous *in vivo* observations by Midgely et al on 5Gy-irradiated normal mouse livers [297], or in proliferating primary cultures (Figure 36). Thus there is no evidence for a change in p53 protein level or transactivation activity in cultured hepatocytes after γ-irradiation.

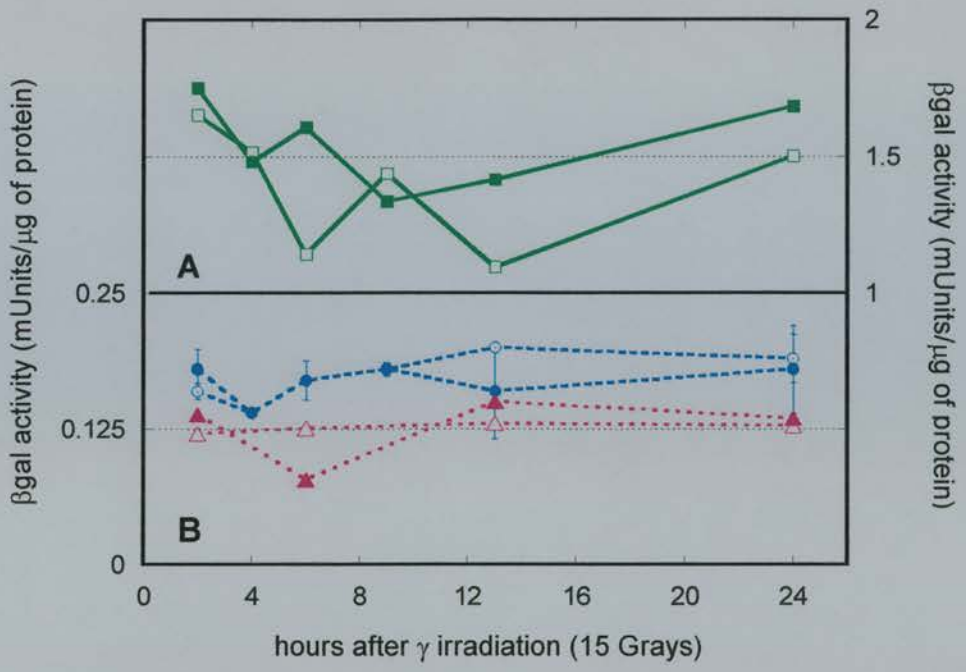



*Figure 35. β-galactosidase activity after 15Gy γ-irradiation.*
*(a) □■ constitutive control plasmid (pCMVβ), (b) ○● p53 reporter plasmid (RGCΔFosLacZ), △▲ negative control plasmid (ΔFosLacZ).Open symbols unirradiated, solid symbols irradiated.*

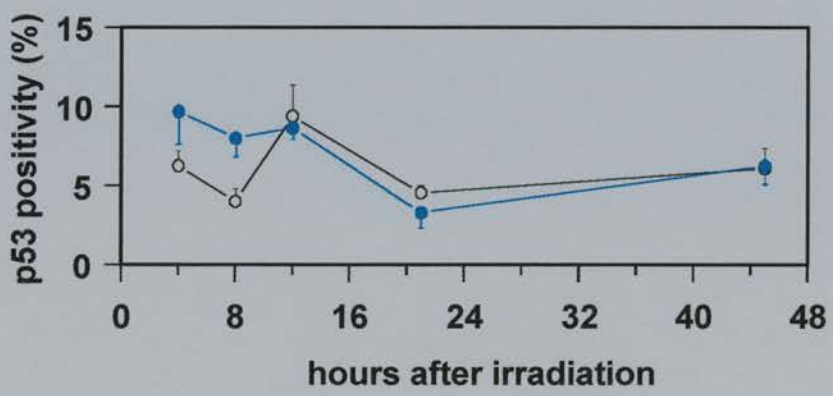



*Figure 36. p53 immunopositivity (pAb 421) of cultured hepatocytes after γ-irradiation (15Gy). Cells were cultured for 71 hours before irradiation.(n=4)*
***Key**: ○ open symbols unirradiated, ● solid symbols irradiated.*

## 5.3.b

## 5.3.c Apoptosis in DNA-damaged hepatocytes

### *5.3.c.1 uv-c produces p53-independent apoptosis*

uv-c irradiation of both wild type and p53-deficient hepatocyte cultures produced delayed, dose-dependent apoptosis, appearing from about 48 hours after irradiation (Figure 37). However there was no significant difference between genotypes in the apoptotic responses to uv-c.

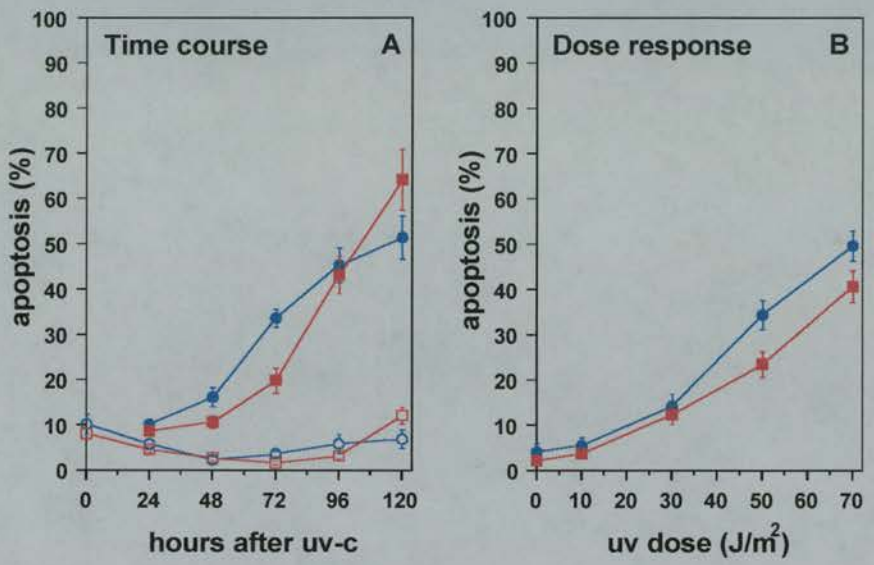



*Figure 37.Apoptosis in primary hepatocyte cultures after uv-c irradiation.*
*(a) Time course of apoptosis after 50J/m² uv-c.*
*(b) Dose response of apoptosis at 72 hours after irradiation.*
*Cells were cultured in Chee's medium with 100nM dexamethasone, 1.5μM insulin, 2% serum. Results are the mean with standard error for duplicate cultures from 3 mice of each genotype (i.e. n=6).*
***Key**: p53 wild type unirradiated ○; irradiated ●; p53 homozygous null unirradiated □; irradiated ■*

### *5.3.c.2 Survival factors in culture medium confer resistance to uv-induced apoptosis*

The apoptosis induced by uv-irradiation could be inhibited by supplementation of medium with survival factors (dexamethasone, insulin, serum). With high levels of supplementation hepatocytes became moderately resistant to uv cytotoxicity, requiring uv fluxes above 15–20 J/m² to show significant induction of apoptosis (Figure 38). The effects of survival factors on uv-induced apoptosis were next compared between p53 genotypes. A dose-response experiment comparison 48 hours after uv injury suggested that p53-deficient cells were less sensitive than wild type to removal of survival factors from culture medium (Figure 39). However subsequent time course experiments showed

no significant difference between genotypes in the sensitivities to uv irradiation under different levels of medium supplementation (Figure 40).

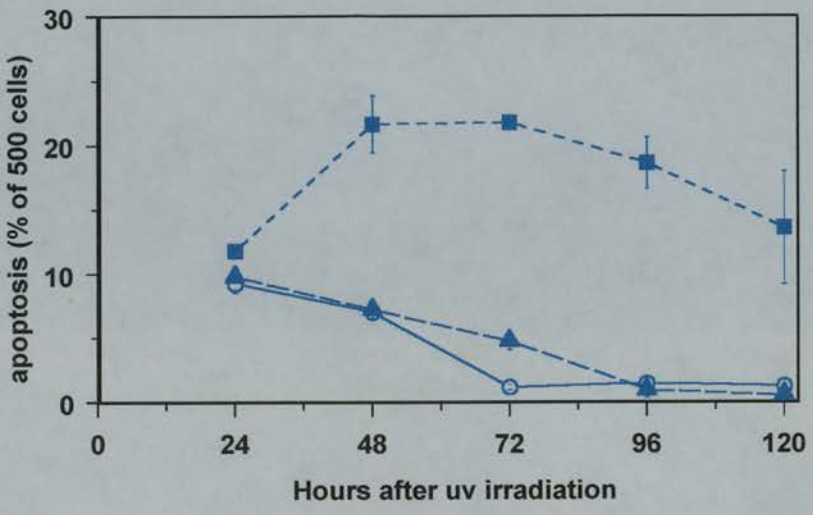



*Figure 38. Apoptosis after uv-c irradiation under high levels of medium supplementation. Wild type hepatocytes were cultured in Chee's medium, supplemented with 1μM dexamethasone, 1.5μM insulin, 25ng/ml EGF and 2% serum. Results are the mean with standard error of duplicate observations.*
***Key**: ● unirradiated; ▲ uv-irradiated (15J/m²); ■ uv-irradiated (50J/m²).*

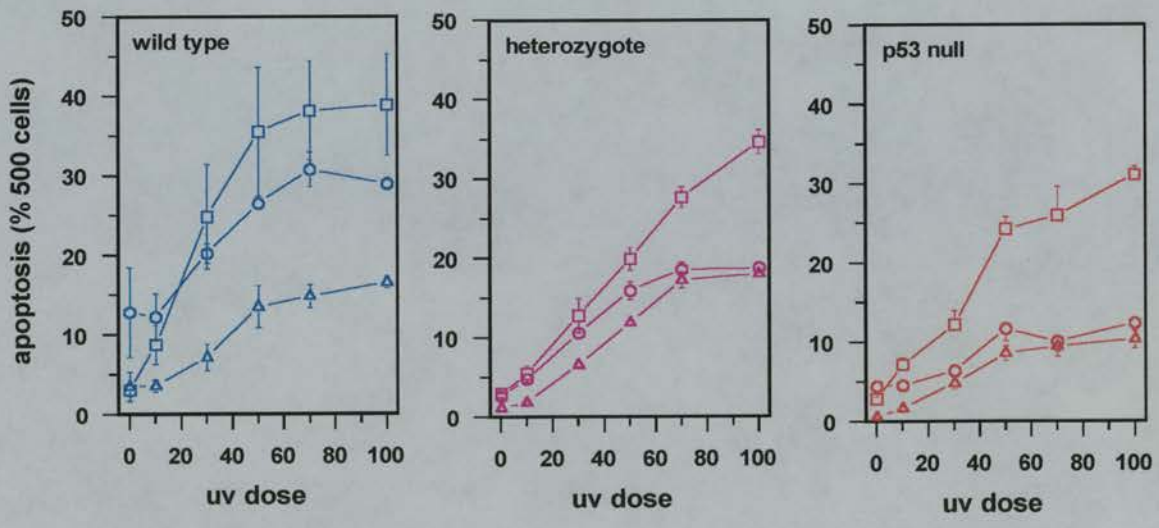



*Figure 39. Effect of p53 genotype on the reduction by survival factors of uv-c-induced apoptosis: dose-response of apoptosis at 48 hours after irradiation. Cultures were either unsupplemented □; supplemented with 30nM dexamethasone and 100nM insulin ○; or with 500nM dexamethasone, 1.5μM insulin and 2% serum Δ. Results shown as mean (± standard error) of duplicate to quadruplicate cultures from each of 3 wild type (n=7), 1 p53 heterozygous (n=4), and 2 p53-deficient mice (n=5).*

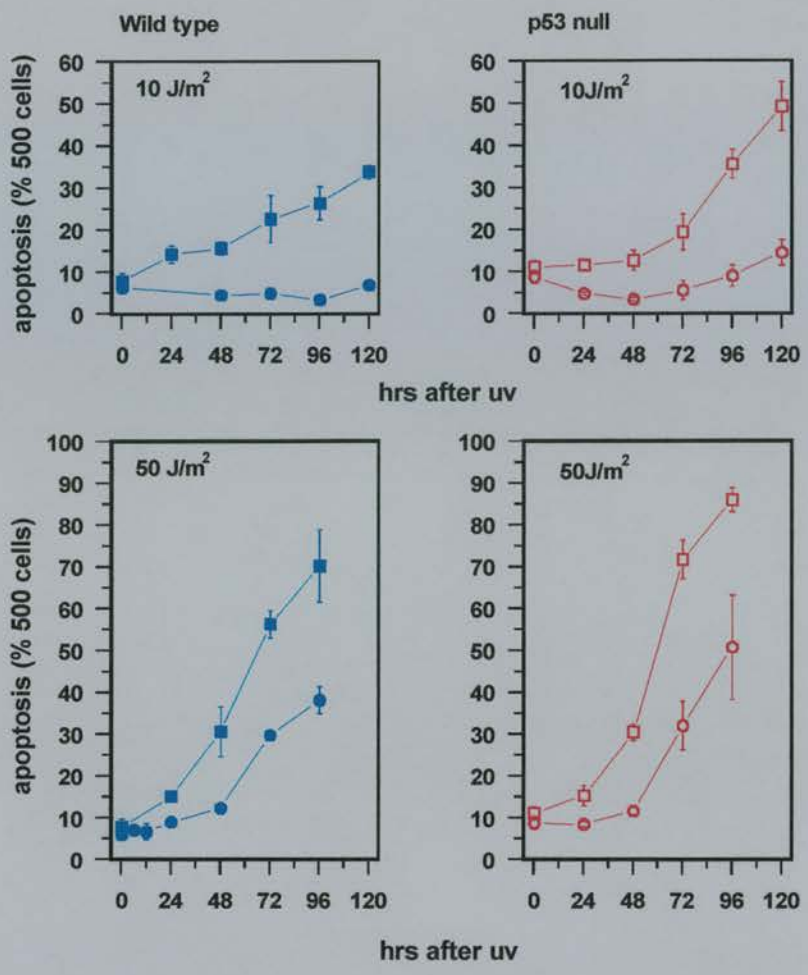



*Figure 40. Effect of p53 genotype on the reduction of uv-c-induced apoptosis by survival factors: time-course of apoptosis after 10 or 50J/m² uv-c. Cultures were either unsupplemented* □; or *supplemented with 500nM dexamethasone, 1.5μM insulin, 2% serum* ○. *Results are mean (± standard error) for duplicate cultures from each of 2 wild type and 2 p53-deficient mice (i.e. n=4).*

### *5.3.c.3 γ-irradiation does not trigger hepatocyte apoptosis*

Gamma irradiation (5Gy) readily induces p53-dependent apoptosis in cells such as thymocytes and intestinal crypt epithelium [291,458], but doses up to 15Gy did not induce hepatocyte apoptosis *in vivo* — in neither quiescent nor regenerating liver (Figure 41c,e,g,h), nor in proliferating primary cultures (Figure 41a). By contrast, apoptosis appeared in irradiated hepatic sinusoid lining cells (endothelial/Kupffer cells), demonstrating adequate dose delivery (Figure 41e).

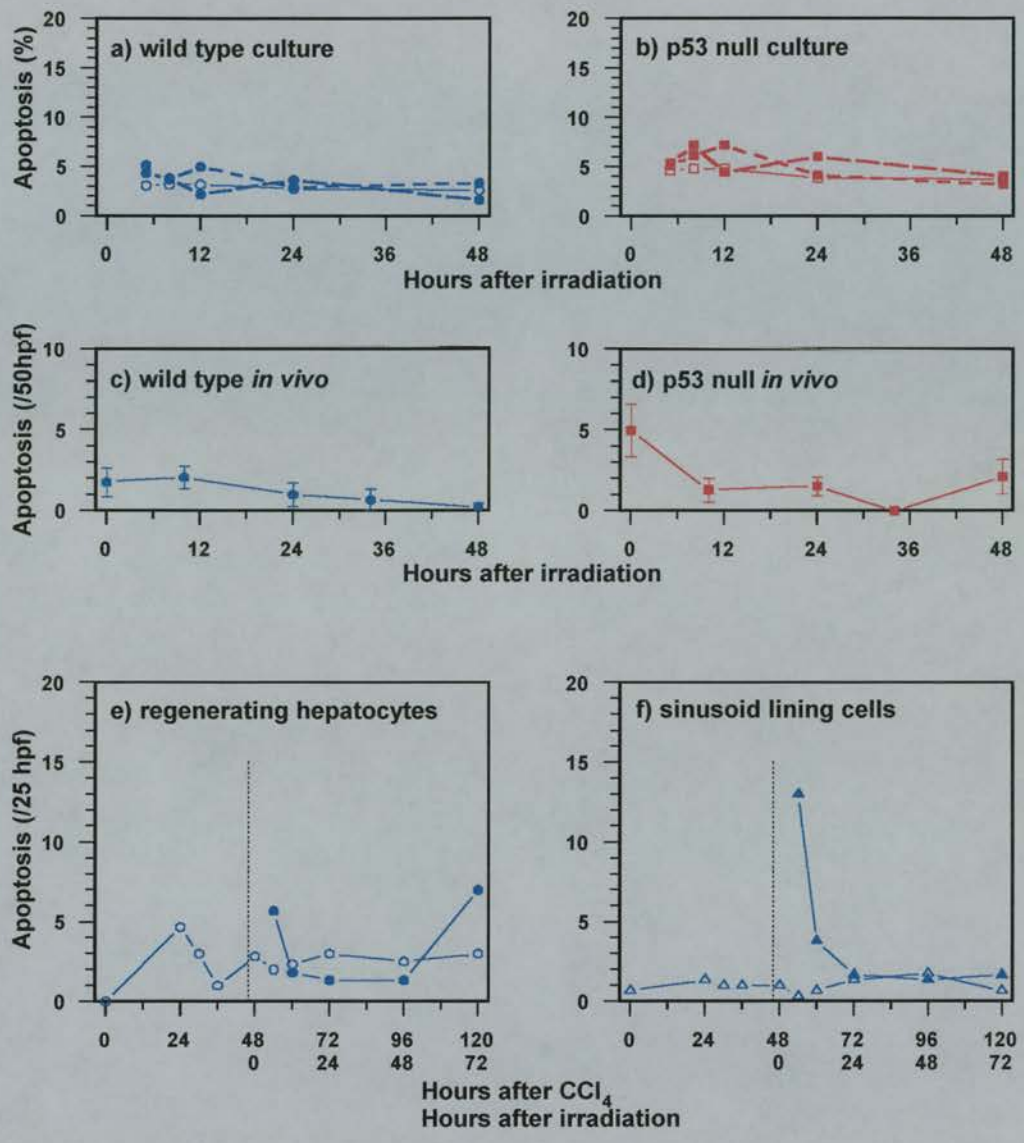
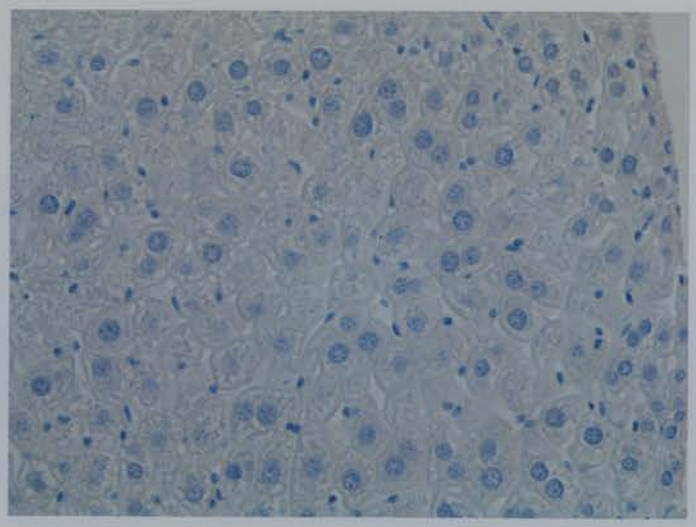



*Figure 41. Effect of γ-irradiation on hepatocyte apoptosis.*
*(a,b) Cultured hepatocytes after 0 ——, 5 - - - or 15 – – – Gy γ-irradiation.*
*(c,d) Hepatocyte apoptosis in vivo after whole body γ-irradiation (15Gy)*
*(e) Hepatocyte and (f) Hepatic sinusoid lining cell apoptosis in vivo, in regenerating wild type mouse liver. Mice were given $CCl_4$ at time zero, as described in Methods, and γ-irradiated or mock-irradiated 48 hours later (timepoint indicated by vertical dashed line).*
*Each point represents the mean (± standard error) of observations on 3-6 mice.*
*Key: open symbols, unirradiated controls; closed symbols, irradiated. Wild type, blue; p53-deficient, red.*



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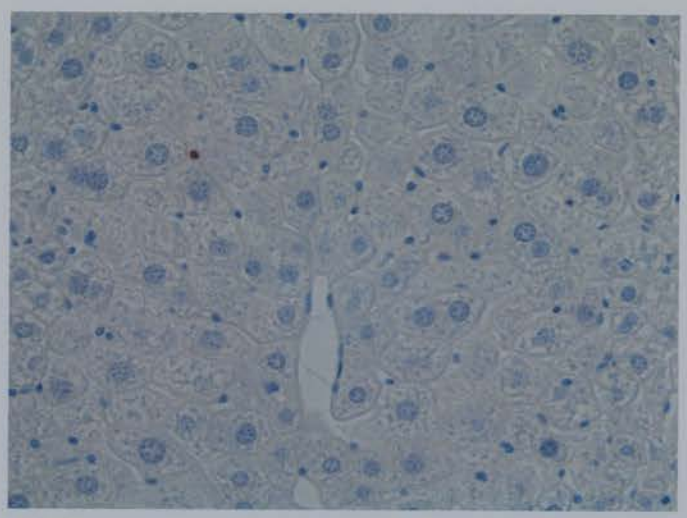


h

*Fig 41 continued. Failure of 15Gy γ-irradiation to produce accumulation of immunoreactive p53 in wild type mouse hepatocytes .*
*Mice were γ-irradiated as described in Materials and Methods. (g) shows immunohistochemistry for p53 on γ-irradiated liver, 6 hours after irradiation; (h) shows unirradiated control liver at the same timepoint.*

## 5.3.d Cell cycle activity of hepatocytes after DNA-damage

### *5.3.d.1 uv-c injury produces p53-dependent growth arrest*

Proliferating hepatocyte cultures were uv-c irradiated 60 hours after plating (shortly before the normal peak of entry to replicative DNA synthesis (see Figure 22)), and the effects of uv treatment on replicative DNA synthesis were evaluated by BrdU immunocytochemistry following serial 6 hour incubations in BrdU-containing medium. The results showed a significant decrease in BrdU uptake in wild type hepatocytes after uv-treatment that did not occur in uv-treated p53-deficient cultures (Figure 42).

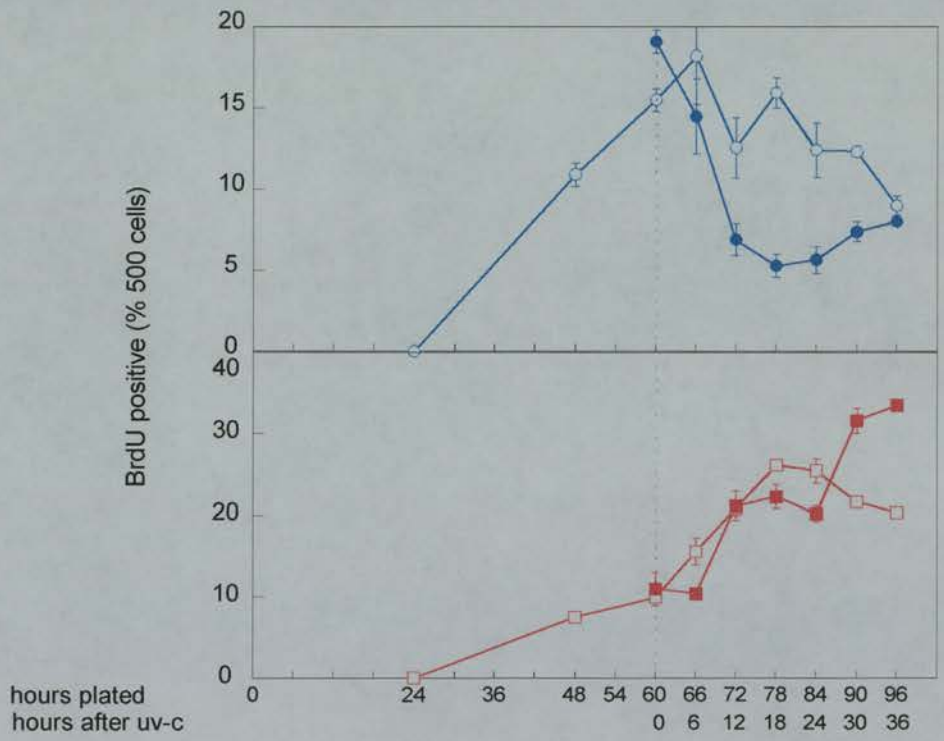



*Figure 42. Effect of uv-c treatment (10J/m²) on BrdU incorporation by cultured hepatocytes.*
*Cells were uv-irradiated 60 hours after plating, and the proportion of cells incorporating BrdU (6 hour pulse) at serial intervals was assessed using immunocytochemistry.*
*Results are the mean with standard error for duplicate cultures.*
***Key**: wild type unirradiated ○; irradiated ●; p53-null unirradiated □; irradiated ■.*

Thus, hepatocytes show a uv-induced, p53-dependent $G_1$/S phase cell cycle arrest, confirming a previous report on rat hepatocytes using antisense p53 [301], and further demonstrating that p53 in hepatocytes mediates growth arrest but not apoptosis after DNA damage.

### *5.3.d.2 γ-irradiation in vivo produces p53-dependent growth arrest, and aberrant mitoses in p53-deficient livers*

Since γ-irradiation induces a p53-dependent growth arrest in other cell types [459,460], and is described to inhibit liver regeneration [461], the effects of irradiation on regenerating wild type and p53-deficient livers were compared. Livers were γ-irradiated (15Gy) 48 hours after administration of $CCl_4$, when most hepatocytes were in late $G_1$, just before entry to S phase (Figure 43a,b). It is evident from Figure 43a and c that γ-irradiation of wild type mice suppressed both hepatocyte entry to S phase (Figure 43a) and the subsequent passage through mitosis at 72 hours after $CCl_4$ (Figure 43c). These results are in keeping with an efficient $G_1$/S cycle arrest, from which release is not seen in the course of this experiment. By contrast, γ-irradiation of p53-null livers reduced by less and did not delay the subsequent S phase peak of BrdU positivity, indicating a much reduced $G_1$/S arrest (Figure 43b). The reduced peak and prolonged fall in BrdU positivity shown by the irradiated p53-null livers may be due to slowed progression through S phase — well described after γ-irradiation [462]. This was followed by a striking rise in the mitotic index 24 hours after irradiation (mitotic counts of 103, 77 and 205, compared with 15, 13 and 8 in unirradiated mice; Figure 43d). Clearly, as well as defective $G_1$/S arrest, there was no significant $G_2$ arrest. Many of the mitotic figures at this time were abnormal, with aberrant forms, isolated chromosomes and chromosomal bridges (Figure 44).

It is therefore likely that this mitotic peak was due to a population of extensively damaged hepatocytes proceeding to and arresting within mitosis. Even a slight increase in the normally brief duration of mitosis (30–45 minutes) would cause the observed mitotic index to rise considerably, as cells accumulated in this phase. The excess in mitotic figures was not evident at later timepoints, suggesting that the cells had undergone either catastrophe and elimination, or delayed and possibly aberrant completion of mitosis. $G_2$ arrest by wild type irradiated livers could not be demonstrated in the present study because release from the $G_1$/S arrest was not observed. However, $G_2$ arrest by regenerating rat liver after γ-irradiation has been reported [463,464]. Therefore, these experiments suggest that in liver both $G_1$ and $G_2$ checkpoint arrests after γ-irradiation are p53-dependent

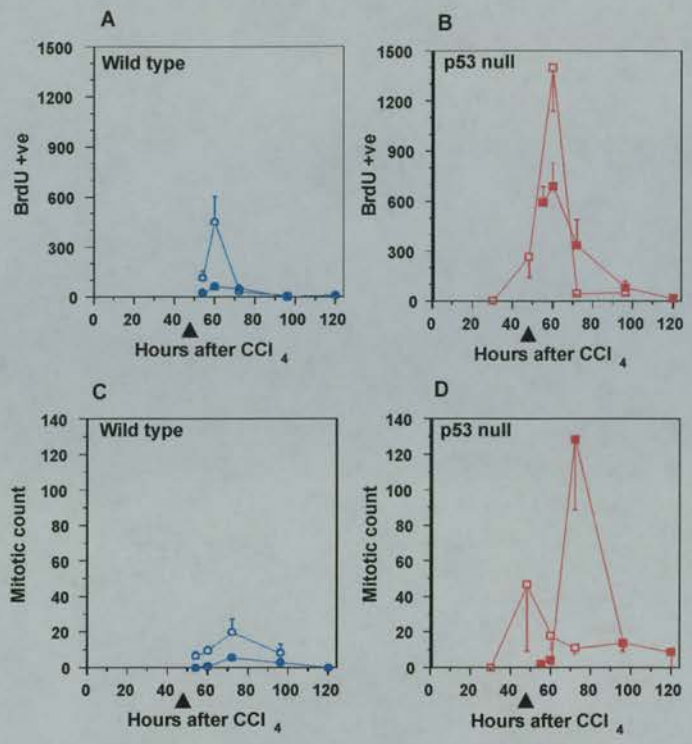



*Figure 43. Effects of 15Gy γ-irradiation on DNA synthesis (a,b) and mitotic activity (c,d) by regenerating wild type and p53 null livers. Mice given $CCl_4$ at time zero as described in Methods, were γ-irradiated or mock-irradiated 48 hours later (arrowheads). Mitotic activity and BrdU incorporation into hepatocytes was measured at the indicated times after $CCl_4$ injury. Results are expressed as mean ± standard error of the mean (3-5 mice per observation). The large error bar for the mitotic count of unirradiated null mice at 48 hours is due to a single exceptional animal that is unexplained. Two other animals at this timepoint had mitotic counts of 10 or below.*
***Key**: wild type unirradiated ○; irradiated ●; p53 null unirradiated □; irradiated ■.*

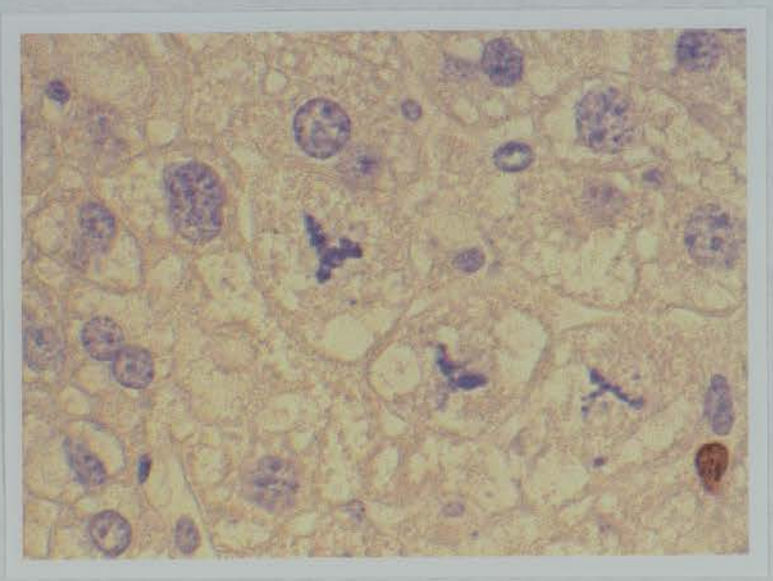


*Figure 44. γ-irradiated regenerating p53-deficient mouse livers, 72 hours after $CCl_4$ (24 hours after 15Gy γ-irradiation). Note the abnormal mitotic figures. Haematoxylin counterstain. Original magnification 100x.*

## 5.4 Effects of p53-deficiency on responses to a liver carcinogen, diethylnitrosamine

The data presented so far suggested that hepatocytes without functional p53 were defective in normal responses to DNA damage, and this increased the susceptibility to develop genetic abnormality after exposure to a DNA damaging agent. Therefore it was decided to test whether p53-deficiency might affect the consequences of exposure to a single dose of a genotoxic liver carcinogen (diethylnitrosamine) *in vivo*.

The results of histological analyses of livers taken from mice 10 weeks after exposure to a dose of diethylnitrosamine (10 or 50mg/kg) are shown in Table 16 and summarised graphically in Figure 46. No mouse developed hepatocellular carcinoma by 10 weeks after carcinogen treatment, however, both morphologically-altered foci and diffuse cytological atypia were observed (Figure 45). Untreated mice did not develop altered foci, and rarely had diffuse atypia. By contrast, after DEN treatment, p53-null livers were more likely than wild type to develop diffuse atypia; p53-heterozygous mice had an intermediate likelihood of showing diffuse atypia, suggesting gene-dose-dependent susceptibility. This trend was demonstrated at both doses of DEN (significant difference between genotypes at 10mg/kg DEN, $p<0.001$, Chi Square). Moreover, after 10mg/kg DEN, p53-null mice were also more likely to have altered foci than heterozygous or wild type mice — although the numbers are too small for meaningful statistical analysis.

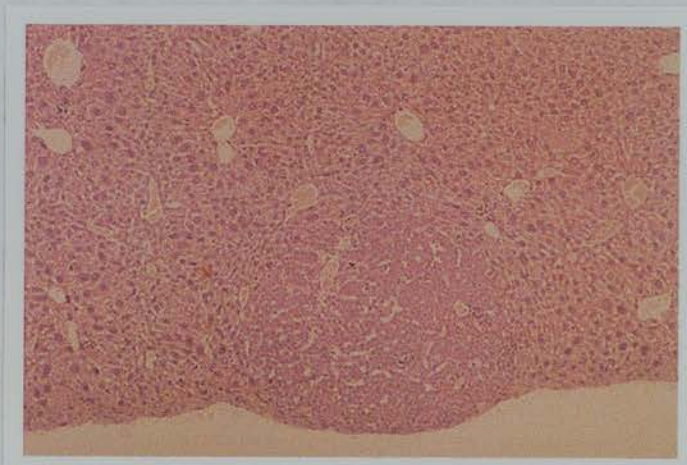


*Figure 45: Liver of a p53 null mouse exposed to DEN (50mg/kg) 10 weeks previously. A morphologically altered focus is present just under the liver capsule, which is elevated. (H & E, original magnification x100). The foci consist of small, predominantly mononuclear hepatocytes with increased cytoplasmic basophilia. Smaller foci were often centrally placed in the liver lobules, adjacent to the central vein (acinar zone 3), with slight distortion of adjacent hepatic plates.*

*Table 16. Liver abnormalities in mice 10 or 16 weeks after intraperitoneal DEN on day 12.*
*a) phenotypically altered foci.*

| p53 status | DEN dose: / Weeks after DEN: | No DEN 10 | 10mg/kg 10 | 50mg/kg 10 |
|---|---|---|---|---|
| **wild type** | affected/total **[%]** | — | 3/32 **[9%]** | 2/6 **[33%]** |
| | females: affected /total | | 0/7 | |
| | foci in each affected liver | | 1,2,3 | 1,3 |
| **heterozygous** | affected/total **[%]** | 0/10 | 4/37 **[11%]** | — |
| | females: affected /total | 0/5 | 0/14 | |
| | foci in each affected liver | | 1,1,2,3 | |
| **null** | affected/total **[%]** | 0/17 | 7/29 **[24%]** | 3/12 **[25%]** |
| | females: affected /total | 0/7 | 2/6 | |
| | foci in each affected liver | | 1,1,3,4,4 | 1,1,3 |

*Red indicates that the foci occurred in a liver with diffuse cytological atypia.*

*a) diffuse cytological atypia.*

| p53 status | DEN dose: / Weeks after DEN: | No DEN 10 | 10mg/kg 10 | 50mg/kg 10 |
|---|---|---|---|---|
| **wild type** | affected/total **[%]** | — | 5/32 **[16%]** | 3/6 **[50%]** |
| | females: affected /total | | 0/7 | |
| **heterozygous** | affected/total **[%]** | 1/10 **[10%]** | 15/37 **[41%]** | — |
| | females: affected /total | (1/5) | 4/14 | |
| **null** | affected/total **[%]** | 0/17 | 23/29 **[79%]** | 12/12 **[100%]** |
| | females: affected /total | 0/7 | 6/6 | |

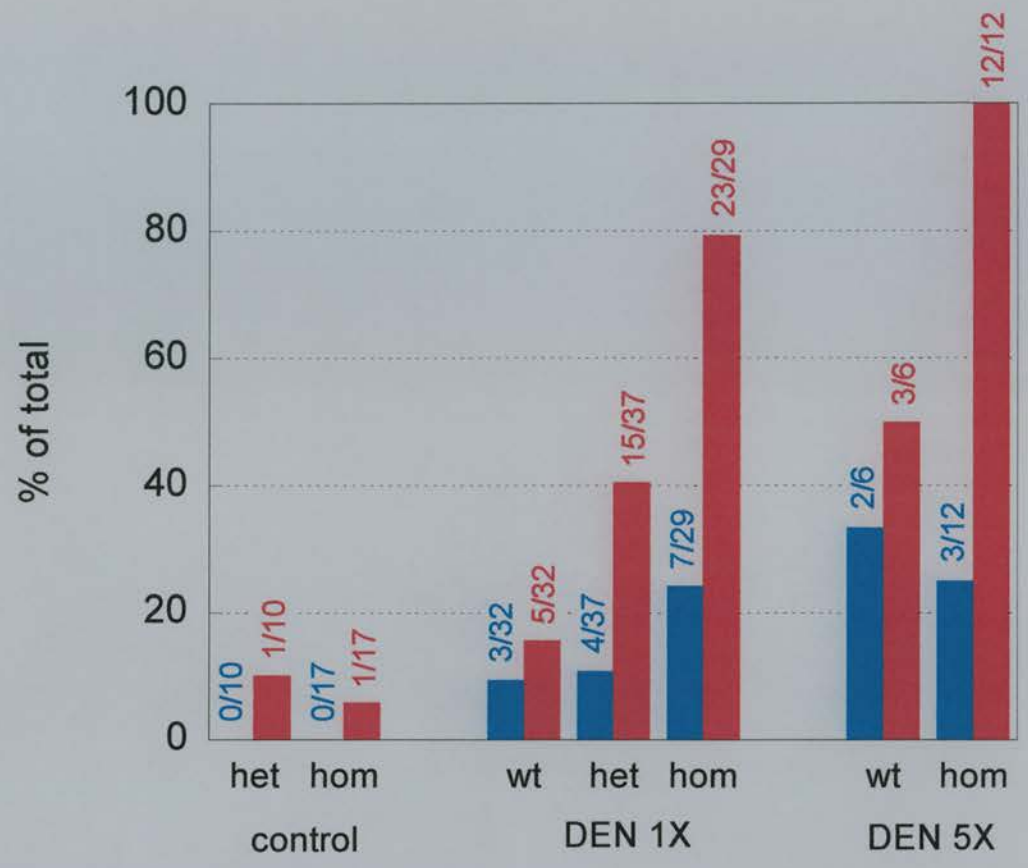



*Figure 46. Prevalences of diffuse cytological atypia and morphologically-atypical foci in DEN experiments.*
***key**: red bars, cytological atypia; blue bars, altered foci. Proportions given are number affected/total.*
*wt, het, hom indicate results for wild type, p53-heterozygous and p53-null mice, respectively.*
*DEN 1x, 10mg/kg DEN; 5x, 50mg/kg DEN.*

# 6. Discussion of results

## 6.1.a p53 sensitises hepatocytes to cytokine regulators of proliferation and apoptosis

There were no apparent consequences of p53 deficiency for unstimulated livers, which were quiescent and had similar histology, ploidy distributions and liver:body weight ratios to wild type. Differences in both proliferation and cell death only became apparent after hepatocytes were released from quiescence ($G_0$) by culture, xenobiotic (TCPOBOP) or $CCl_4$-induced liver necrosis. In cells thus stimulated p53 deficiency increased the proportion of hepatocytes that proceeded through S phase. Moreover the increase in cycling fraction was shown in culture to be relatively independent of and unaffected by mitogens. In parallel, loss of p53 reduced dependence on cytokine support for survival. Thus cells with no p53 were able to survive and proliferate under conditions that caused wild type cells to undergo growth arrest and apoptosis. Therefore there is no evidence that p53 is necessary for replicative quiescence, indeed it does not appear to have a role in normal liver, however p53 becomes important once hepatocytes are released from $G_0$, sensitising them to mitogen and cytokine regulators of cell cycle progression and apoptosis.

This model is consistent with data that normal rat hepatocytes, stimulated by isolation and primary culture (or by partial hepatectomy), progress apparently autonomously from $G_0$ to mid-$G_1$, but are critically dependent upon mitogens for further progression through the cell cycle [51]. The passage from $G_0$ to $G_1$ has been defined in hepatocytes by the sequential expression of certain oncogenes, ending with p53 expression in mid-late $G_1$ [37]. Although no significance has been previously ascribed to this physiological elevation of p53 during liver regeneration, the present results suggest that p53 is a critical effector of the mitogen restriction point. Absence of p53 disables the restriction, allowing appropriately stimulated hepatocytes to cycle independently of mitogens.

The reduced ratio between peak labelling and mitotic indices in p53-null mice, compared with wild type, suggests a prolonged S phase and/or $G_2$ phase in the regenerating p53-deficient hepatocytes. Although this is not a well recognised feature of p53 deficiency, I have also observed a prolongation of S phase in p53-deficient embryonic stem cells compared with wild type (S. Prost et al., submitted). Whether the increased cycling fraction is restricted to particular ploidy subsets of hepatocytes, which differ in relative sensitivity to mitogens [70], remains to be determined.

After liver regeneration, cytokines are believed to be important to stop hepatocyte proliferation, and $TGF\beta_1$ is perhaps the most potent [44]. However, although loss of p53 made cultured hepatocytes less responsive to $TGF\beta_1$ mitoinhibition, regenerating p53-

deficient livers did not show abnormally persistent proliferation. Therefore mechanisms other than $TGF\beta_1$ are important to terminate proliferation *in vivo*. p53 probably sensitises hepatocytes to cell cycle inhibition by $TGF\beta_1$ through co-operative activation of $p21^{WAF1}$ [141]. The potential for $p21^{WAF1}$ to inhibit liver growth has been shown in mice with a liver-specific $p21^{WAF1}$ transgene [149]. The mice had small livers with hypoplastic lobules and did not show the normal compensatory hyperplasia of remaining lobes after partial hepatectomy.

There is no comparable published data on the proliferative characteristics of hepatocytes from p53-null mice. Tsukada has stated that p53-null hepatocytes had no enhanced proliferative potential in culture, compared with wild type, but provided no data [294]. Albrecht recently reported that tritiated thymidine uptake by the liver remnant 36 hours after partial hepatectomy (the peak of DNA synthesis in wild type mice) did not differ between wild type and p53-deficient mice [7]. The methodology and data were not presented, and so it is difficult to make a comparison with the present results, however the discrepancy is interesting. The stress stimulus of $CCl_4$ may provide a greater oppurtunity for p53 deficiency to manifest; indeed Columbano has described unpublished data that $CCl_4$ induces very high levels of p53 mRNA expression in mouse liver [295]. The present results can also usefully be compared with a report of the effects of introducing mutant p53 into a well-differentiated hepatocyte cell line: Dumenco found that transfection into a TGFα-transgenic hepatocyte cell line of a transactivation-deficient mutant p53 (ser246), reduced the serum-dependence for growth, in keeping with the present results [358]. By contrast, the mutant transfectants retained sensitivity to TGFβ mito-inhibition and did not have a growth advantage in serum-enriched media. These differences to p53-deficient primary hepatocytes may be due to the TGFα-transgene in the cell line, or other unidentified genetic changes arising during immortalisation. Alternatively there may be differences between the phenotype due to p53 deletion and that of the p53 mutant (see Introduction) [356].

There is no other published data on the apoptotic characteristics of hepatocytes from p53-null mice. The present results show for the first time that insulin, EGF or serum decrease apoptosis in mouse hepatocytes and so are survival factors for these cells. The reduced need of p53-deficient hepatocytes for these survival factors is concordant with data that stable transfection of a mutant p53 into hepatocyte cell lines increased colony formation and enhanced survival, although these studies did not assess apoptosis [356,358]. It is possible that p53 does not directly trigger apoptosis of wild-type hepatocytes in the absence of survival factors, but is acting only as a modulator of sensitivity to undergo apoptosis through other, cytokine-regulated pathways.

## 6.1.b Hepatocyte responses to DNA injury

### *6.1.b.1 p53*

p53 protein levels, although characteristically altered during the course of various p53-dependent responses, are not specific to a particular response, and in other cell types do not necessarily correlate with changes in functional activity. Therefore, when it became evident that in hepatocytes, p53-dependent responses could be observed without immunocytochemical evidence of increased p53 concentration (i.e. γ-irradiation-induced growth arrest), a functional assessment of p53 was made (of transactivation activity), using a transiently-transfected p53-reporter plasmid.

#### Transient reporter plasmid assay for p53

The results show that transient transfection is a suitable method to evaluate p53 function in normal cells after DNA damage, and that the method is sufficiently stable and reliable to be used to provide a time course of the p53 response. Previous studies have used stably transfected carcinoma cell lines and found increased p53 reporter expression after a variety of genotoxic treatments, including uv and γ-irradiation [465,466] and etoposide [467]. Zhan reported a single time point at 24 hours whilst Lutzker used pooled clones carrying 3 different reporter constructs and found expression to steadily increase over a time course of 2-12 hours after treatment. However stable transfection is clearly not feasible for primary culture work. Zhan *et al* did attempt transient transfection of a $PG_{13}$-CAT p53 reporter plasmid but could not demonstrate significant induction of expression after the same genotoxic injuries that produced responses in stable transfectants. This could be due to low transfection efficiencies or to an attenuated p53 response by the carcinoma cells to DNA injury compared with hepatocytes, although the latter seems unlikely given the efficient induction in stable transfectants. Stabilisation of p53 after calcium phosphate transfection has been reported and might have masked p53 responses to genotoxic treatment in their study [468]. No such effect was apparent for lipofection in the present study, since β-galactosidase activity was similar (baseline) in untreated p53 reporter and control plasmid transfectants. The relative levels of p53 reporter induction are comparable with those observed using a transiently transfected $PG_{13}$-CAT reporter to evaluate changes in p53 function during primary keratinocyte differentiation [469].

In conclusion, although potentially less sensitive than study of clones with a stably transfected reporter, this transient reporter assay is adequate to evaluate authentic p53 responses to levels of DNA injury commonly applied in studies of p53, and moreover can be applied to primary and non-clonogenic cell populations, giving the power of greater relevance to *in vivo* responses, combined with a defined cellular environment.

#### p53 after DNA injury

p53 protein accumulation after **UV irradiation** has been reported in a variety of cell types *in vivo* or primary culture, including human skin keratinocytes, dermal fibroblasts

and mouse primary prostatic fibroblasts [470-475]. Indeed there is no report of failure to produce p53 changes by UV treatment. The present results for p53 immunopositivity in uv-treated hepatocytes are comparable with recently published time courses of p53 immunopositivity in UV-b treated mouse and rat hepatocytes [232,301,476]. It is of interest, and not previously reported, that occasionally only one nucleus of a binucleate hepatocyte showed p53 immunopositivity; however, the significance of this observation is unknown.

After uv-c treatment, p53 transactivation activity (reporter plasmid) had already peaked and returned to baseline when p53 protein levels were at peak. Therefore increased p53 transactivational activity after uv-c was not simply a reflection of increased protein levels, and so may be independently regulable in hepatocytes, as suggested for some other cell types [467,469,477,478]. If so, the relative importance of these two changes for initiation of a p53-dependent response has yet to be determined. By contrast to the present results, Lu reported that p53 transactivation activity in a mouse prostate fibroblast cell line was decreased after uv-c irradiation (16 hours after 10 and 50J/m$^2$), whilst protein levels increased [479]. That study used the same reporter plasmid construct (RGCΔFosLacZ) as used here. However, whilst the data may reflect a tissue-specific difference in p53 regulation, it is difficult to exclude an artefact of transcriptional suppression by uv and the relatively insensitive immunofluorescence method used to detect reporter plasmid expression.

Assessment of p53 immunopositivity in liver after whole body **γ-irradiation** of normal mice gave similar negative results to Midgely [297] and Ogawa [283], here using a higher dose (15Gy) and extending the observation to proliferating hepatocytes in culture (showing that cell cycle status did not influence the findings) (fig 41, p97 and p97a). γ-irradiation also failed to alter reporter gene activity, at a dose adequate to arrest liver regeneration *in vivo* and that induces hepatic O$^6$ alkyl transferase expression, both by p53-dependent mechanisms. The data are supported by MacCallum, who recently reported no increased p53 transactivation activity in γ-irradiated liver, as assessed by activity at a RGC reporter transgene (the same construct as used here) [298]. Therefore, by contrast with uv-injury, p53-dependent responses to γ-irradiation were accomplished without detectable increase in p53 concentration or evidence of p53-dependent transactivation.

This suggests that the basal state of p53 in hepatocytes is sufficient to permit the responses, or that a different property of p53 is stimulated by γ-induced DNA injury [480]. It is possible that the lack of reporter response merely reflects a lower sensitivity than given by endogenous biological activities such as growth arrest [481], but a third intriguing possibility remains — that p53 is transcriptionally activating genes through only a subset of its response elements which does not include the RGC motif. p53 binds to its response elements with different affinities that can be changed selectively and in opposite ways by altering its phosphorylation state [482]. It would therefore be necessary to use several reporter constructs to accurately assess changes in p53 transactivation activity. Nevertheless, these results suggest that a common p53-dependent response (i.e. growth

arrest) to two different types of DNA injury can proceed through different activities of p53. Since p53 mutants vary in their ability to activate specific response elements [483-486], different p53 mutations in carcinogenesis might have consequences for susceptibility to different types of DNA damage and to assessments of specific transcriptional transactivation.

In conclusion, p53 responses to genotoxic injury varied with agent. The observation of distinct p53 transactivational and immunopositivity responses in hepatocytes to the two different types of DNA injury is of particular interest since both agents caused p53 dependent growth arrest. Thus qualitatively different genotoxic stimuli acted differently through p53 yet had a common consequence in growth arrest.

### *6.1.b.2 p53 couples DNA damage to growth arrest but not apoptosis*

Hepatocyte p53-dependent growth arrest after uv was also observed by Tsuji, using an antisense strategy [301], whilst no comparable *in vivo* data to that presented here is available. The suggestion that p53 regulates both $G_1$ and $G_2$ checkpoints of proliferating hepatocytes has been made for some non-epithelial cell lines, but not demonstrated *in vivo*, and implies a critical importance for p53 in preventing the passage to mitosis of DNA-damaged hepatocytes.

p53 coupled genotoxic injury to growth arrest, but there was no direct relationship between p53 status and hepatocyte apoptosis after DNA damage. γ-irradiation did not produce hepatocyte apoptosis at doses effective on other cell types, often in a p53-dependent manner, including intestinal crypt epithelium [458], thymocytes [291], peripheral T lymphocytes, mitogen activated T lymphocytes [487,488], cardiac and pulmonary endothelium and mesothelium [489], splenocytes [297] and spermatocytes [490]. Midgley has also observed that 5 Gy γ-irradiation *in vivo* did not induce hepatocyte apoptosis [297], and the present results extend that finding to proliferating hepatocytes in culture, regenerating liver and a higher dose (15Gy). Moreover, *Bax*, a downstream promoter of p53-dependent apoptosis, was not induced in liver by γ-irradiation [491]. By contrast, uv treatment did produce apoptosis, as also recently observed on primary rat hepatocytes by Worner [232], but this was not p53-dependent despite stabilisation and activation of p53. There was no early peak of apoptosis within the first 48 hours after uv, as is characteristic of p53-dependent apoptosis in other cell types [291,458,492,493]. This suggests that p53-dependent apoptosis due to DNA damage is not enabled in normal hepatocytes and therefore other genes may be more critical to such pathways in liver. Indeed, the time course of hepatocyte apoptosis after uv treatment was similar to that for the IRF-1-dependent apoptosis of mitogen-activated T lymphocytes after γ-irradiation [488], although recent experiments using the present culture system have shown that IRF-1 does not regulate the hepatocyte apoptosis induced by uv-irradiation (S. Prost, C.O.C. Bellamy, D.J. Harrison *et al.*, in preparation). Nevertheless, other genes may be more critical to such pathways in liver, and represent better targets for novel cancer chemotherapeutic strategies. In particular, the observation that cytokines reduced apoptosis of DNA-

damaged hepatocytes has implications for genotoxic drug therapy of liver neoplasms, suggesting a utility for cytokines to either manipulate tumour cell chemosensitivity, or to promote normal cell survival, as has also been noted for other cell types exposed to different genotoxins and cytokines [494,495]. The pathways of protective cytokine action on DNA-damaged hepatocytes are unclear but there was no evidence for p53 involvement. This contrasts with the effect of cytokines to reduce hepatocyte apoptosis in unirradiated cultures, where p53 status regulated cytokine dependence. Since the same cytokines were used to prevent apoptosis in both circumstances, this implies that different pathways are involved. Taken together, the data illustrate the tissue specificity of different p53-dependent responses and provide further evidence that apoptosis and cycle arrest mediated by p53 are independent pathways.

In conclusion, hepatocyte responses to genotoxic injury varied with agent, but the role of p53 was limited to cell cycle arrest whilst other factors (including the type of injury, the cytokine environment and perhaps other genes), determined cell survival.

## 6.1.c Ploidy regulation is p53-independent in unstimulated livers

Polyploidisation occurs in a variety of non-neoplastic cell types, either constitutively or in response to defined pathophysiological stresses, and is a feature of normal postnatal liver development [2,496]. It is a tightly regulated process in which only integral multiples of diploid karyotype are permitted, but neither the purpose nor the regulation of this irreversible process are well understood [73,75,497]. Indeed the mechanisms underlying the normal couplings of S phase to M phase and of M phase to cytokinesis are rather poorly defined in eukaryotic cells other than yeast [73,75,497]. Murine hepatocyte polyploidisation occurs to a relatively high degree, reportedly predominantly through acytokinetic mitosis to produce binucleate cells, followed by nuclear fusion (thus doubling cell ploidy) in subsequent cell cycles [2]. Other unusual patterns of mitosis also occur, particularly for high ploidy cells, and indeed, during the analyses of BrdU immunocytochemistry, bizarre labelling patterns were seen, including labelling of only one of a pair of nuclei and labelling of only a half or a quarter segment of a mitotic spindle. It seemed reasonable to hypothesize that p53 could influence liver polyploidy, given observations linking loss of p53 function to centrosome amplification, loss of $G_2$/mitotic checkpoints and abnormal tetraploidisation in other cell types [454,498-505]. p53 also transcriptionally activates p21, a broad spectrum inhibitor of cdk-cyclin complexes, certain of which probably regulate polyploidisation [506,507]. Dysregulation of p21 (both under- and over-activation) has been linked to abnormal ploidy control and dysfunctional cytokinesis in carcinoma cell lines [74,508] and to formation of giant polyploid hepatocytes in transgenic mice [149]. Moreover, expression in mouse liver of an SV40 T transgene (known to inhibit function of p53 and the pRb family) produced hyperpolyploidy prior to carcinoma development [509]. However the present results show that p53 genotype did not influence the onset, rate or degree of normal hepatocyte polyploidisation. This suggests that the excessive polyploidisation of SV40 T transgenic hepatocytes was due to interference by the transgene with the pRb

family, rather than p53. Indeed hepatocytes of adult chimaeric Rb-deficient mice had abnormal nuclear pleomorphism, suggestive of hyperpolyploidy [510].

p53 did not regulate liver ploidy under basal conditions, but these findings in unstimulated livers do not exclude a role for a p53-dependent pathway in determining altered polyploidisation following DNA damage [511]. Indeed, hepatocyte polyploidisation has been reported to increase in the months following γ-irradiation [463,512], and ERCC1-deficient mice that are defective in DNA repair show persistent p53 accumulation in hepatocytes and develop abnormal liver polyploidy [296]. The relationship of polyploidy to aneuploidy is not well defined, and whether polyploidy represents an adaptive phenomenon to genotoxic stress is unclear, but the present findings that p53 deficiency might allow generation of abnormal hepatocyte ploidy after DNA injury suggest it will be of interest to compare polyploidisation responses to DNA damage of wild type and p53-deficient livers. In this way, as for regulation of proliferation and cell death, p53 may be important for ploidy regulation only when the hepatocyte is stressed.

## *6.2 Final discussion*

### 6.2.a What does p53 do in the liver?

p53 does not appear to have a role in normal liver growth and function. However, hepatocytes are competent to induce p53 protein and function, and the present results have shown differences between p53-deficient and wild type hepatocyte responses to trophic and genotoxic stimuli. It seems that p53 has an important modulatory role on both proliferation and cell death, but only when the hepatocyte is stressed: hepatocytes with no p53 survive and proliferate in culture under conditions of minimal trophic support, whereas wild type cells will growth arrest and undergo apoptosis. Thus, p53 appears to sensitise and so link stimulated hepatocytes to their environment — by inflicting a requirement for suitable cytokine stimuli in order that cell proliferation can proceed and cells continue to survive. In this way, p53 imposes "social control" upon stimulated hepatocytes.

In DNA-damaged hepatocytes, by contrast with several other cell types, p53 promotes recovery rather than deletion (apoptosis). This is achieved through growth arrest if hepatocytes are proliferating — p53-deficiency allowed γ-irradiated proliferating hepatocytes to proceed unchecked from G1 to mitosis, and the observation of abnormal mitotic figures directly demonstrates the increased potential for chromosomal disruption. p53 may also directly enhance hepatocyte DNA repair activity: the data of Rafferty et al show that induction of at least one type of hepatic DNA repair activity ($O^6$-alkyl guanyl transferase) after γ-irradiation is p53-regulable [300]. However the significance of those data is uncertain because that enzyme does not directly repair γ-irradiation-DNA damage, and so is a rather non-specific response. Nevertheless, the role of p53 in regulation of hepatic DNA repair is of great interest. Recent studies in our laboratory in these primary

hepatocytes have provided some evidence that p53 could regulate hepatocyte DNA nucleotide-excision repair.

Thus, the phenotype of p53-deficiency in liver is latent, but under conditions of excessive tissue damage and repeated induction of compensatory proliferation p53 is likely to become a major player. The implications for carcinogenesis will now be discussed.

## 6.2.b Implications for hepatocarcinogenesis

The liver carcinogens Hepatitis B x protein and aflatoxin interact directly with p53 protein and gene respectively, suggesting a role for p53 dysfunction in early human hepatocarcinogenesis which the present study has sought to understand. The results define a latent phenotype for p53 deficiency in hepatocytes that is likely to be revealed under pathological conditions involving sustained tissue damage and regeneration, such as are found in chronic active hepatitis or cirrhosis, and that allows sustained survival, increased proliferation and impaired damage responses that could accelerate carcinogenesis. These observations are of particular relevance to human hepatocarcinogenesis, which almost always occurs on a background of chronic liver damage and regeneration, and could explain a selection for p53 dysfunction in clones of hepatocytes in an unfavorable environment within inflamed liver. This concept accords with the observations of Graeber *et al* that in an unfavourable environment (hypoxia), p53 deficiency conferred a survival advantage to transformed fibroblasts through decreased apoptosis [513]. By downregulating dependence upon local cytokines for survival and proliferation, p53 deficiency could have similar consequences for chronically-stressed hepatocytes.

It is perhaps not surprising therefore that others report no increased frequency of hepatocellular carcinoma in untreated or single dose chemical carcinogen-treated p53 heterozygous and homozygous deficient mice [292,293,364]. Such models do not produce the chronically disturbed hepatic environment that precedes virtually all human hepatocellular carcinomas and that is probably necessary to fully manifest the latent phenotype of p53 dysfunction described here. A more appropriate model might be to evaluate the effects of a cross between p53-deficient and Hepatitis B surface antigen transgenic mice. The latter develop a chronic necroinflammatory hepatitis, with regenerative hyperplasia in high producer lineages that leads eventually to development of hepatocellular carcinoma [9].

Nevertheless, results in the present study suggest that p53-deficiency does change the response of hepatocytes to DEN treatment, although no conclusions can be made about carcinoma development. Cytological atypia in p53-deficient liver has not been described, and has no known significance. However the changes observed are characteristic in humans of epithelial dysplasia, and together with the abnormal appearence of γ-irradiated regenerating livers, are strongly suggestive of a predisposition to pre-neoplastic change in p53-deficient hepatocytes, after DNA damage. The increased prevalence of morphologically atypical foci with reduction in p53 gene doseage supports this idea.

# 7. Appendices

## Hepatocyte isolation and culture

### *Perfusion medium*

Hepatocyte perfusion medium (Life Technologies), **or**
$Ca^{++}$ and $Mg^{++}$-free Hank's buffered saline (Sigma)
EGTA 0.5mM
EDTA 1mM
Gentamicin (Life Technologies) 50mg/ml
pH 7.4

### *Digestion medium*

*Stock solution:*
L-15 medium (Sigma)
L-glutamine 4mM
Gentamicin (Life Technologies) 50mg/ml
HEPES (Sigma) 40mM
Insulin (Bovine pancreatic, Life Technologies) 100nM
pH 7.5
*Immediately before use, add:*
Collagenase 0.06–0.065%, final concentration (filter-sterilised)
DNAse I (Boerhinger) 40μg/ml, final concentration

### *Plating medium*

L15 medium (Sigma), or Chee's medium, or DMEM
L-glutamine 4mM
Gentamicin 50mg/ml
HEPES 25mM
Aprotinin (Sigma) 1μg/ml
Insulin (Life Technologies) 300nM
Dexamethasone (Sigma) 100nM

### *Percoll stock solution*

Percoll (Sigma) 100ml
10x Hanks' buffered saline solution (Life Technologies) 11 ml
pH 7.5

### *Modified Chee's medium*

Chee's medium [436], modified as follows (Gibco Custom Media):

arginine-free
ornithine 0.4mM
thymidine 10mg/l
phenol red, Na 10mg/l
HEPES 30mM
osmolality 330 mOsm/kg, by adjusting NaCl concentration
pH 7.45

***Supplemented prior to use with:***

L-glutamine 4mM
Gentamicin (Life Technologies) 50mg/ml
Sodium selenite (Sigma) 30nM
Transferrin (Sigma) 6.25μg/ml

***Hormone supplements, if not stated specifically:***

Dexamethasone (Sigma) 100nM
Epidermal growth factor (mouse submaxillary gland, Sigma) 25ng/ml
Insulin (Life Technologies) 300nM

## *Coating of culture surfaces*

### *Fibronectin*

Fibronectin (Sigma) was allowed to adsorb overnight at 4°C from a 10μg/ml sterile solution in PBS, calculated to give 1–2μg fibronectin /$cm^2$. The solution was aspirated just prior to plating of hepatocytes.

### *Collagen type 1*

A 0.01% solution of type I collagen (Sigma, rat tail) in 0.1M acetic acid, sterilised over chloroform, was allowed to air dry onto culture surfaces at 37°C to give 10–20μg/$cm^2$. Dishes were stored at 4°C and if necessary washed to neutral pH by PBS prior to plating of hepatocytes.

# Fixation and staining

### *Phosphate Buffered Saline (PBS)*

137mM NaCl
2.7mM KCl
4.3mM Na2HPO4
1.47mM KH2PO4
pH 7.1

### *Boum's fixative*

5% v/v glacial acetic acid
85% v/v methanol
10% v/v pure formalin solution (40% formaldehyde in water)

### *Schiff's solution*

Dissolve 2.5g Fuchsin Basic (99% pure grade) in 500ml water, just boiled
Cool to 50°C. Add 50ml 1M HCl
Cool to room temperature. Add 5g potassium metabisulphite and leave in the dark for 24hours
Add 5g activated charcoal and agitate for 2 minutes
Filter (Whatman No 1) to a clear colourless solution
Store at 4°C in the dark

### *5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) solution*

In PBS (pH 7.1):
10mM $K_3Fe(CN)_6$ (potassium ferricyanide)
10mM $K_3Fe(CN)_6$.3H2O (potassium forrocyanide)
1mM $MgCl_2$
1mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Sigma, from 100mg/ml stock solution in dimethylformamide), added just before use.

# Molecular biology

### *SOC medium*

0.5% bacto-yeast extract (w/v)
2% bacto-tryptone (w/v)
10mM NaCl
2.5mM KCl
10mM $MgCl_2$
10mM $MgSO_4$
20mM glucose
filter sterilised and stored at 4°C

### *LB medium (Luria-Bertani medium)*

10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
DDW to 1 litre
pH 7
Autoclaved

### *LB agar (Luria-Bertani agar)*

500ml LB medium
7.5g agar
autoclaved, cooled to 50°C
antibiotics added as appropriate:
100mg/ml ampicillin or 50mg/ml Kanamycin Sulphate (Sigma)
Poured onto a microbiological 10cm petri dish

### *gel loading buffer*

0.25% bromophenol blue (w/v)
1% Ficoll
50% glycerol
0.1% sodium dodecyl sulphate
25mM EDTA
pH8

### *Tris-borate EDTA buffer (TBE)*

0.089M Tris.HCl
0.089M boric acid
1mM EDTA
pH 8

### *Tris EDTA buffer (TE)*

10mM Tris
1mM EDTA
pH 8

# p53 genotyping by polymerase chain reaction.

The reaction mix contained three primers, one wild type p53-specific (binding within exon 6), one targeting construct-specific (binding within neo) and one common to both wild type p53 and targeting construct (binding within intron 7).

**Primers (5'→ 3'):**
Intron 7 (3') CAAAGAGCGTTGGGCATGTG (5')

Exon 6 (5') GTGGTGGTACCTTATGAGCC (3')

Neo (5') CATCGCCTTCTATCGCCTTC (3')

**Reaction mix**
1x Taq polymerase buffer (Life Technologies)
2mM $MgCl_2$ (Life Technologies)
0.025% Detergent (WI, Life Technologies)
0.2mM dNTPs
5% DMSO
0.5μM Primers
1.25 units Taq polymerase (Life Technologies)
2μl genomic DNA

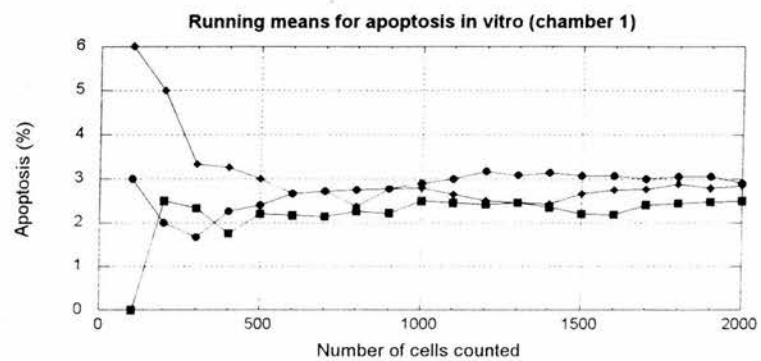
**Polymerase chain reaction conditions**
94°C for 2 minutes, then 30 cycles of 94°C for 1 minute, 62°C for 1 minute and 72°C for 1 minute. Finally 72°C for 10 minutes.

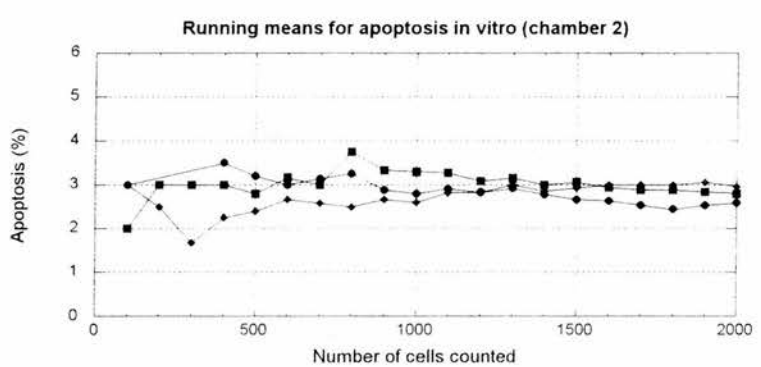
**Reaction products**
p53 wild type: 642 bp
p53 target construct: 510 bp

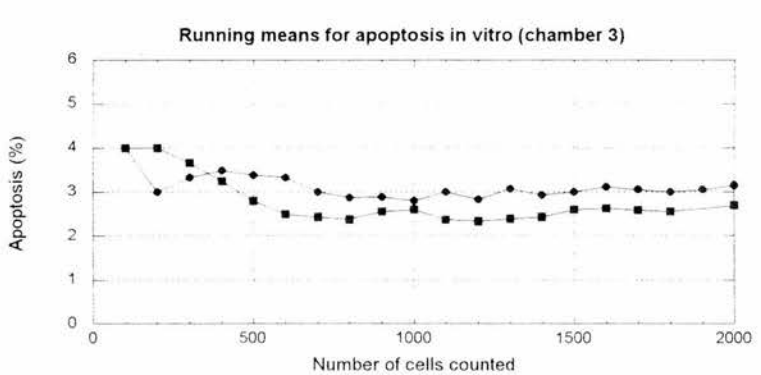
# Running means.

***Running means for the rate of hepatocyte apoptosis in monolayer cultures.***

Running means were established on three independent replicate cultures of hepatocytes (chambers 1–3), fixed and stained by the Feulgen method. Each running mean was recounted on each culture, one or two times, as shown.

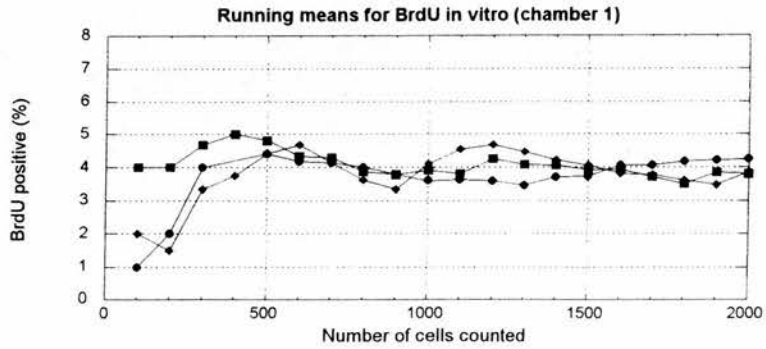


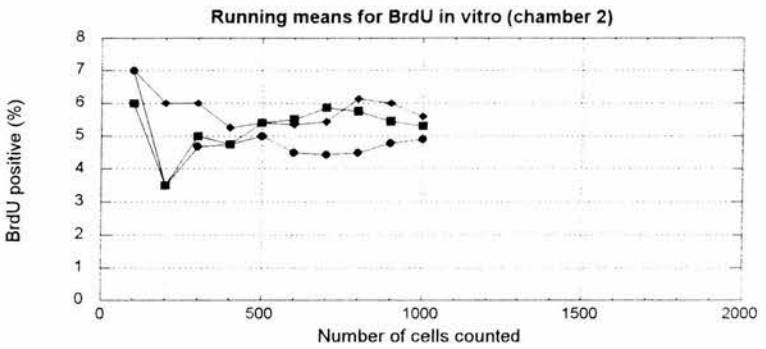



***Running means for the proportion of BrdU immunopositive hepatocytes in monolayer cultures.***

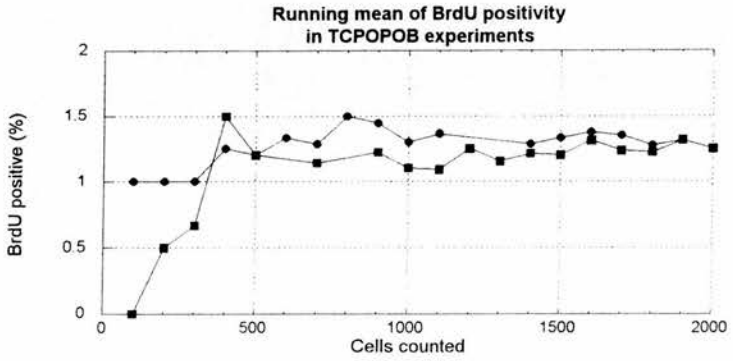
Running means for BrdU immunopositivity were established on 2 independent replicate hepatocyte cultures, fixed and stained as described in Methods. Each running mean was recounted 2 times, as shown.



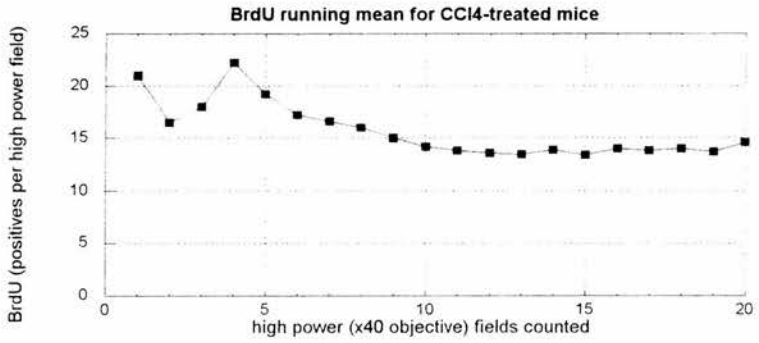



***Running means for the proportion of BrdU immunopositive hepatocytes in tissue sections from TCPOBOP-treated mice.***

A running mean was established and recounted once, as shown.




***Running mean for the proportion of BrdU immunopositive hepatocytes in tissue sections from $CCl_4$-treated mice.***



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*seminars in* **CANCER BIOLOGY**, Vol 6, 1995: pp 3–16

# Cell death in health and disease: the biology and regulation of apoptosis

*Christopher O.C. Bellamy, Roger D.G. Malcomson, David J. Harrison and Andrew H. Wyllie*

*Apoptosis is a morphologically stereotyped form of cell death, prevalent in multicellular organisms, by which single cells are deleted from the midst of living tissues. Recognition of the cellular corpses and their removal by phagocytosis occurs without disturbance to tissue architecture or function and without initiating inflammation. Apoptosis is regulable and is of fundamental importance to tissue development and homeostasis. Cellular susceptibility to apoptosis is determined by a variety of signals, of both extracellular and internal origin, including proliferative status. Dysregulated apoptosis is important in the pathogenesis of several important human diseases including neoplasia, and recognition of the defects involved is prompting development of new therapeutic strategies.*

**Key words:** apoptosis / genetic regulations / homeostasis

PHYSIOLOGICAL CELL DEATH is an inconspicuous yet prevalent phenomenon in complex multicellular organisms. It is characterized by the deletion of scattered, single cells from the midst of a living tissue without disturbance to the continuity of tissue architecture or function. With few exceptions cellular death is accomplished by a process with a structural stereotype, termed apoptosis, that strongly suggests a common underlying effector mechanism.[1] Apoptosis is observed in circumstances as diverse as embryogenesis, normal adult tissue turnover and organ atrophy; it is also fundamental to the regulation and operation of pathophysiological processes such as the immune response, inflammation and the elimination of cells after genotoxic injury (Figure 1). The realisations that apoptosis represents an innate cellular defence against carcinogenesis, that the regulatory pathways to apoptosis (but not the effector mechanisms) are frequently disabled in malignant neoplasms and that many cancer chemotherapeutic agents may act by induction of apoptosis have stimulated intense investigation into the underlying molecular controls.

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## The structural changes of apoptosis

In apoptosis coordinated changes occur in the nucleus, the cytoplasm and at the cell surface (Figure 2).[2] The time to onset of apoptosis after a lethal stimulus is variable but the changes are rapid (a few minutes duration): cells lose contact with their neighbours and round up. The endoplasmic reticulum dilates and superficial cisternae fuse with the plasma membrane. Other cytoplasmic organelles remain largely unaffected. At the same time there is a striking loss of cell volume, apparently due to voiding of water and ions with consequent compaction of the organelles and an increase in cell density. The nucleus condenses and chromatin marginates to form dense granular caps under the intact nuclear membrane. The nucleolar fibrillar centre dissociates from its transcriptional complexes. The cell surface starts to bleb violently and time lapse phase contrast studies show an extraordinary bubbling appearance.[3] Around this time the nucleus breaks up into several membrane-bound fragments. The cell itself then splits into multiple membrane-bound 'apoptotic bodies', some of which contain nuclear fragments. The apoptotic bodies are phagocytosed almost immediately by neighbouring cells or by macrophages without eliciting an inflammatory reaction, in contrast to necrosis as described below. Apoptotic bodies in phagosomes remain recognizable by light microscopy for up to a few hours and are consequently the predominant form of apoptosis recognized in tissue sections. In epithelia or cultured monolayers apoptotic bodies can instead be shed into lumina or the culture medium where they degenerate within a couple of hours: they gradually lose membrane integrity (becoming permeable to vital dyes) and metabolic activity ceases.

Apoptosis is not the only possible mode of cell death. Necrosis is a non-specific term for a variety of

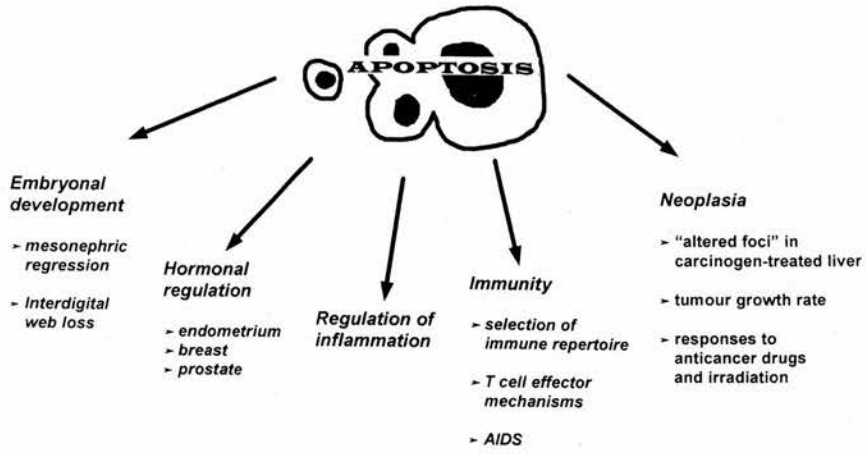


**Figure 1**. Prevalence of apoptosis in physiological and disease contexts (modified and reproduced with permission of John Wiley and Sons, Chichester, UK).

other modes of death in which, in contrast to apoptosis, the cell plays only a passive role while it is destroyed. Cell lysis during necrosis releases intracellular contents into the extracellular space, resulting in inflammation and further secondary tissue damage. Necrosis typically involves contiguous cells and is associated with tissue architectural disruption whilst apoptosis generally affects scattered single cells and preserves the tissue architecture (except in special circumstances such as embryonic remodelling).[1] Necrosis is always pathological whereas apoptosis is a physiological process that may also be triggered in pathological situations.

## Kinetic considerations

The 'gold standard' for identification of apoptosis is

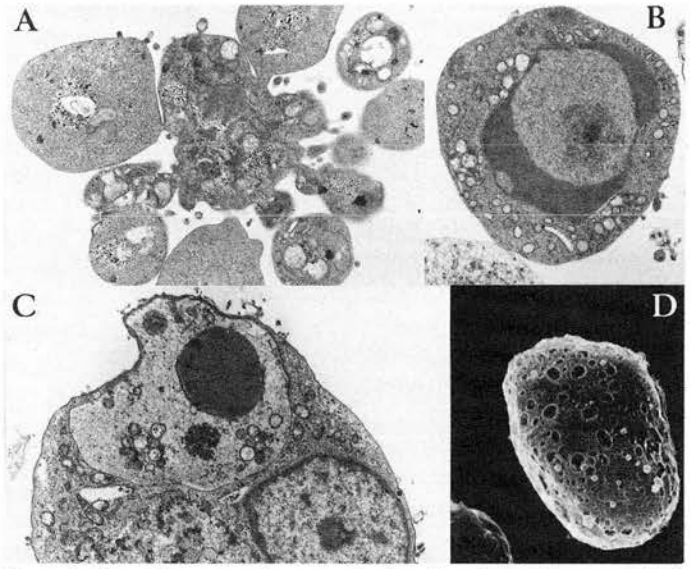


**Figure 2**. (A) Transmission electron micrograph showing late stage apoptotic fibroblast with fragmentation of the cell into membrane-bound apoptotic bodies. (B) Early apoptosis showing distinct peripheral chromosome condensation against the intact nuclear membrane. (C) Phagocytosed apoptotic body within a phagosome. (D) Cell surface changes in apoptosis: scanning electron micrograph of an apoptotic thymocyte, showing loss of microvilli and gaping cisternal pits, formed by fusion of dilated endoplasmic reticulum with the surface. (Reproduced with permission of Kluwer Academic Publishers, Dordtrecht).

morphological assessment. A comment on methods of evaluation of apoptosis has been given elsewhere[4] but the importance of quantitation is worth stressing. The speed of apoptosis and the rapidity of clearance of apoptotic bodies *in vivo* (a few hours at most)[5] mean that the identification of only a few apoptotic bodies in a tissue section can represent a considerable degree of cumulative cell loss. Numerically small differences in 'apoptotic indices' (i.e. the percentage of cells that are apoptotic) can therefore be of great biological import. For example, an intravenous bolus of anti-CD4 antibody increases the apoptotic index in murine lymph nodes from 0·06% to 1·33%, and this is sufficient to halve the total cell count of the lymph nodes within 48 h.[6] Unfortunately studies of this nature require that large numbers of cells be counted in order to achieve running means for apoptotic indices and allow statistical evaluation of differences. Furthermore, to be able to ascribe changes in tissue or tumour size to altered rates of apoptosis, parallel quantitative evaluation of cell proliferation is necessary. Other factors such as the rate of disposal of apoptotic bodies will also affect the perceived apoptotic index.

## Underlying cell biology of the effector processes

### *Cytoplasmic events*

The abrupt increase in **cell density** is due to voiding of water and ions, possibly channelled to the cell surface through the endoplasmic reticulum.[2] As yet no mechanism has been identified to account for this profound and sudden fluid shift. The cell size and shape changes during apoptosis require major **cytoskeletal reorganization** that is still largely uncharacterized, although actin polymerization (in part stimulated by protein kinase C) is essential for the budding that generates apoptotic bodies.[7] Some apoptotic cells, e.g. hepatocytes, also activate **tissue transglutaminase** to produce an insoluble shell of cross-linked protein.[8] Although increased transcription of specific mRNAs frequently precedes apoptosis (see below), a site-specific endogenous **RNAse** activity late in apoptosis is suggested by the rapid degradation of ribosomal and messenger RNA in apoptotic cells.[9,10] The role of **proteolysis** (e.g. of terminin proteins)[11] is unclear but specific regulatory proteases are consider later.

### *Nuclear events*

As mentioned above, the nuclear membrane is not lost during apoptosis, unlike mitosis. However the subjacent **nuclear lamina** (which anchors chromatin) is dissassembled by depolymerization of constituent lamin filaments.[12,13] Lamin phosphorylation and depolymerization also occurs during mitosis, catalysed by cdc2 kinase, and it is possible the same mechanism operates during apoptosis.[14] That elements are common to mitosis and apoptosis is as likely to reflect a general role in regulation of nuclear structure as to indicate any deeper similarity between these distinct processes. In contrast to mitosis the depolymerized lamins subsequently undergo proteolysis in apoptosis, perhaps making lamina dissassembly irreversible.[13] **Ubiquitin conjugation** of nuclear proteins appears to be important in some (but not all) examples of apoptosis, and has been suggested to regulate some of the chromatin structural changes.[15] A specific feature of apoptosis is rapid **DNA cleavage**. Initially large transient 50 kbp and 300 kbp fragments are detectable that probably represent chromatin loops and rosettes detached from the anchoring nuclear matrix.[16] In many cell types there is further rapid and extensive double strand cleavage of internucleosomal DNA to yield a series of oligonucleosome chains of 180-200 bp multiples (180 bp is the length of DNA in a single nucleosome). These are visualised as the characteristic 'DNA ladder' on agarose gel electrophoresis.[2] The DNA cleavage is not sequence specific and the precise identity of the nuclease(s) responsible is still unknown (see ref 17, A. Eastman this issue, pp45-52, for a detailed discussion). It is sufficient to say here that nuclease is constitutively present in some cell types but is induced in others prior to apoptosis. The nuclear changes of apoptosis can occur without oligonucleosome generation (e.g. TGF β1-induced rat hepatocyte apoptosis), which when present may be a very late event, after chromatin margination.[16,18,19] Of note is that mitochondrial DNA is not fragmented in apoptosis, indicating that DNA fragmentation is a specific nuclear event.[20,21]

### *Surface changes*

A key property of apoptotic bodies is their rapid recognition and phagocytosis by adjacent cells or professional phagocytes. Cell surface changes during apoptosis that promote recognition are best characterized for apoptotic inflammatory cells, and include loss of sialic acid (thereby exposing glycoprotein side-

chain sugars) and exposure of membrane phosphatidylserine.[22] A third mechanism involves thrombospondin secreted by macrophages to form a molecular bridge between apoptotic cell and macrophage surface CD36 or $\alpha_3\beta_3$ integrin.[22] Importantly phagocytosis of apoptotic cells does not activate macrophages to produce an inflammatory response.[1]

### *Organization of effector events*

An ordered set of molecular events underlies the phenomena of apoptosis. To what extent these events are organized as a linear cascade or as parallel independent processes at multiple subcellular sites is unclear. It is of interest however that the nucleus is not essential for the cytoplasmic changes of apoptosis.[23] The degree of redundancy built into effector mechanisms is also unknown, although no obligate gene (i.e. without which apoptosis cannot occur at all) has yet been identified. **Expression of effector molecules** in non-apoptotic cells is variable: thymocytes must synthesize protein and RNA in order to undergo apoptosis in response to some stimuli,[24] but other cell types constitutively possess the effector molecules, as demonstrated by apoptosis in the presence of protein or RNA synthesis inhibitors. For other cells inhibition of protein synthesis itself triggers apoptosis, suggesting the presence of a short-lived protein inhibitor of cell death. These observations have led to the concept of states of readiness ('priming') for apoptosis, determined by the dynamic activity of regulatory pathways to induce or deplete effector molecules.[2] The idea has implications for chemotherapy in that cells in a highly primed state (with all the apoptotic machinery in place) might be more susceptible than unprimed companions to the triggering of apoptosis by cytotoxic stimuli. The increased sensitivity to cytotoxic agents of cells in 'proliferative' compartments of tissues when compared with non-proliferative compartments has been cited as an example of priming, although whether this difference is due simply to accumulation of effector molecules is unproven. In fact current models suggest that cell cycle activation is actually lethal itself, unless specific rescue factors prevail (see next section).

## The regulation of apoptosis

Broadly speaking, physiological apoptosis can be externally triggered or a cell-autonomous event (Figure 3). **Cell-autonomous apoptosis** is a hard-wired phenomenon, often termed 'programmed cell death'. It is exemplified in morphogenesis by the coordinated death of web space cells that sculpts digits from the coarsely-shaped limb bud,[1] and by the chronologically and spatially invariant death of 131 cells (12% of total, excluding germ cells) that occurs during development of the nematode *Caenorhabditis elegans*.[25] The nature of the internal clock or switch that activates death is not known, although study of mutant *C elegans* with abnormal cell death phenotypes has led to characterisation of some of the genes involved, as

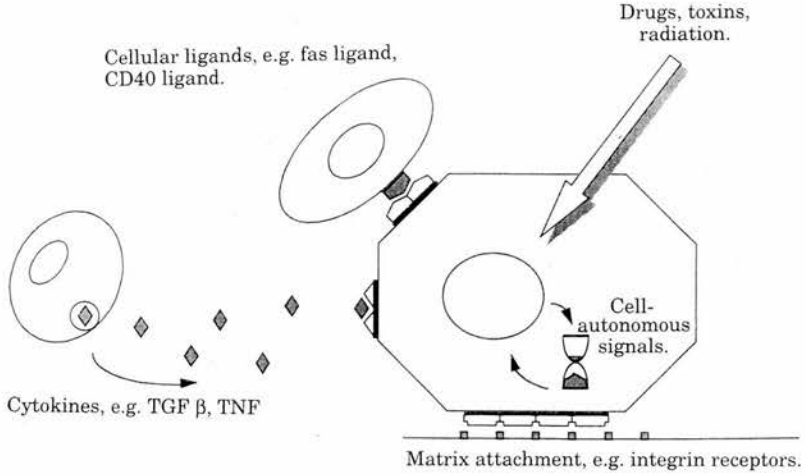


**Figure 3**. Cartoon illustrating the diverse sources of signals that regulate susceptibility to apoptosis.

discussed below. The rationale for this sort of inevitable cell death is easy to appreciate in limb morphogenesis but is less clear for *C elegans*, where mutant animals that lack programmed cell deaths survive and appear grossly normal. Observed minor behavioural changes and slower maturation have been hypothesized to put the cell death-deficient animals at a selective disadvantage compared with wild-type.[25]

### *External signals*

Specific ligand-receptor binding can transduce both death and survival signals. It is of relevance that a 'survival factor' need not be a mitogen and also that mitogens are not necessarily survival factors; indeed there appears to be benefit in separating the two functions. The TNF receptor superfamily includes several members that participate in positive and negative regulation of cell death, for example TNFRI, CD40, NGFR, CD30 and the fas antigen.[26-28] Receptor activation is not necessarily determinate of outcome and *signal context* is critical. For example, TNFα-induced apoptosis is blocked by expression of the zinc finger peptide *A20*[29], and fas stimulation kills chronically but not recently activated T cells, (a distinction postulated as a mechanism for limiting the extent of normal immune responses).[30] The importance of context is well illustrated by the dual signal model of B cell responses to crosslinking of surface immunoglobulin by antigen.[27] Crosslinking induces apoptosis, but not if a second signal is provided by costimulation of surface CD40. The ligand for CD40 is present on activated (but not resting) T helper cells, so apoptosis is only blocked during an antigen-specific immune response. Therefore, according to the model, self-reactive B cells will normally be deleted on encountering antigen, as a result of the absence of activated self-reactive T cells that could provide a rescuing signal. Thus the humoral signal of antigen binding is read in the context of a cellular signal (CD40 binding). Some agents have **dual effects** on the same cell population, for example TGFβ1 is a cytokine that both inhibits DNA synthesis and stimulates apoptosis of hepatocytes and endometrial stromal cells.[31,32] It is not clear what determines the particular response at individual cell level.

### *Competitive selection*

When apoptosis is the default fate of newly generated cells, competitive selection for survival factors is a powerful strategy to ensure that only those cells survive that are best suited to perform a particular function. Evidence from central nervous and haemopoietic systems suggests there are specific factors for different cell types and 'windows of susceptibility' during which cells are dependent upon particular factors.[33-35] This type of scenario is in contrast to the fixed, programmed cell death of *C. elegans*. For example, in neuromuscular development lower motor neurones are generated in excess and deleted by apoptosis if their axons fail to contact muscle end plates, the source of neurotrophic survival or rescue factors.[34] Likewise, during nephrogenesis, metanephric mesenchyme converts to epithelium under the influence of an inductive signal from the ureteric bud.[36] This signal rescues from the apoptosis that is the fate of uninduced cells. A similar principle brings about the increase in affinity of specific antibody produced during a humoral immune response (affinity maturation). This is achieved by selection for long term survival of only a small subpopulation of centroblasts, based upon the relative affinity of their surface immunoglobulin for the antigen, presented on follicular dendritic cells.[37] The remainder die by apoptosis in the light zone of the lymph node germinal centre, being seen as 'tingible bodies' within adjacent macrophages. Accessory survival signals are provided by cell adhesion molecules on the follicular dendritic cells, ICAM-1 and VCAM-1, which bind centroblast LFA-1 and CD49d respectively.[38]

Dependence on survival factors is also a key method of cell **population size regulation**. In kinetic terms a continuously renewing tissue (e.g. gut epithelium, bone marrow) consists of stem cells, progeny transit cells (which may or may not divide a few times) and post-mitotic, differentiated cells. There is normally overproduction of transit cells of which only a proportion survive to maturity, through the action of specific rescue factors, e.g. IL-1α, IL-6 for myeloid progenitor cells. The concentration of transit cell rescue factors is thus limiting and a determinant of final population size. Paracrine survival factors also maintain tissue localisation, e.g. within bone marrow. A requirement for survival factors by mature cells, which may differ qualitatively from that of immature cells, is illustrated by the atrophy of thyroid or adrenal glands after hypophysectomy, prostatic regression after castration and involution of post-lactational breast parenchyma that are all characterised by increased apoptosis.[1,2] The social control hypothesis provocatively takes the concept of survival factors to

its limit by suggesting that *all* cells (except blastomeres) are *continuously* dependent on survival signals from other cells to avert an intrinsic death program.[35]

This section has so far described how soluble factors and cell-cell interactions can control cell death, but cell-matrix interactions also regulate apoptosis. Specific contact of surface integrin receptors with extracellular matrix molecules is an important survival signal for differentiated endothelial and epithelial cells.[39,40] Without integrin-matrix binding these cells undergo apoptosis, a phenomenon that has been termed anoikis ('homelessness').[40] Such cells cannot therefore survive out of position if the appropriate matrix requirements are not fulfilled. Thus matrix composition localizes seed to soil in an unforgiving manner, a concept of critical importance to understanding mechanisms of neoplastic progression. In contrast, fibroblasts do not show integrin dependence,[40] in keeping with the need to rove across tissue boundaries during repair of injury.

### *Signal transduction*

The signal generated by a stimulus must be transmitted to effector molecules that often lie in different subcellular compartments. Transduction pathways vary according to the trigger stimulus but two major areas of focus are cytosolic calcium and protein kinases. Apoptosis is usually preceeded by a rise in cytosolic calcium concentration and possible downstream targets include calmodulin and calpain.[41,42] Calmodulin and calpain are themselves pleiotropic molecules whose precise roles in apoptosis need to be clarified. Protein kinase C (PKC) has been identified as both a positive and negative regulator of apoptosis,[42] while a protein kinase A pathway is active in thymocyte apoptosis.[43] It is possible, though without proof, that the conflicting roles of the PKC family may reflect the selective involvement of PKC isotypes[44] or interaction with other signalling pathways such as sphingomyelin-ceramide.[45] The *ras* pathway has long been known to transduce growth signals from plasma membrane to nucleus but is also a negative regulator of apoptosis when overexpressed in fibroblast cell lines.[46,47] Overexpression of activated *raf* kinase, a downstream element in the *ras* pathway, was also found to prevent apoptosis induced by IL-3 withdrawal.[48] New evidence for crosstalk between these different pathways is revealing a complex network of vertical and horizontal interactions that integrates different signals to determine the outcome of a stimulus.[49] The machinery for context-dependent responses is thus coming to light. A major unanswered question is how these pathways converge on the common effector processes of apoptosis.

### *Genetic regulation of apoptosis: strategies for investigation*

Simple observational studies on tissues can be very informative, providing physiological paradigms for cell death. These studies indicate the tissue compartments most susceptible to apoptosis under basal conditions, and can also provide prima facie evidence for a role for apoptosis in specific disease.[2,50] In *C. elegans* and *Drosophila*, observation of abnormal cell death phenotypes in mutant animals has allowed genetic analysis to identify novel genes controlling cell death.[25,51,52] The characterization of mammalian homologues of such genes and the use of double mutant animals to order the genes in a 'death pathway' show the power of the model.[25,53,54] Conversely, specific genetic manipulation of animals or cells offers clues to the functions of known genes of interest. Antisense aligonucleotides are a potentially powerful tool to abrogate a particular gene's function, although in practice a number of confounding factors present themselves. In fact each strategy inevitably has significant advantages and disadvantages: for example, drugs are powerful tools for the study of apoptosis; many are cancer chemotherapeutic drugs and as they reveal more about the regulation of cell death so that information in turn will suggest novel strategies of chemotherapy (see ref 55, this issue). However, interpretation of the effects of pharmacological agents must discriminate action at specific points on physiological death pathways from nonspecific activation of a suicide program. Likewise, genetic experiments that use the unregulated overexpression of genes in (genetically abnormal) cell lines are susceptible to criticisms of physiological relevance. Animals with germline targeted knockout of selected genes are a more physiological model, yet unique gene functions will still be much more readily apparent than those less critical or redundant, but which have relevance. Transgenic animals carrying extra genes in the germline that are constitutively overexpressed are also powerful models but still difficult to interpret in terms of normal function.

Together these different approaches have provided information that forms a surprisingly concordant picture of the internal regulation of apoptosis. They have delineated genes of three broad classes in a

common mechanism — those suppressing apoptosis, those defining the final common activation elements and those 'upstream' of the suppressors, but 'downstream' of the signal transduction described earlier, which we call here intermediate genes.

### *Genes suppressing apoptosis*

Induction of the *bcl-2* gene is often critical in the action of survival factors. *Bcl-2* is a mammalian homologue of the *C. elegans ced-9* gene, whose function it can partially replace,[53] and it can prevent apoptosis caused by a variety of physiological, pathological and pharmacological stimuli.[56] How *bcl-2* works is uncertain, although presumably it acts close to the final irreversible steps of apoptosis on which afferent pathways converge. There is evidence that *bcl-2* affects calcium partitioning and cellular redox status, although this is still controversial (see ref 57, RW Craig, this issue, pp35-43, also 58,59). However *Bcl-2* is not a universal antidote to cell death. For example, ectopic *bcl-2* expression by cortical thymocytes can prevent the induction of apoptosis by irradiation, glucocorticoids and antibodies to CD3, but does not affect negative selection.[60] This may be partly due to the presence of *bcl-2* antagonists such as $bcl_{XS}$ which is expressed at high levels in immature ($CD4^+CD8^+$) thymocytes.[61] $Bcl_{XS}$ has not been shown to interact directly with *bcl-2* but another antagonist, *bax*, seems to exist in dynamic equilibrium between *bax-bax* homodimers that are permissive for apoptosis, and *bax-bcl-2* heterodimers which may be a biologically active form of *bcl-2* that suppresses apoptosis.[62,63] It is not yet clear whether the *bcl-2* antagonists simply quench survival functions of *bcl-2* or whether they have intrinsic lethal properties that *bcl-2* must hold in check. In either case the relative amounts of such molecules in a cell could predetermine its response to a lethal stimulus. In tissues where *bcl-2* is not essential for survival such as the CNS (which develops normally in mice lacking *bcl-2*),[64] functional analogues exist, such as $bcl_{XL}$ which is expressed at high level in brain.[61] The antiapoptotic actions of the *ras* pathway were discussed earlier and it will be of interest to determine whether the *bcl-2* family interacts with *ras/raf* transduction pathways. *Abl* is another antiapoptotic gene that in contrast to *bcl-2* is biochemically well defined. The *abl* product is a tyrosine protein kinase and a constitutively activated form, retroviral *v-abl*, can suppress the apoptosis that follows growth factor withdrawal *in vitro*.[65] Protein kinase C and phospholipid hydrolysis may be mediators of this effect.[66] An *abl* fusion product (*bcr-abl*) in Philadelphia chromosome-positive human leukaemic cells is also constitutively activated and probably suppresses apoptosis in the malignant cells.[67] In addition to *v-abl* viruses have evolved a number of strategies to suppress apoptosis and these are discussed separately below.

### *Distal activating genes*

The *ced3* and *ced4* genes of *C. elegans* are strong candidates for distal activating genes if not ultimate effectors of apoptosis.[25] One vertebrate structural homologue of *ced3* is ICE (interleukin-1β converting enzyme), a cysteine protease that when overexpressed in fibroblasts induces apoptosis and inhibitors of which prevent the neuronal apoptosis that follows NGF withdrawal.[54] The universality of ICE's ability to induce death and its actual relevance to physiological apoptosis of normal cells are yet to be tested and it is likely that a family of ICE-related proteases will be identified. In some systems *bcl-2* is able to inhibit ICE-induced cell death, placing it downstream of ICE-like proteases in regulation of apoptosis.[54] Analogous attempts to order the *ced3/ced4* and *ced9* genes in a *C. elegans* putative death pathway are more speculative. Animals with *ced9* gain of function do not show *ced3/ced4*-dependent cell deaths, whilst animals lacking *ced9* function show excessive and abnormal *ced3/ced4*-dependent cell deaths.[52] If the three genes lie along one linear pathway then these observations place *ced9* proximal to *ced3/ced4*.[52] However, if *ced9* activation is a separate pathway to a *ced3/ced4* death cascade then *ced9* action could be either proximal or distal to *ced3/ced4* and still be consistent with the data above. Given the many similarities of *ced9* to *bcl-2* and of *ced3* to ICE it would seem reasonable at present to expect *ced9* to act downstream of *ced3*. The reaper peptide of *Drosophila* also seems to be a distal activator for apoptosis.[51] *Reaper* expression precedes apoptosis by 1-2 hours and is detectable in apoptotic bodies. Deficiency of *reaper* blocks developmental and radiation-induced apoptosis, although not completely, showing that *reaper* is not obligate for apoptosis to occur. Reaper has no homology to known peptides and the effect of expression in vertebrate cells is not yet reported.

### *Intermediate genes*

Many genes that regulate apoptosis were first identified as oncogenes or oncosuppressor genes. The c-*myc* oncogene is classically associated with promotion of

cell division and one might expect that constitutive activation of *myc* would be sufficient to induce autonomous cell proliferation. However, this is not so; in culture, activation of *myc* induces apoptosis unless specific rescue factors are also present, for example IGF-1 for fibroblasts or IL-3 for myeloid cells.[3,68] Thus *myc* activation simultaneously generates two possible outcomes in these cells: death (by default) or proliferation (if rescued) (Figure 4). Deregulated expression of the *myb* oncogene, at least in myeloid cells, may have a similar effect.[69] Such coupling of oncogene activation to survival factor dependence may serve two purposes. It opens a window of susceptibility to apoptosis (a 'high turnover' state), allowing competitive selection of susceptible cells to regulate population size as described above. It also forms an inbuilt safety mechanism to delete cells in the event of incongruous oncogene activity. A corollary of the 'high turnover' state model is that because proliferating cells in general are poised for apoptosis they will be susceptible to a variety of triggering agents, including cancer chemotherapeutic agents. In support of this idea epithelial cells in the proliferative zone of gastrointestinal crypts are more sensitive to irradiation and cytotoxic agents than adjacent non-proliferating crypt cells.[70] Tissue compartments in a high turnover state are therefore in a precarious balance between population expansion (excess proliferation) and regression (excess apoptosis), determined by the availability of mitogens, survival factors, cytotoxic stimuli and recruitment into or out of the high turnover state, e.g. differentiation. At a molecular level it is unclear whether the coupling of cell cycle activation to apoptosis is specifically mediated by *myc* or by a downstream component of an active cell cycle.

The *p53* oncosuppressor gene is the most frequently mutated gene in human malignancy (see ref 71, C.E. Canman, M.B. Kastan, this issue, pp17-25). It is critical for a DNA damage response to DNA strand breaks, produced either directly or during the excision-repair of other DNA lesions. In different cell types, such breaks result in G1 arrest or apoptosis.[72,73] What determines the differences in response is unclear, although of interest is the observation that overexpression in a cell line of transcription factor E2F-1 (a positive regulator of *myc* expression[74], involved in cell cycle regulation), switched a *p53*-dependent G1 arrest to a high turnover state with excess apoptosis.[75] E2F is shut off by hypophosphorylated pRb (the active retinoblastoma gene product), a negative regulator of cell cycle progression that is inactivated by cyclin-dependent kinases (cdk) in G1, themselves a target in *p53*-induced G1 arrest (via WAF1).[76-78] An interesting pathway is thus sketched out, in which *p53*/WAF1 growth arrest is achieved through cdk inhibition to activate pRb, which then shuts off E2F-1. However defects such as loss of pRb

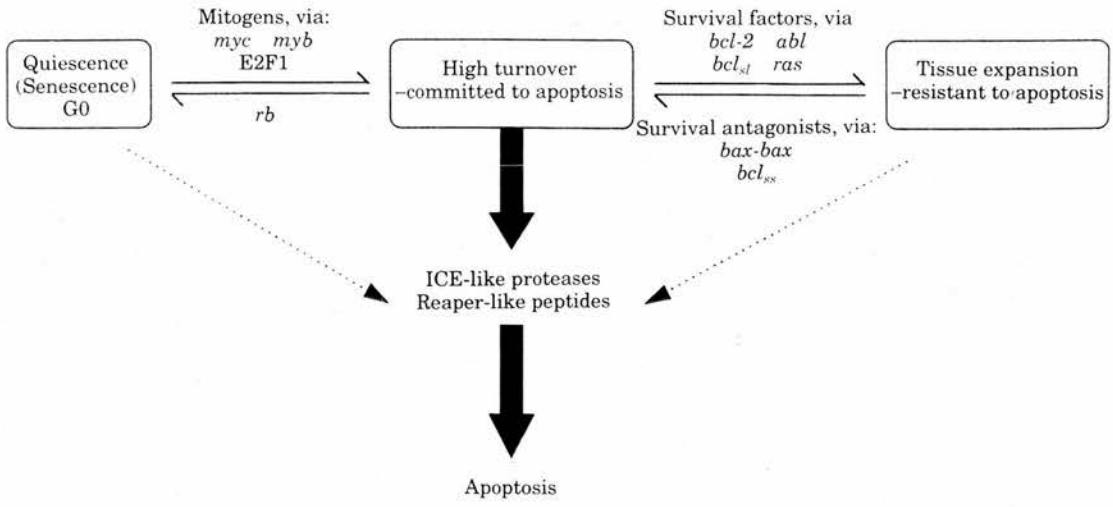


**Figure 4.** Schematic diagram to illustrate the differential susceptibility to apoptosis of proliferation-competent cells: cell cycle activation invokes a committment to apoptosis (a 'high turnover state') that is modulated by the dynamic action of 'survival genes' and their antagonists to determine net tissue growth or contraction. Note that apoptosis *can* occur in all the states of activation depicted. This scheme is not concerned with apoptosis following cellular injury (e.g. *p53*-dependent death), nor with regulation of apoptosis in non-proliferating cells.

function would prevent shut-down of E2F-1, which could then induce a high turnover state, as described above. This model is supported by the example of mice deficient in the retinoblastoma gene product. They die *in utero* with CNS and haemopoietic abnormalities, characterized by excessive proliferation and apoptosis.[79] The direct mechanisms of *p53*-dependent apoptosis are also becoming clearer. p53 downregulates *bcl-2* expression and also upregulates its antagonist *bax*.[80,81] This suppresses a major anti-apoptotic pathway and places *p53* proximal to *bcl-2/bax* in the regulation of apoptosis.[82]

The purpose of the decision to enter G1 arrest after DNA damage is unclear but one attractive hypothesis suggests that it provides an opportunity to repair DNA lesions before DNA replication occurs.[83] If the damage is in some way recognized as irreparable, the hypothesis suggests that *p53*-dependent apoptosis would be triggered, to prevent the replication of damaged DNA and a gradual accumulation of genetic defects that might result in carcinogenesis. In support of this hypothesis thymocytes and gastrointestinal crypt cells of p53-deficient mice lack the normal apoptotic response to DNA damaging agents, intriguingly in a gene-dose dependent fashion.[84,85] In addition *p53* has been shown to be critical for the maintenance of genomic integrity in serially passaged cells, and mice without functional *p53* genes die prematurely from malignancies.[86,87] Thus *p53* is involved in the policing, the ministration and the execution of cells with DNA damage. It is hardly surprising therefore that *p53* mutations are common in malignancy, given the advantages for neoplastic progression that disabling of *p53* confers.

*p53* is not critical for all apoptotic pathways (for example murine thymocytes from *p53*-deficient animals retain a normal apoptotic response to glucocorticoid),[84] but it is implicated in apoptosis effected *in vitro* by mechanisms apparently unrelated to DNA damage. Thus *p53* is required for normal sensitivity *in vitro* of haemopoietic cells to survival factors and loss of even a single allele reduces (but does not remove) the requirement for survival factors.[88] Introduction of wild-type *p53* into a leukaemic cell line has also been reported to generate dependence on IL-6 for survival, and p53 was necessary for the apoptotic response to serum withdrawal in a cell line cotransfected with adenovirus E1A and *ras* oncogenes.[89-91] Therefore, although mice deficient in *p53* develop normally[84] and *p53* is clearly not essential for developmental cell death, altered survival factor thresholds may influence population selection during development and provide a more favourable environment for carcinogenesis.

A number of other gene products are implicated in the control of apoptosis (e.g. clusterin, *c-rel, fos,* cyclin D1[92-95]), but in most instances it still remains to sort primary regulators from secondary perturbations.[96,97] Recent evidence from a myeloid leukaemia cell line has implicated the *Myd118* gene as a downstream mediator of TGFβ-induced apoptosis and has suggested that *bcl-2* inhibits apoptosis of these cells via downregulation of *Myd118* expression.[69]

## Disease connotations: apoptosis in disease and the responses to disease

It is evident that apoptosis provides a powerful regulatory mechanism for many aspects of normal tissue growth and function. This section extends the discussion to describe how apoptosis regulates the responses to disease and how defective regulation of apoptosis may be central to the pathogenesis of many important disorders.

### *Inflammation*

The response to injury or infection has itself considerable potential to damage tissue and it is therefore tightly regulated. Neutrophils, eosinophils and monocytes die by apoptosis within a relatively short period (e.g. 3-4 days for eosinophils in culture), however death can be significantly delayed by proinflammatory cytokines such as C5a (neutrophils), IL-1β, TNF-α, IFN-γ (monocytes) and IL-5 (eosinophils).[98,99] In contrast TGFβ and TNFα accelerate eosinophil and neutrophil apoptosis respectively.[100,101] This suggests a potential mechanism *in vivo* for control of the survival and ultimately the removal of these potentially dangerous cells from sites of inflammation when the inflammatory stimulus subsides. Defects in these mechanisms or in the clearance of apoptotic cells may underlie some chronic inflammatory diseases (e.g. hypereosinophilic syndromes) due to inappropriate persistence of inflammatory cells with continued release of toxic cellular contents perpetuating tissue injury and inflammation. Of interest in this regard is the multifocal inflammatory disease and tissue necrosis that occurs in mice without functional TGFβ1.[102]

### *Autoimmunity*

Apoptosis is critical for the development, function and regulation of the immune system (see ref 103, B.A. Osborne, this issue, pp27-33). One instructive example is thymocyte maturation. A competent immune system must recognise a wide variety of foreign antigens but provision must be made to delete self-reactive effector cells that would otherwise cause autoimmune disease. In the thymus, 97% of thymocytes are deleted in their first few days of life.[104] This occurs as a result of a complex weeding-out process in which immature thymocytes die by apoptosis unless able to recognise and bind antigen presented in association with self MHC molecules (*positive selection* for MHC restriction) and yet die also if the receptor occupancy is too great (*negative selection*).[105] The principle is that high receptor occupancy is likely to reflect reactivity to self-antigen since most antigen presented in the thymus is self-derived. However, negative selection is certainly more complex than that, and further contributory mechanisms involving other surface signals are likely.[105-108] In concert with thymic selection, peripheral deletion of mature T cells is also important to prevent autoimmune tissue damage. One mechanism involves apoptosis induced by activation of surface fas molecules. Fas (CD95, Apo-1 receptor) is a member of the TNF receptor superfamily and like some other members can transduce a signal for either apoptosis or proliferation. Mice without functional fas or fas ligand accumulate abnormal $CD4^-CD8^-$T cells and develop autoimmune disease resembling human systemic lupus erythematosus (SLE).[94] These mice have lost the antigen-driven fas-dependent apoptosis of mature T cells that maintains tolerance to self.[110] Further compelling evidence for a direct pathogenetic role of fas-mediated cell death is provided by the recent observations in SLE patients of elevated serum levels of a soluble form of fas at concentrations that blocked induction of fas-mediated apoptosis *in vitro*, and that in mice caused altered lymphocyte development and proliferation responses to self antigen.[111]

### *Cytotoxic lymphocyte (CTL) killing*

Cell-mediated cytotoxicity is an integral component of specific host defences, for example against virally infected cells, and apoptosis is believed to be the mode of death in a proportion of CTL-induced target cell killing.[26] Evidence suggests that activation of target cell fas by the CTL is an important mechanism for this cell-mediated apoptosis, although engagement of target cell TNF receptors may also act to trigger apoptosis in some instances.[112,113] The finding that activation of surface fas on hepatocytes triggers apoptosis[114] suggests a potential pathogenetic mechanism for viral or perhaps autoimmune hepatitis that may have implications for new strategies of therapy. It is of relevance to note that CTL can kill by a mechanism that involves perforin insertion into target cell membranes and granule exocytosis.[26] The relative importance and interactions of these different modes of killing is not established.

### *AIDS*

The gradual depletion of $CD4^+$T cells during HIV infection that leads to clinical AIDS is thought to be due to excessive apoptosis.[114] HIV-infected cells express a viral envelope transmembrane gp120-gp41 complex which binds the CD4 D1 domain of uninfected T cells and triggers apoptosis directly.[115] Furthermore HIV particles shed gp120 which, although unable to trigger apoptosis itself, can bind CD4 and program uninfected T cells for apoptosis (instead of proliferation) in response to subsequent T cell receptor stimulation by antigen.[116] The deletion of naive and memory T cell clones on encountering their specific antigen abolishes the individual's ability to mount a specific immune response to infections.[6,116]

### *Neoplasia*

Escape by cells from normal activation of apoptosis allows survival of 'forbidden clones' that would otherwise have been deleted. This might allow the propagation of, for example, new genetic defects that would have been eliminated through p53-dependent apoptosis. In addition, reduced dependence on survival factors may be important in the early stages of carcinogenesis to allow expansion of subpopulations of cells capable of subsequent progression to malignancy. An example is experimental liver tumour promotors such as nafenopin and phenobarbital that reversibly inhibit apoptosis with consequent hyperplasia and development of preneoplastic foci.[117] Constitutive bcl-2 expression by follicular lymphomas is an example of a death suppression strategy in human neoplasia.[118] The qualities that allow survival of malignant cells in foreign tissues have received little attention but are a *sine qua non* for invasion and

metastasis. It is likely that escape from integrin dependence is one such necessary event.

### *Oncogenic viruses*

Oncogenic viruses have developed strategies to prevent host cell apoptosis that have shed light on control pathways. The Epstein Barr virus BHRFI protein is a *bcl-2* homologue, whilst the LMP-1 protein upregulates *bcl-2* expression and induces the *A20* zinc finger protein that confers resistance to TNFα cytotoxicity.[119-121] The adenovirus E1B gene encodes a functional homologue of *bcl-2* and a protein that inactivates the *p53* oncosuppressor.[122,123] In fact several viruses inhibit *p53* function in different ways, including SV40 (large T antigen), Epstein Barr virus (EBNA 5), human papillomavirus types 16 and 18 (E6 protein) and hepatitis B virus (HBx protein) (see ref 124 for references). This is a testament to the importance of that molecule in countering abnormal cell proliferation. Interestingly, many oncogenic viruses contain genes that activate cells from the growth arrested state (SV40 T antigen, adenovirus E1A, HPV E7) probably via inactivation of Rb protein, release of transcription factor E2F and activation of c-*myc*. At least some of these changes also imply increased susceptibility to apoptosis, as discussed earlier. The combination therefore of pro-apoptotic oncogenes with others having anti-apoptotic activity appears to be an essential part of the viral strategy to induce cell proliferation without also activating cell death.

### *Cancer therapy implications*

The ability to modify sensitivity to apoptosis through the regulatory pathways has clear implications for the treatment of malignancy.[125] Potential strategies fall into three categories — direct triggering of apoptosis by cytotoxic agents, enhancing susceptibility to apoptosis to increase the efficacy of other therapies, and boosting the resistance of normal cells to apoptosis (with survival factors). Restoration of function of interrupted apoptotic pathways, e.g. *p53*-dependent apoptosis, with consequent self-deletion by tumour cells would be a most attractive strategy. Bcl-2 antagonists might likewise be expected to cause regression of follicular lymphomas or at least to increase their radio- or chemosensitivity. Induction of a high turnover state (with survival factor dependence) or antagonism of tumour survival factors (e.g. antiandrogens for prostate carcinoma, tamoxifen for oestrogen receptor-expressing breast carcinomas) are other approaches to therapy. Boosting normal cell resistance to apoptosis with exogenous survival factors can be used after ablative therapy to improve restoration of the normal cell population,[126] reducing treatment morbidity and allowing greater frequency of cytotoxic treatments.

## Conclusion

Apoptosis has an importance in physiology and pathology that has only recently become fully appreciated. Its purpose is to rapidly delete single cells from living tissue without interrupting tissue function or structural integrity. Diverse fields of developmental, cell and molecular biology are linking to contribute to a deeper understanding of apoptosis that has a direct relevance to a variety of human diseases and in some instances suggests new therapeutic strategies. Much of the basic biology of the regulation and effector events of apoptosis remains obscure but the field is now subject to such an intense level of investigation that it is not unrealistic to expect great advances within a very short time.

## Acknowledgements

COCB is a Cancer Research Campaign Gordon Hamilton Fairley Clinical Fellow. The authors' research is supported by the Cancer Research Campaign.

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# p53 and apoptosis

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Loss of function of the p53 tumour suppressor gene is a frequent and important event in the genesis or progression of many human malignancies. Loss of p53 dependent apoptosis is believed to be critical to carcinogenesis in many of these cases, suggesting the possibility to therapeutically restore this pathway and directly eliminate malignant cells or increase or restore their sensitivity to chemotherapeutic agents.

The regulation of p53-dependent responses is complex and variable between cell types, and whether a cell undergoes apoptosis after activation of p53 is highly sensitive to signal context, including environmental and cell intrinsic influences. This article focuses upon p53-dependent apoptosis, considering current understanding of the biochemical steps involved, the factors determining selection of apoptosis over other p53-dependent responses, the significance of p53-dependent apoptosis for the genesis, progression and drug resistance of human cancers, and finally the prospects for clinical manipulation of this pathway in cancer therapy.

The new interest in apoptosis has touched many fields, but none more so than cancer biology. Apoptosis is envisaged as eliminating cells with DNA damage or growth dysregulation that could become precursors of malignant clones. In this way it complements growth arrest and DNA repair as mechanisms to preserve the genetic integrity of tissues. Until recently these mechanisms represented distinct fields of research. However, exciting new evidence suggests that a small set of common regulatory molecules are involved, and the integration of previously complimentary fields is bringing a new depth of understanding to cancer biology. The p53 tumour suppressor gene product is central to this new focus. Roles for p53 have been identified in aspects of DNA damage recognition, DNA repair, cell cycle regulation and most particularly in triggering apoptosis after genetic injury (see Ko and Prives[1], for overview).

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p53 is the most commonly mutated gene in human malignancy, prevalent in cancers of a wide variety of histogeneses and primary sites. This wide occurrence of defective p53 derives from 3 properties. First, wild type p53 is highly vulnerable to dysfunction caused by even a single base change in the coding sequence. Second, in contrast with classical tumour suppressor gene theory a single abnormal p53 allele or allele loss can alter phenotype. Depending on the gene lesion, this manifests by a

gene-dose dependent reduction in certain p53 functions, a dominant negative inhibition of the remaining wild type allele's function, or gain of a novel function(s) not associated with wild type. Third, the participation of p53 in multiple pathways of fundamental importance to carcinogenesis makes it an Achilles' heel of cancer suppression, a defect in which can radically diminish cellular defences against carcinogenesis.

This article focuses upon p53-dependent apoptosis, highlighting differences between cell types and reviewing current understanding of the biochemical steps involved, the factors determining selection of apoptosis over other p53-dependent responses, the significance of p53-dependent apoptosis for the genesis, progression and drug resistance of human cancers, and finally the prospects for clinical manipulation of this pathway in cancer therapy.

## The p53 protein

The biochemistry and molecular genetics of p53 have been reviewed in detail elsewhere[2]. p53 is a nuclear DNA-binding phosphoprotein that normally exists as a homotetramer or complex of tetramers. It is a transcriptional activator of a specific set of target genes, and can exert transcriptional repression, probably by interaction with transcription factors or the general transcription machinery. p53 also interacts directly with cellular proteins and is itself a target of several viral proteins. It is present *in vivo* in a biochemically latent form and is normally rapidly degraded ($t_{1/2}$ ~30 min), probably by ubiquitin-dependent proteolysis. p53 activity and stability are regulated post-transcriptionally and post-translationally by still incompletely understood mechanisms that include alternative splicing, conformational change, phosphorylation, protein-protein associations and regulation of nuclear localisation. p53 also negatively regulates its own transcriptional activity through induction of the mdm2 oncogene, forming a negative feedback loop. The activity and stability of p53 protein vary in a cell cycle dependent manner, although p53 is not required for normal mitotic or meiotic cycles.

## p53-dependent apoptosis

### *Upstream events*

Triggers for p53-dependent apoptosis include DNA damage, inappropriate oncogene activation, certain cytokines[3] or cytokine deprivation[4], hypoxia[5] and heat shock[5]. The biochemical pathways converging onto

p53 are not clearly defined, although there is stimulus specificity, for example thymocyte apoptosis triggered by DNA damage is p53-dependent but that triggered by dexamethasone is not[6].

DNA damage is the best defined stimulus of p53-dependent responses and in several cell types is coupled to stabilisation and accumulation of p53 protein. Indeed, analysis of cells and tissues of mice homozygous-deficient for p53 has definitively shown that p53 is required for DNA damage-induced apoptosis of cortical thymocytes[6], myeloid progenitor cells[7], marrow pre-B cells[8], quiescent peripheral B and T lymphocytes[8], cerebellar granule neurones[9], keratinocytes[10] and proliferating crypt epithelial cells of small and large intestine[11]. In these situations p53 itself may be the sensor of DNA damage. The full range of DNA lesions that provoke a p53 response is not known, but primary lesions to which p53 binds directly *in vitro* include insertion/deletion mismatches and DNA strand breaks. p53 binds avidly to strand breaks and this stabilises and activates the otherwise short-lived protein. Whereas γ-irradiation and some chemical agents produce strand breaks directly, for many genotoxins the strand breaks are only generated indirectly, during DNA repair, when short patches of DNA bearing the damaged nucleotides are excised. In this way, p53 is made sensitive to a broad range of different DNA lesions. As yet poorly defined response-enabling pathways are probably important for a normal p53 response to DNA damage, since cells from patients with the inherited radiosensitivity syndrome ataxia telangiectasia or Fanconi anaemia show an attenuated and delayed p53 response to γ-irradiation[12,13].

Aside from DNA strand breaks, little is known about how the other triggers of p53-dependent apoptosis couple to p53, but there is evidence for distinct pathways[5]. One novel stimulus for p53 induction is depletion of ribonucleotide triphosphates (rNTP), necessary for RNA synthesis[14]. So far, this pathway has been linked only to p53-dependent growth arrest rather than apoptosis, but the possibility that p53 could be responsive to alterations in RNA as well as DNA is intriguing.

## *Signal transduction through p53*

Within 3 h of γ-irradiation *in vivo*, murine splenocytes, thymocytes and osteocytes show dramatic accumulation of immunoreactive p53, lasting for over 48 h[15]. UV irradiation of human skin sufficient to produce mild sunburn generates similar kinetics of p53 protein accumulation in keratinocytes and dermal fibroblasts[16]. But although p53 accumulation in normal cells is characteristic of p53-dependent responses, it is not specific for any particular response, and indeed may not be the critical

event that precipitates engagement of downstream pathways. *In vitro*, large changes in transcriptional transactivation by p53 are achievable independently of protein levels[17,18] and p53 responses can be triggered without changes in protein concentration, suggesting that accumulation is not always sufficient or perhaps even necessary for engagement of p53 downstream responses. Instead 'activation' of p53, for example by phosphorylation, could determine initiation of downstream events. p53 activates transcription of its target genes by interactions with specific p53-response motifs and these interactions are selectively regulable through changes in p53 phosphorylation[19]. Simultaneous up- and down-regulation of p53 interactions with different response elements is possible, and hence to better understand p53 function, experiments using panels of reporter constructs for p53 response elements may be necessary to dissect out qualitative changes in patterns of p53 transcriptional transactivation.

The binding of p53 by endogenous regulatory proteins can also regulate function; for example the Wilms tumour suppressor gene product, WT1, inhibits p53-dependent apoptosis without affecting p53-dependent growth arrest[20]. Moreover, a significant proportion of p53 protein in at least some cell types is alternatively spliced, and almost certainly functionally different to the whole protein[21]. However, the role of alternative splicing in regulating p53 is not understood at present.

In summary, p53 is more than a simple link in a chain of signalling, and there is a tremendous complexity of signal transduction possibilities through p53. But whilst *in vitro* work is informative, still little is known about how signals are processed through p53 *in vivo*.

## Downstream events

Cells with only one functioning copy of the p53 gene have intermediate rates of apoptosis between null and wild type cells (a gene dose effect), suggesting an induction threshold for apoptosis that lies within the upper range of physiological p53 activity, and a subtlety to regulation *in vivo* that constitutive expression systems do not interrogate. The particular p53 activity most critical for apoptosis is still a matter for debate. Interpretation of various apparently contradictory findings is complicated by the use of expression systems generating supraphysiological amounts of protein, or in which endogenous p53 or viral proteins that target p53 are present. Nevertheless, *in vitro* there appear to be distinct mechanisms by which p53 can be made to engage apoptosis[22,23]. One mechanism requires specific transcriptional transactivation by p53, perhaps of *bax*, a member of the *bcl-2* family that in relative excess to

*bcl-2* permits apoptosis. However, *bax* is dispensable for p53-dependent apoptosis in γ-irradiated thymocytes[24], suggesting that other factors are more important. Transcriptional repression by p53, for example of *bcl-2*, may also contribute to apoptosis[25] and modifying influences by other members of the bcl-2 family are probably relevant, for example *bcl-x*$_L$ can inhibit p53-mediated apoptosis *in vitro*[26].

Additional to effects on gene expression, p53 interacts directly with proteins and there is evidence that binding of the TFIIH protein complex is critical for a pathway of p53-dependent apoptosis that does not involve specific transcriptional transactivation[23]. p53 inhibits the helicase activity of this complex by binding to the XPB (ERCC3) and XPD (ERCC2) subunits, both of which are required in this apoptotic pathway[23,27]. Thus, a single protein defect can compromise both repair and apoptosis responses to DNA damage. The data are particularly provocative since TFIIH participates in basal transcription, nucleotide excision repair, and probably also in cell cycle control. Thus, a core element is identified, through which dynamic regulation and coupling of these critical cellular processes can be achieved and the balance shifted according to circumstance. Moreover there is a basis for understanding how defects in one pathway can have effects on the others and the balance between them.

Once p53-dependent apoptosis is triggered, there is no evidence that it differs in any way from apoptosis induced by other means, and it is not doubted that downstream events feed into a common effector pathway of apoptosis. In summary, the biochemical steps by which p53 triggers apoptosis are still incompletely defined, but there is evidence for distinct mechanisms that predominate according to cell type and may be interactive[22,23,28].

## *What determines the outcome?*

When normal proliferating cells sustain DNA damage they respond in one of two ways: cell cycle arrest or apoptosis, and p53 is implicated in both. For example after 5 Gy γ-irradiation, proliferating fibroblasts growth arrest[29], whereas proliferating intestinal crypt epithelium undergoes apoptosis[11], both by p53-dependent mechanisms. These and other experiments show that cell type is an important determinant of the outcome of p53 activation. However, in culture, it is possible to switch one response to another (see below), suggesting a potential flexibility of outcome and the existence of cellular decision mechanism(s) that determine the predominant p53 response pathway. Greater understanding of these issues would better define the contributions of p53 to

tumour suppression in different tissues, and the data available will now be discussed.

*Growth arrest and apoptosis:* p53 can cause cells to growth arrest at certain stages in the cell cycle ('checkpoints'), of which the best understood is late $G_1$ phase arrest. p53 triggers $G_1$ arrest through specific transcriptional activation of the cyclin-dependent kinase inhibitor, p21. Embryo fibroblasts from p21-null mice are correspondingly severely deficient in p53-dependent $G_1$ arrest[30,31] (although not completely so, suggesting that other minor p53-responsive pathways to $G_1$ arrest exist, perhaps involving the p53 target gene GADD45[32]). By contrast, p21-deficient mice retain p53-dependent apoptosis in tissues such as thymus and small intestinal epithelium[30,31]. These and other experiments indicate that the p21 growth arrest pathway is distinct from pathways to p53-dependent apoptosis[22,23].

It is possible that, in some circumstances, p53 simultaneously signals growth arrest and apoptosis, and downstream suppression of apoptosis is necessary to prevent that outcome. For example, use of a bcl-2 transgene to delay p53-dependent apoptosis in a myeloid leukaemia cell line revealed a p53-dependent growth arrest[34]. Moreover, the suppression of apoptosis may derive from the growth arrest pathway, as suggested by experiments in which removal of p21 from colorectal carcinoma cell lines that normally engaged p53-dependent growth arrest, caused them instead to undergo apoptosis[35]. Intriguingly, carcinoma cells heterozygous for p21 showed a split response between growth arrest and apoptosis, suggesting that the switch of response occurred within a narrow physiological range of p21 expression. Therefore, although mechanistically distinct, the pathways of p53-dependent growth arrest and apoptosis may communicate.

Commitment to p53-dependent apoptosis is also regulated by extrinsic influences from the local environment; for example cytokine survival factors can inhibit p53-dependent apoptosis[4,7,36]. Indeed, signal context is a critical determinant of the response to p53 activation. Experiments on cultured fibroblasts have shown that growth arrest switches to apoptosis if the growth arrest signals (e.g. serum depletion or activated p53) are challenged with forced growth activation signals (e.g. from deregulated c-myc or E2F oncogenes)[33,37,38]. This link between deregulated proliferation signals and apoptosis has also been observed *in vivo*, in embryos null for the retinoblastoma (Rb) tumour suppressor gene product, $p105^{Rb}$, that inhibits cell cycle progression. The embryos die in mid-gestation with excessive, uncontrolled proliferation but also apoptosis of cells in the developing nervous and haemopoietic systems. Study of lens epithelium has shown that the apoptosis is p53-dependent[39]. The coupling of contextually inappropriate proliferation

signals to apoptosis probably represents a defence against autonomous oncogene-driven proliferation, deleting the offending cell by apoptosis unless other permissive factors are present. Such models are clearly relevant to understanding tumour suppression (see below), although it is still not certain how closely they reproduce mechanisms of choice within normal cells.

*DNA repair and apoptosis:* p53 was originally suggested to facilitate DNA repair simply through establishing growth arrest and so providing extra time for completion of repair before DNA replication or mitosis. However, a more active contribution is now evident. A detailed consideration of this aspect of p53 function is beyond the scope of this article, but it is clearly of great importance to clarify whether p53-dependent apoptosis is coupled to its repair activities, as hinted by the interaction with XPB and XPD described earlier. For example, do repair and apoptosis after DNA damage share damage sensing mechanisms? Is apoptosis triggered by repair rather than damage? One influential hypothesis to explain responses to DNA damage suggests that cells have the capacity to recognise when DNA damage is too profound to be repaired completely or sufficiently rapidly, whereupon p53-dependent apoptosis is triggered, preventing replication of a damaged genome[40]. This remains unproven, but the observation of proteins common to repair and p53-dependent apoptosis demonstrate potential for such a decision mechanism.

## p53-independent apoptosis

p53-independent pathways can determine apoptosis and growth arrest responses to DNA damaging agents. Even cell types such as intestinal crypt epithelium which show p53-dependent apoptosis (usually manifested within 24 h of genotoxic insult), can have additional late phase p53-independent apoptosis (Clarke AR, unpublished results). These pathways to apoptosis may be triggered by the DNA damage itself or alternatively, genotoxins can affect cellular components other than DNA and trigger apoptosis through pathways quite unrelated to the genetic injury. For example activation of membrane sphingolipase by $\gamma$-irradiation is not related to the DNA damage[41], but triggers apoptosis in some tissue types through a ceramide pathway. This pathway is probably p53-independent and may be responsible for most of the pulmonary endothelial apoptosis observed after $\gamma$-radiation injury[42].

### *IRF-1 and p53*

In some cell types, apoptosis or growth arrest following γ-irradiation has been shown to be dependent upon the transcription factor interferon regulatory factor 1 (IRF-1). For example, apoptosis of activated peripheral T lymphocytes after γ-irradiation is IRF-1 dependent and not p53-dependent[43]. By contrast, IRF-1 is not required for the p53-dependent apoptosis of γ-irradiated thymocytes and, together, these data suggest that p53 and IRF-1 pathways to apoptosis are independent and operate in distinct cell populations[43]. The situation for growth arrest is more complex: p21 expression can be up-regulated by several p53-independent pathways, and at least one, requiring IRF-1, is also stimulated by DNA damaging agents[44]. Intriguingly, experiments using p53-null and IRF-1-null mouse embryo fibroblasts have shown that each pathway alone is insufficient to trigger growth arrest and that they must act in concert[44]. This suggests that a threshold of p21 activation exists that is not easily achievable by a single damage response pathway. In this way, p53-dependent growth arrest is mutually dependent upon co-activation of one or more p53-independent pathways. Thus, once again, the context in which an individual signal acts is seen to be critical to the outcome of p53 activation.

## Contributions of defective p53-dependent apoptosis to cancer development

It is difficult or impossible to understand the contribution to tumour suppression of different p53 functions without the use of model systems, of which transgenic and gene-targeted mice continue to provide critical data. Even so, the overlapping contributions to genomic stability and tumour suppression of growth arrest, DNA repair, apoptosis and other functions are probably not completely separable, and it may be the interaction and coupling between these activities which will prove critical in cancer development. Nevertheless, there is considerable evidence that defective p53-dependent apoptosis has pathogenic significance for human cancer.

### *Oncogene-triggered apoptosis*

As described earlier, p53 can limit the carcinogenic potential of aberrant oncogene activation, by triggering apoptosis. This is an effective protection against not only endogenous oncogene activation but also

viral oncogene expression in host cells. Not surprisingly, however, oncogenic viruses such as EBV, adenovirus and HPV have evolved counterstrategies to evade or block the induction of apoptosis, *ipso facto* evidence for its importance. For example, the adenovirus E1A oncogene is unable alone to transform primary cells since the affected cells die by p53-dependent apoptosis. However, adenovirus produces a second protein, E1B, that inhibits p53-dependent apoptosis, and this allows sustained proliferation and transformation to occur[45].

The significance of oncogene-activated, p53-dependent apoptosis for suppression of carcinogenesis has been directly tested *in vivo*: In a transgenic mouse model of experimental choroid plexus tumours, a variant of the SV40 T antigen that functionally disrupts only the retinoblastoma family of proteins (leading to aberrant E2F oncogene activation) but not p53, produced atypical hyperplasia associated with increased apoptosis[46]. The added effect of p53 inactivation was shown to reduce the excess apoptosis, without affecting proliferation rates, leading to the rapid development of highly malignant tumours. A similar demonstration was provided by mice bearing an HPV16 E7 transgene that was expressed in photoreceptor cells[47]. The transgene caused functional Rb-1 inactivation, leading to abnormal activation of the E2F oncogene. In these transgenic mice the photoreceptor cells failed to terminally differentiate and instead underwent apoptosis. However, when the analysis was repeated on a p53-deficient genetic background, a similar pattern of apoptosis was not observed and the transgenic animals developed neoplasms arising from the photoreceptor cell layer.

## *DNA damage-triggered apoptosis*

The elegant experimental systems described above show that p53-dependent apoptosis in response to inappropriately activated proliferation signals inhibits carcinogenesis at an early stage by deleting the potentially neoplastic cells. They do not, however, test the hypothesis that DNA damage-induced p53-dependent apoptosis suppresses carcinogenesis. Indeed, this is difficult to do because of difficulty in isolating p53-dependent apoptosis from its repair or growth arrest functions. For example, p53 deficient keratinocytes from gene targeted mice were reported to show a gene dose dependent reduction in the normal apoptotic response to UV-irradiation, the prime aetiological agent of squamous carcinoma of the skin[10]. This leads to survival of clones which, over successive exposures to UV, should predominate over wild type in the epidermis and acquire further mutations. However, p53 dysfunction also renders keratinocytes deficient in repair of UV-induced

DNA damage[48]. Moreover, keratinocytes from mice carrying a mutant p53 transgene showed only decreased DNA repair but no alteration in apoptosis following UV irradiation, despite increased susceptibility of these mice to UV-induced squamous skin carcinomas[48]. Thus the role of p53-dependent apoptosis in UV-induced skin carcinogenesis remains uncertain.

The intestine provides an alternative tissue to explore this question since p53-dependent apoptosis following genotoxic damage of crypt stem cells has been independently documented by different groups[11,49]. However, in γ-irradiated murine small intestine at doses adequate to trigger p53-dependent stem cell apoptosis, p53 deficiency was not associated with increased numbers of mutated stem cells compared with wild type (as assessed in an endogenous indicator gene), suggesting that inappropriate survival of genetically damaged cells does not necessarily equate to increased tissue mutability *in vivo* (Clarke AR *et al*, in preparation).

The thymus presents another tissue model for DNA damage induced apoptosis in tumour suppression. p53 null thymocytes are deficient in apoptosis after DNA damage but not other physiological stimuli (e.g. corticosteroid) and are at a greatly increased risk of lymphomagenesis that is still further accelerated by γ-irradiation[6,50]. By contrast, p21-deficient mice (which have defective p53-dependent $G_1$ growth arrest but preserved p53-dependent apoptosis) do not show increased susceptibility to spontaneous cancers, including thymic lymphomas[31]. However, whilst retention of p53-dependent apoptosis in these mice may explain the preservation of tumour suppression, p53-dependent repair and other cell cycle checkpoints could also be intact. Susceptibility of p21-null mice to DNA damage-induced carcinogenesis has not yet been reported.

Thus, whilst DNA damage-induced p53-dependent apoptosis is likely to contribute to suppression of carcinogenesis, particularly in haemopoietic or lymphoid tissues[50], and in the embryo is critical to suppress radiation-induced teratogenesis[51], it can be difficult to separate such a contribution from other p53-dependent activities.

## *Sensitivity to local environment*

p53 regulates dependence on cytokine survival factors, as demonstrated for haemopoietic cells[7], prostate[52] and hepatocytes (Bellamy COC *et al*, submitted). A potential contribution of p53 dysfunction to carcinogenesis and tumour progression is, therefore, through increased survival (decreased apoptosis) in competitive or unfavourable environments, for example within solid neoplasms or during neoplastic spread to other

tissues. Hypoxia and associated necrosis are prevalent in solid malignant neoplasms, and the latter has often been correlated with a poor prognosis. Although normal cells were relatively resistant to hypoxia, transformation was found to make them sensitive to apoptosis under conditions of extreme hypoxia[53]. However, p53 deficiency (or Bcl-2 overexpression) protected against hypoxia-induced cell death both *in vitro* and *in vivo*, and over successive exposures to hypoxia resulted in overgrowth of cultures by an initially small fraction of p53-deficient cells[53]. Hypoxia was thus shown to exert a selective pressure for loss of p53-dependent apoptosis from neoplastic cells.

Wilms' tumour provides an authentic human example in which p53-dependent apoptosis may be relevant to tumour progression. p53 mutations are rare in this paediatric malignancy except in the poor prognosis anaplastic variant, characterised by focal areas of anaplastic morphology within the neoplasm. In these tumours the p53 mutations are restricted to the histologically anaplastic tissue, which was also shown to display much reduced apoptosis compared with surrounding non-anaplastic tumour[54]. These studies, therefore, imply a pathogenetic role for p53 inactivation in Wilms' tumour progression and suggest that loss of p53-dependent apoptosis may be the critical event, analogous to the mouse models described above.

## p53 and responses to cancer therapy: sensitivity or resistance?

Irradiation and many drugs used to treat cancers are primarily genotoxic, either directly or by disrupting DNA metabolism and, at therapeutic doses, trigger apoptosis in the target cells. If the apoptosis is due to DNA damage then the p53 status of the neoplastic cells might be expected to modify the drug effect. Two contrasting scenarios are envisaged. Firstly, in cells that readily undergo p53-dependent apoptosis as the preferred response to DNA damage, p53 dysfunction could allow survival and, therefore, resistance to treatment. Indeed the surviving cells may have acquired further mutations as a result of exposure to the treatment agent and behave more aggressively than before. However, if p53-dependent apoptosis is not a readily invoked consequence of DNA damage, and instead growth arrest and repair activities are compromised by loss of p53 pathways, then neoplastic cells are more likely to enter S phase and mitosis bearing high levels of unrepaired damage and viability could be decreased. Moreover, the cycling fraction of p53 deficient cell populations is often high and, if the drug is one that preferentially acts during S phase or mitosis, lethality would be increased, simply because a

greater proportion of neoplastic cells pass into susceptible cell cycle phases during exposure of the patient to the drug. The consequences of altered p53 status for drug efficacy in cancer therapy are, therefore, difficult to predict. Indeed, *in vitro* evidence exists for sensitisation, resistance and no effect conferred by loss of wild type p53 on the efficacy of chemo- or radiotherapy[55–58]. Given these caveats to broad generalisations, some of the experimental evidence that p53 is relevant to cancer therapy will now be discussed.

As discussed earlier, transformation of normal cells by oncogenes sensitises them to triggering of apoptosis that is often p53-dependent. This lowers the threshold at which irradiation and many drugs used in cancer chemotherapy induce apoptosis, as demonstrated *in vitro* for fibroblasts transformed by E1A+ras oncogenes. In that system, the p53 genetic background of the transformed fibroblasts was a critical determinant of drug-induced apoptosis; p53 null transformed cells were resistant to doses of γ-irradiation or adriamycin that efficiently killed p53 wild type transformed fibroblasts by apoptosis[56]. Significantly, identical results were found *in vivo* when tumours derived from the transformed cells were grown in mice and γ-irradiated or adriamycin-treated[59]. In other systems, oncogenes such as c-myc[60] and HPV E7+ras[61] have been similarly shown to induce p53-dependent sensitivity to irradiation or chemotherapeutic drugs.

The elegant model systems just outlined are informative, but the role of p53 in the efficacy of human cancer therapy is likely to be less clear-cut due to other gene products also affecting chemosensitivity. One important prediction of these observations, however, is that where cytotoxic agents commonly induce p53-dependent apoptosis in transformed cells, loss of p53 pathways can produce a **multiresistant** phenotype. This concept has implications for both *de novo* resistance to treatment and the development of acquired resistance in recurrent or relapsing malignancy. Indeed, in the murine E1A/ras-transformed fibroblast tumour model, described in the previous paragraph, Lowe *et al* found that over 50% of the initially treatment-resistant or recurrent tumours derived from transformed fibroblasts on a p53 wild type background had acquired p53 gene mutations[59]. Thus the cytotoxic treatment (γ-irradiation) had selected for apoptosis resistance, and consequently enriched the tumour population in cells with defective p53, which would be predicted to show resistance to other cytotoxic agents. It also follows that reintroduction of wild type p53 function to such cancers should restore sensitivity to therapy, and the prospects for achieving this will be discussed in the next section.

As well as determining responses to genotoxic therapies for malignancy, p53 status may affect responses to hormone ablation therapy, such as anti-androgen therapy of prostate carcinoma[52]. The

principle of this type of treatment is that some element of hormone responsiveness or dependence is retained by the neoplasm and is therefore a target for slowing tumour growth or induction of regression by shifting the balance between cell proliferation and apoptosis. Since p53 can regulate dependence of some cell types on survival factors, loss of wild type p53 may confer resistance to hormone ablation therapy.

## Gene specific therapy

As can readily be appreciated from the discussion so far, the ability to reactivate p53-dependent pathways in neoplasms is a potentially powerful therapeutic tool, by either directly provoking apoptosis or by returning sensitivity to cytotoxic cancer chemotherapeutic drugs. Indeed, retention of p53-dependent apoptosis probably explains the sensitivity to chemotherapy of testicular neoplasms[18,65]. *In vivo* testing of this hypothesis is clearly a priority, but it is too early yet for substantial data to have accumulated[62,63]. The availability of inducible p53 gene constructs, cloned into tumorigenic cell lines or introduced into the germline, will allow p53 expression to be suddenly switched on within neoplasms by exposure of the cells or tissue to the pharmacological inducing agent, and permit better experimental evaluation of the mechanisms and potential benefits of p53 therapy.

If benefit is shown for p53-specific therapy, the options for clinical intervention are manifold. They include gene delivery systems by use of lipid vehicles or viral vectors, or alternatively peptides designed to mimic or activate particular aspects of p53 function and tagged for target cell specificity might be used. Finally, structural mutants of p53 that are unable to maintain a stable wild type conformation might be stabilised by specific pharmacological agents, or perhaps even vaccinated against, using mutant specific epitopes as immunogens. The results of a recent study of the effects of retrovirally introduced wild type p53 on non-small cell lung cancer in 9 patients are encouraging preliminary evidence that reintroduction of p53-dependent apoptosis is a viable option for human cancer therapy[64].

## Conclusion

The position of p53 at the head of key cellular pathways, without actually being essential for life, and its susceptibility to dysfunction through loss or mutation of a single allele, make a powerful but fragile instrument of tumour suppression. The downstream links to central

molecular regulators like TFIIH provide mechanisms for the co-regulation of apoptosis, cell cycle and DNA repair after DNA damage, and also illustrate how defects in one pathway could impinge upon the others. p53 is pleiotropic and there are important differences between cell types in both the upstream induction of p53 and the downstream responses evoked. Thus the consequences of p53 dysfunction for carcinogenesis must be read in the context of the specific lesion, the cell type, differentiation state, genetic background and cellular environment.

p53-dependent apoptosis is important for tumour suppression in some tissues, such as thymus, or in particular biochemical situations, such as forced oncogene activation. However, apoptosis may not be the important tumour suppressor function of p53 in other tissues and in some cell types p53 may be partially or completely redundant to pathways dependent on other genes such as IRF-1. Moreover, the particular balance of p53 downstream pathways that operates in normal cells may be distorted in neoplasia, perhaps for example giving the potential to therapeutically trigger p53-dependent apoptosis in a cell type that would not normally engage this response. Achieving better understanding of these issues is essential to more fully comprehend the contribution of p53 to tumour suppression in different tissues, and is a major goal of cancer biology.

Despite these complexities, evidence for a clinical utility of gene-specific therapy of human cancer in specific situations is accumulating, and the practicalities of implementing such therapy are already being addressed.

## Acknowledgements

I should like to thank S. Prost for comments and discussion, and the Cancer Research Campaign for my Gordon Hamilton Fairley CRC Clinical Research Fellowship.

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JOURNAL OF PATHOLOGY, VOL. 183: 177–181 (1997)

# UV BUT NOT γ-IRRADIATION INDUCES SPECIFIC TRANSCRIPTIONAL ACTIVITY OF p53 IN PRIMARY HEPATOCYTES

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## SUMMARY

**The mechanisms are poorly understood by which p53 can stimulate different downstream events, including growth arrest, DNA repair, and apoptosis, after DNA damage. Changes in protein levels do not predict a particular p53 response, but it is possible that differences in functional activities such as transactivation are important. The present report describes the successful use of a specific p53 reporter plasmid transfected into primary murine hepatocytes to evaluate p53 transactivation activity over time after two different genotoxic injuries (γ-irradiation, 15 Gy and UV-c irradiation, 10 J/m²) known to produce p53-dependent growth arrest in this cell type. The results show that UV injury to hepatocytes was followed by a transient increase in transcriptional activation of the reporter plasmid by p53 and that this response preceded changes in p53 protein levels, as assessed by immunocytochemistry. By contrast, γ-irradiation injury failed to induce detectable changes in either transactivation activity or hepatocyte p53 protein levels. The data show that p53 responses to DNA damage are dependent on both cell and injury type and suggest that in hepatocytes they can be independent of protein concentration and specific transcriptional activity. The results have implications for how particular dysfunctional p53 mutations in carcinogenesis could alter hepatocyte responses to different DNA injuries.** © 1997 John Wiley & Sons, Ltd.

J. Pathol. 183: 177–181, 1997.
No. of Figures 4. No. of Tables 0. No. of References 26.

KEY WORDS—liver; p53; DNA damage; UV irradiation; gamma irradiation; reporter plasmid

## INTRODUCTION

The tumour suppressor protein p53 is a critical mediator of DNA damage response pathways that couple to growth arrest, apoptosis, and DNA repair.[1] Cell lineages differ in the particular p53 response pathway engaged after DNA injury, but whether the choice is regulated through p53, and by what mechanism, is poorly understood. Accumulation of p53 protein is not specific to any particular p53-dependent pathway, but phosphorylation or alternative splicing could determine the downstream events initiated by p53 by selectively regulating transactivation and transrepression activities.[2–5]. It is therefore essential to complement protein quantitation with functional assays. Greater understanding of these issues would better define the contribution of p53 to tumour suppression in different tissues.

Despite good evidence for p53 dysfunction in hepatocellular carcinomas,[6,7] rather little is known about p53 in normal hepatocytes. Proliferating hepatocytes respond to both UV and γ-irradiation-induced DNA injuries by p53-dependent growth arrest.[8,9] However, although hepatocytes become strongly immunoreactive for p53 protein after UV irradiation,[9,10] this is not seen after-γ-irradiation.[11] The observation suggests that qualitative differences in the character of the p53 response underlie the common biological response to these two injuries; hence it was of interest to extend the comparison to assessment of p53 function. The present report describes the use of a p53 reporter plasmid transfected into primary murine hepatocytes to investigate p53 transactivation activity at the ribosomal gene cluster (RGC) response element following γ- and UV-c irradiation. The transient reporter assay is shown to be sufficiently sensitive and reliable to evaluate authentic p53 responses to DNA damage in primary culture.

## MATERIALS AND METHODS

### *Plasmids*

pCMVβ (Clontech) is a mammalian expression vector designed for the constitutive expression of β-galactosidase from the *Escherichia coli lacZ* gene in mammalian cells. pRGCΔFosLacZ is a reporter plasmid for transcriptionally active wild-type p53.[12] It is based on the pBSK plasmid into which have been cloned oligonucleotides containing two copies of a p53 RGC consensus binding site, a transcriptionally inactive mutant murine *fos* promoter, the *lacZ* gene (open reading frame), and the small t intron with the polyadenylation signals of SV40. Wild-type p53 binds to the RGC elements and stimulates expression of β-galactosidase. pRGCΔFosLacZ and a negative control plasmid pΔFosLacZ, which is identical except that it lacks the

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CCC 0022–3417/97/100177–05 $17.50


*Received 13 January 1997*
*Revised 26 March 1997*
*Accepted 21 April 1997*

RGC fragments necessary for p53 binding, were the kind gifts of Professor S. H. Friend.

### *Hepatocyte isolation, culture, cationic liposome-mediated transfection, and irradiation*

Primary hepatocytes from adult male mice 6–10 weeks old were isolated by a retrograde two-step perfusion procedure[13] and purified by centrifugation through Percoll.[14] The hepatocytes were plated into fibronectin-coated 24- or 12-well tissue culture dishes in serum-free modified Chee's culture medium at a density of 0·2–0·3 × $10^5$ per $cm^2$ and transfection was performed after approximately 40 h culture. The monolayers were fed with fresh, serum-free medium a few hours prior to transfection. The quantities of reagents given here are optimized for a single 1·90 $cm^2$ culture well and were scaled up appropriately for larger well sizes. Lipofectin reagent (6 $\mu$l of 1 mg/ml, Life Technologies) was diluted into 100 $\mu$l of serum-free, antibiotic-free culture medium and incubated at room temperature for 45 min. This suspension was then mixed gently with 100 $\mu$l of serum-free, antibiotic-free medium containing purified plasmid DNA (1 $\mu$g), incubated for a further 45 min at room temperature, and then made up to 300 $\mu$l with further medium. Hepatocytes were incubated with the DNA–lipid suspension for 6 h in a humid 5 per cent $CO_2$/95 per cent air atmosphere at 37°C, after which the DNA-containing medium was replaced with 500 $\mu$l of standard culture medium. Twenty-four hours after commencing transfection, the cultures were treated with either $\gamma$-irradiation (15 Gy) or UV-c (10 J/$m^2$). Cultures were $\gamma$-irradiated by a $Cs^{137}$ source at 0·33 Gy/min or UV-c-irradiated (254 nm) after complete removal of culture medium, using a Spectrolinker XL-1500 (Spectronics Corporation). Unirradiated controls were otherwise transported and handled identically.

### *Quantitation of β-galactosidase activity by colorimetric assay*

$\beta$-Galactosidase activity in transfected hepatocytes was quantified using the colorimetric Beta-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega), following the manufacturer's instructions. The assay measures the amount of yellow hydrolysis product (*o*-nitrophenol) generated from ONPG substrate (*o*-nitrophyl-$\beta$-D-galactopyranoside), compared with a series of known standards incubated in parallel. The negative control was untransfected hepatocytes. The reaction was stopped by adding 1 M $Na_2CO_3$ and the absorbance at 420 nm read by an ELISA plate reader (MR 5000, Dynatech). To account for variability in the number of cells recovered from each well for estimation of $\beta$-galactosidase activity, the protein concentration of each cell extract was estimated using the Biorad Protein Assay kit. The negative control used lysis buffer. A standard curve was constructed using a bovine serum albumin standard solution (Biorad), diluted to final concentrations of 1–8 $\mu$g/ml and incubated in parallel with the test samples. The sample absorbance was read at 595 nm.

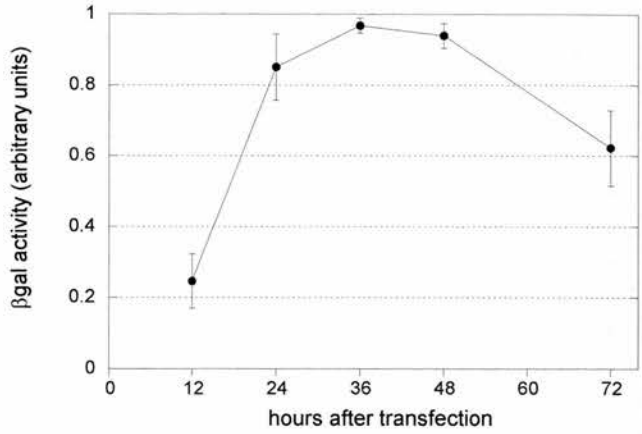


Fig. 1—$\beta$-Galactosidase activity in primary hepatocytes transfected with pCMV$\beta$ plasmid that constitutively expresses *LacZ*

### *p53 protein immunocytochemistry*

Hepatocyte monolayers were fixed in ice cold acetone/methanol (1:1, v/v) for 10 min and stored dry at − 80°C. Immunocytochemistry was performed using a routine avidin–biotin complex labelling technique after blocking of endogenous biotin using a kit (Vector Laboratories). Cells were incubated in a humid chamber overnight at 4°C with the primary antibody [mouse monoclonal pAb421 (Oncogene Science)] at 1/1000 dilution in 5 per cent serum, 0·5 per cent Tween 20, and phosphate-buffered saline (PBS). Negative controls omitted the primary antibody. The secondary antibody [biotinylated rabbit anti-mouse $F(ab')_2$ (Dako)] was applied for 30 min at 1/400 dilution and positive cells were visualized using DAB chromogen. For each observation, 300 cells were counted and the results were expressed as the percentage of cells showing nuclear p53 positivity.

## RESULTS

Plasmids were introduced into primary cultures of adult murine hepatocytes by lipofection, since this method is efficient and there was no evidence of toxicity with the protocol used. Transfection efficiency using cationic liposomes was consistently between 15 and 20 per cent, as assessed on pCMV$\beta$-transfected cells by estimating the proportion of stained, i.e. expressing *LacZ*, cells after incubating in 1 mg/ml X-Gal, 1 mM $MgCl_2$, 10 mM $Fe_3(CN)_6$, 10 mM $Fe_4(CN)_6$ in PBS.

The kinetics of plasmid expression were assessed in cells transfected with the constitutively expressing pCMV$\beta$ plasmid. In these cells, $\beta$-galactosidase activity was detectable by ONPG colorimetric assay within 12 h, with relatively stable expression levels from 24 to 48 h, slightly decreasing by 72 h (Fig. 1). Subsequent experiments on transfected cells were therefore performed between 24 and 48 h after transfection.

Cells transfected with the p53-reporter plasmid (pRGC$\Delta$FosLacZ) or its control plasmid (p$\Delta$FosLacZ) showed no significant $\beta$-galactosidase activity, equivalent to untransfected controls. However, after treatment with 10 J/$m^2$ UV-c, there was specific induction of

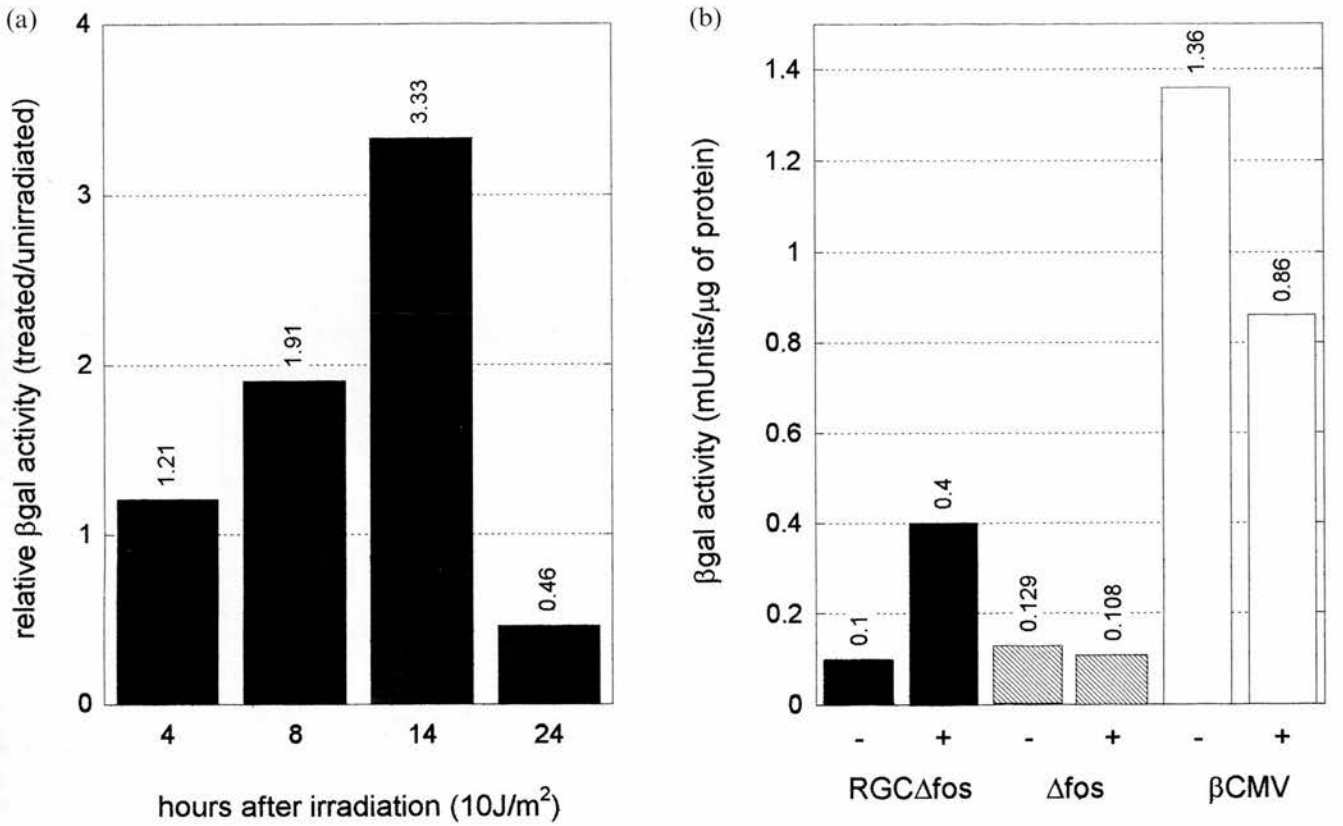


Fig. 2—p53 reporter plasmid β-galactosidase expression following UV-c treatment of transfected primary hepatocytes. (a) p53 reporter activity in hepatocytes following 10 J/m² UV-c. β-galactosidase activity is expressed relative to untreated transfected hepatocytes. (b) Effect of UV-c treatment on β-galactosidase expression from the p53 reporter plasmid (RGCΔFosLacZ), the negative control plasmid (ΔFosLacZ), and the constitutive pCMVβ plasmid. Results are shown 14 h after UV-c (indicated by +); untreated controls are indicated by −. Note that a and b are results of independent experiments

β-galactosidase expression in cells transfected with the p53 reporter plasmid, peaking at about 14 h after irradiation and subsequently decreasing to control levels by 24 h (Fig. 2a). The activity of the negative control plasmid remained at baseline (Fig. 2b). There was a concomitant 37 per cent decrease in constitutive pCMVβ β-galactosidase activity after UV-c irradiation (Fig. 2b). This was due to non-specific transcriptional suppression by UV, a well-documented effect[15] that makes the observation of increased p53-reporter gene expression following UV injury even more significant.

Immunocytochemical analysis of the UV-treated hepatocytes showed p53 protein accumulation in treated cultures, peaking around 24 h after irradiation (Fig. 3). It is noticeable that baseline p53 immunopositivity increased slowly with time in culture. This is unexplained but may reflect a 'stress response' to the culture environment and the acquisition of undefined cellular injuries.

By contrast to UV injury, γ-irradiation produced no significant change in expression from any plasmid, with the p53-reporter and its control remaining at basal levels from 2 to 24 h after irradiation (Fig. 4A), and pCMVβ expression equivalent to unirradiation control transfectants (Fig. 4B). Furthermore, there was no effect of γ-irradiation on p53 immunopositivity (data not shown), in accord with previous *in vivo* findings of Midgley *et al.*[11]

## DISCUSSION

### *Hepatocytes and p53 after DNA injury*

The present findings confirm reports that p53 protein accumulates in hepatocytes after UV[9,10] but not γ-irradiation[11] and extend these differences to include p53 transactivation activity. The data are supported by

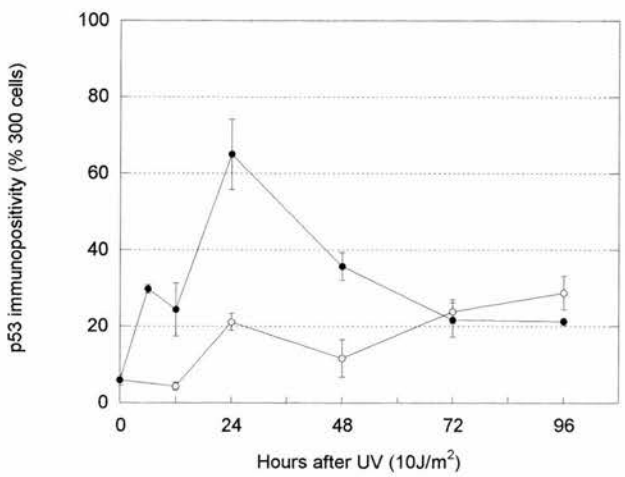


Fig. 3—Time course of p53 immunopositivity after treatment with UV-c at 10 J/m². Results are mean ± SE ($n$=2–4) for replicate cultures in a typical experiment that was repeated three times with similar findings. ○, unirradiated; ●, irradiated

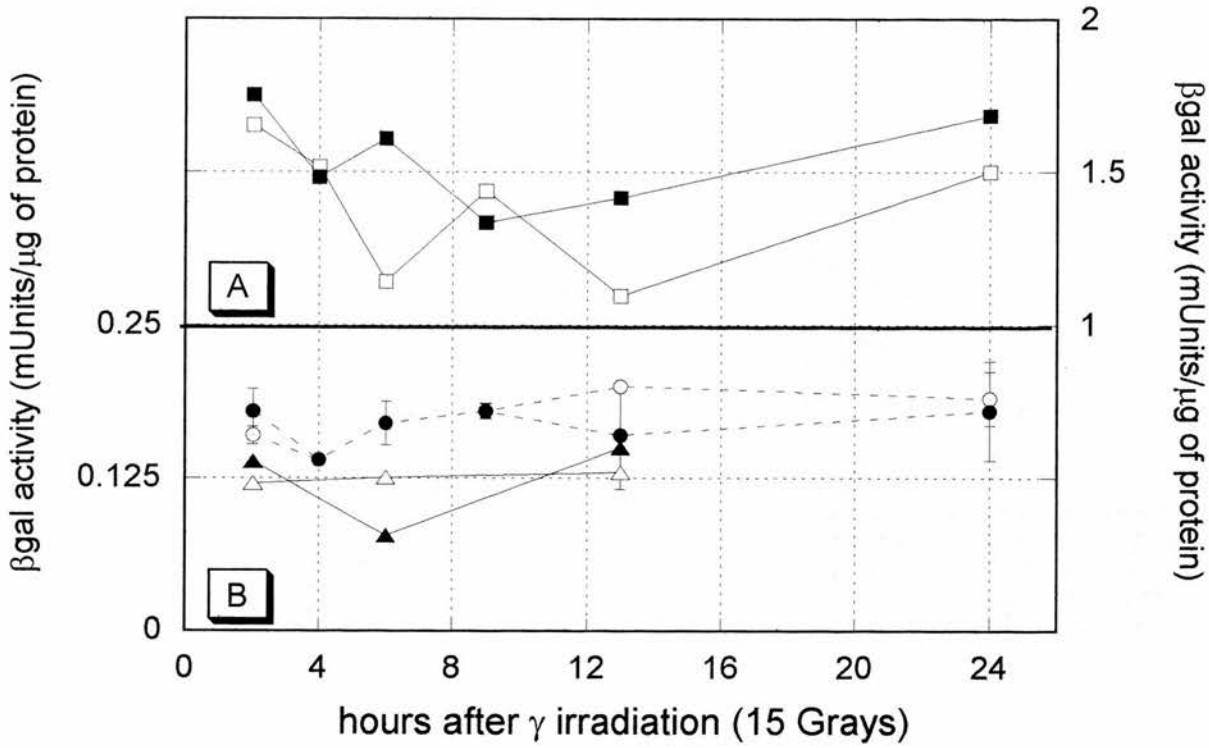


Fig. 4—β-Galactosidase activity after 15 Gy γ-irradiation. (A) □,■ Constitutive control plasmid (pCMVβ); (B) ○,● p53 reporter plasmid (RGCΔFosLacZ); △,▲ negative control plasmid (ΔFosLacZ). Open symbols: unirradiated; solid symbols: irradiated.

MacCallum *et al.*,[16] who recently reported no increased p53 transactivation activity through RGC in liver after γ-irradiation *in vivo*. This observation of distinct p53 transactivational and immunocytochemical responses to two different types of DNA injury in hepatocytes is of particular interest, since at the doses used here both agents induce a p53-dependent growth arrest in proliferating hepatocytes, without significant apoptosis.[8,9] Thus, qualitatively different stimuli act differently through p53, yet have a common consequence.

After UV-c treatment, p53 transactivation activity had already peaked and returned to baseline when p53 protein levels were at peak. Increased p53 transactivational activity after UV-c is not therefore simply a reflection of increased p53 protein levels and may be independently regulable in hepatocytes, as suggested for some other cell types.[2,5,17,18]

In contrast to UV-c, γ-irradiation of hepatocytes failed to alter reporter gene activity at a dose adequate to arrest liver regeneration *in vivo* and that induces hepatic $O^6$-alkyl transferase expression, both by p53-dependent mechanisms.[8,19] These responses to γ-irradiation are therefore accomplished without a detectable increase in p53 concentration or evidence of p53-dependent transactivation. This suggests that the basal state of p53 in hepatocytes is sufficient to permit the responses, or that a different property of p53 is stimulated by γ-irradiation-induced DNA injury. It is, of course, possible that the lack of reporter response merely reflects a lower sensitivity than given by endogenous biological activities such as growth arrest,[20] but a third intriguing possibility remains that p53 is transcriptionally activating genes through only a subset of its response elements which does not include the RGC motif. p53 binds to its response elements with different affinities that can be changed selectively and in opposite ways by altering its phosphorylation state.[4] It would therefore be necessary to use several reporter constructs to assess accurately changes in p53 transactivation activity. Nevertheless, these results suggest that a common p53-dependent response (i.e., growth arrest) to two different types of DNA injury can proceed through different activities of p53. Since p53 mutants vary in their ability to activate specific elements,[21–24] different p53 mutations in carcinogenesis might have consequences for susceptibility to different types of DNA damage.

### *Transient reporter plasmid assay for p53*

The present results show for the first time that transient transfection is a suitable method to evaluate p53 function in normal cells after DNA damage and that the method is sufficiently stable and reliable to be used to provide a time course of the p53 response. Previous studies have used stably transfected carcinoma cell lines and have found increased p53 reporter expression after a variety of genotoxic treatments, including UV and γ-irradiation[25,26] and etoposide.[18] Zhan reported a single time point at 24 h, whilst Lutzker used pooled clones carrying three different reporter constructs and found expression to increase steadily over a time course of 2–12 hours after treatment. However, stable transfection is clearly not feasible for primary culture work. Zhan *et al.* did attempt transient transfection of a $PG_{13}$-CAT p53 reporter plasmid, but could not demonstrate significant

induction of expression after the same genotoxic injuries that produced responses in stable transfectants. This could be due to low transfection efficiencies or to an attenuated p53 response by the carcinoma cells to DNA injury compared with hepatocytes, although the latter seems unlikely given the efficient induction in stable transfectants. Stabilization of p53 after calcium phosphate transfection has been reported and might have masked p53 responses to genotoxic treatment in their study. No such effect was apparent for lipofection in the present study, with $\beta$-galactosidase activity at equivalent levels in p53 reporter and control plasmid transfectants. The relative levels of p53 reporter induction are comparable to those observed using a transiently transfected $PG_{13}$-CAT reporter to evaluate changes in p53 function during primary keratinocyte differentiation.[17] Therefore, although potentially less sensitive than the study of clones with a stably transfected reporter, this transient reporter assay is adequate to evaluate authentic p53 responses to levels of DNA injury commonly applied in studies of p53. Moreover, it can be applied to primary and non-clonogenic cell populations, giving the power of greater relevance to *in vivo* responses, combined with a defined cellular environment.

## ACKNOWLEDGEMENTS

We thank Professor Stephen H. Friend (Department of Pediatrics, Harvard Medical School, U.S.A.) for kindly providing the p53 reporter plasmids. This work was supported by the Scottish Hospital Endowment Research Trust. COCB is a Cancer Research Campaign Gordon Hamilton Fairley Clinical Research Fellow.

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# p53 deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage

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Reprinted from FASEB Journal
VOL. 11, NO. 7 · JUNE 1997

# p53 deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage

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ABSTRACT **Despite good evidence for p53 dysfunction in human hepatocellular carcinomas, little is known of the significance of p53 to normal hepatocytes and whether p53 dysfunction is relevant to early hepatocarcinogenesis. We have therefore examined the consequences of targeted p53 deficiency in hepatocytes for regulation of apoptosis, proliferation, and ploidy. p53 deficiency was silent in normal liver and did not affect progression from diploidy to polyploidy in the aging liver. However, in primary culture the absence of p53 resulted in increased hepatocyte proliferation indices and decreased sensitivity to proliferation inhibition by TGFβ. Moreover, p53-deficient cells continued to survive and proliferate under conditions of minimal trophic support that led to growth arrest and apoptosis of wild-type cells. In vivo, p53-deficient mice had enhanced proliferative responses to both xenobiotic hepatomitogen and $CCl_4$-induced liver necrosis, although lack of persistent proliferation showed that other control mechanisms are important. There was no simple relationship between p53 and apoptosis after DNA damage because UV irradiation led to p53-independent apoptosis, even though p53 was stabilized. However, p53 did couple DNA damage to growth arrest, and abnormal mitoses after γ-irradiation of regenerating p53 null livers demonstrated circumstances where loss of $G_1$ and $G_2$ checkpoints may generate abnormal ploidy. Thus p53 becomes important when hepatocytes are released from $G_0$ and stressed, sensitizing them to mitogen and cytokine regulators of cell cycle progression and apoptosis. Hence p53 deficiency is likely to be significant in an environment of persistent regenerative stimuli and unfavorable trophic support or in the presence of other enabling genetic lesions. This model is relevant to human hepatocarcinogenesis, which almost always occurs against a background of chronic hepatocellular destruction in hepatitis and cirrhosis. In that context, by reducing the need for cytokine support and disabling DNA damage-induced growth arrest, p53 deficiency should facilitate the expansion of preneoplastic clones in chronic liver disease.—Bellamy, C. O. C., Clarke, A. R., Wyllie, A. H., Harrison, D. J. p53 deficiency in liver reduces local control of survival and proliferation, but does not affect apoptosis after DNA damage. *FASEB J.* 11, 591–599 (1997)**

*Key Words: cell cycle · carcinogenesis · hepatocyte · wild-type cell · liver carcinoma*

THE p53 ONCOSUPPRESSOR GENE PRODUCT IS a critical mediator of $G_1$ cell cycle arrest and apoptosis after DNA damage (1). These responses may protect against replication of damaged DNA by permitting DNA repair or deleting the affected cell. Accordingly, loss of p53 function can result in loss of the $G_1$ checkpoint arrest, abrogation of DNA damage-associated apoptosis, karyotype instability, and accelerated carcinogenesis. More recently, p53 has been reported to affect $G_2$ cycle arrest, centrosome replication, mitotic spindle organization, and to regulate DNA repair (see ref 1 for references). It has also become clear that there is tissue variability in the induction of particular p53-regulated responses. For example, after γ-irradiation, fibroblast cell lines undergo p53-dependent $G_1$/S phase arrest, whereas proliferating intestinal crypt epithelial cells undergo p53 gene dose-dependent apoptosis but growth arrest is p53-independent (2, 3). Clearly, therefore, it is fundamentally important to consider the tissue context when evaluating the roles of p53 in suppression of carcinogenesis. This report has investigated the roles of p53 in the liver, specifically the hepatocyte.

Liver carcinoma is a major cause of cancer death worldwide. Mutations in the p53 tumor suppressor gene, usually with loss of the residual wild-type allele, are frequent in human hepatocellular carcinomas (HCC)[2] and correlate positively with increasing histological grade of carcinoma and early recurrence,

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[2] Abbreviations: HCC, hepatocellular carcinoma; TCPOBOP, 1,4 bis 2-(3,5-dichloropyridyloxybenzene); TGF, transforming growth factor; EGF, epidermal growth factor; BrdU, 5-bromo-2′-deoxyuridine; FBS, fetal bovine serum.

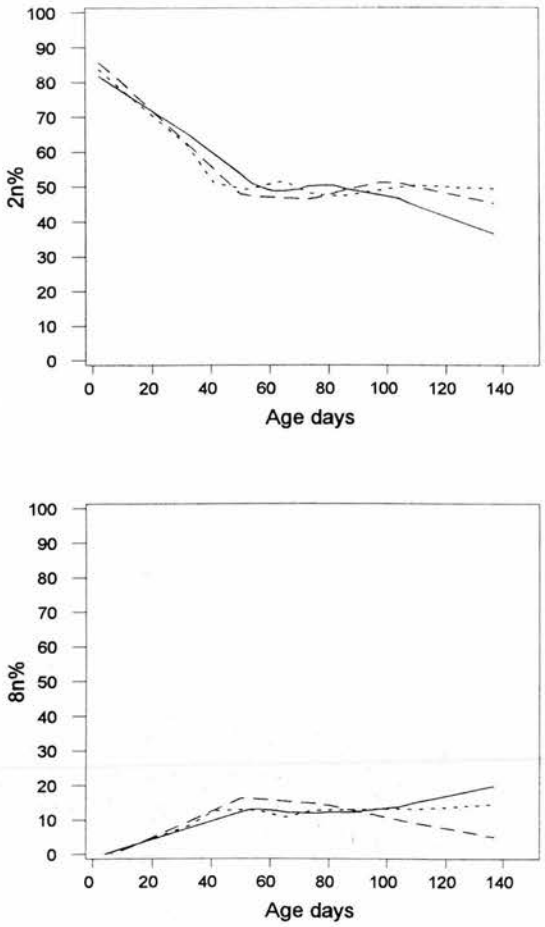



**Figure 1.** Effects of p53 genotype on hepatocyte polyploidization assessed by flow cytometry of propidium-iodide-stained nuclei (40) from wild-type and p53-deficient mice (18). The relative proportions of diploid (2n%), tetraploid (4 n%), and octaploid (8 n%) nuclei at different ages (range 2–137 days) are depicted as Lowess plots. p53 null (—); p53 heterozygous (- - -); wild type (– – –).

suggesting that defective p53 function is of pathogenic significance and confers increased clinical aggression (4, 5). Studies of intratumor heterogeneity have suggested that p53 mutation and allele loss are late events in human HCC, mediating tumor progression but occurring after carcinogenesis (6). However, several lines of evidence indicate that p53 dysfunction can precede hepatocarcinogenesis and have a pathogenic role. First, abnormal accumulation of p53 protein has been observed in hepatocytes of patients with cirrhosis and liver cell dysplasia, high-risk lesions for carcinoma development (7, 8). Second, exposure to aflatoxin $B_1$, a dietary mutagen and a cofactor in human hepatocarcinogenesis, is associated with a characteristic point mutation in p53 (9). This mutation has been demonstrated in nonneoplastic hepatocytes, particularly in areas of high aflatoxin exposure where HCC is prevalent (10). Third, dysfunctional p53 is probably more common in HCC than is appreciated from standard genetic screens of "hotspot" regions because of both mutation distribution (11) and functional inactivation without mutation, best characterized for chronic hepatitis B infection. Hepatitis B virus is the major etiological agent of human HCC: the hepatitis B X protein heterodimerizes with and inactivates p53 in vitro and in vivo; in transgenic mice this is temporally and spatially associated with the development of HCC from preneoplastic lesions, providing strong evidence that p53 dysfunction is relevant to hepatocarcinogenesis (12–14).

However, critical facts remain undetermined. The normal functions of p53 in hepatocytes are unknown. It is not known whether p53 dysfunction in nonneoplastic hepatocytes can confer a selective advantage relevant to early hepatocarcinogenesis nor how such an advantage might be manifested—through dysregulation of cell cycle, apoptosis, DNA repair, or a combination.

This study used a mouse model to examine the effects of targeted disruption of the p53 gene in otherwise normal hepatocytes. In vivo observation and primary hepatocyte culture were used to dissect out the roles of p53 in the regulation of hepatocyte polyploidization, proliferation, apoptosis, and the responses to DNA damage.

## RESULTS

### Constitutive hepatocyte polyploidization is p53-independent

Hepatocyte nuclear DNA content was evaluated by flow cytometry in livers from 130 mice aged between

2 and 137 days (42 wild-type, 50 heterozygous, and 38 p53 null) (**Fig. 1**). In neonatal mice, hepatocytes were diploid. Shortly after weaning and continuing through adult life there was a progressive increase in the relative proportion of polyploid hepatocytes and, concomitantly, increasing degrees of polyploidization. However, it is evident from Fig. 1 that p53 genotype did not influence the timing, trend, or degree of age-related hepatocyte polyploidization. Aneuploidy was not detected in any sample.

### p53-deficient hepatocytes are more likely to proliferate

Before isolation for culture from quiescent adult liver, virtually all hepatocytes are in the $G_0$ phase (15). The populations are thus effectively synchronized before receiving the proliferative stimulus of isolation and primary culture. In culture, both wild-type and p53 null hepatocytes showed a rise in BrdU positivity from near zero at plating to a peak at 72–96 h in culture, but the BrdU positive fraction was consistently greater in the null cells (**Fig. 2**). Thus more null hepatocytes were cycling, but with a similar kinetics of entry from $G_0$ to S phase as wild type. This finding was independent of the presence of mitogens in the culture medium (insulin, epidermal growth factor [EGF], fetal bovine serum [FBS]). After the addition of mitogens, the BrdU indices of wild-type hepatocytes were much increased (Fig. 2). By contrast, the high BrdU indices of null hepatocytes were little influenced by these factors. This suggests that p53 null cells were more likely than wild type to proliferate but were relatively unresponsive to further stimulation by cytokines. Responsiveness to cytokine-induced mitosuppression was also decreased in the p53 null hepatocytes. When incubated in high concentrations of TGFβ sufficient to abrogate wild-type hepatocyte proliferation, p53 null hepatocyte proliferation was only reduced to the level of untreated wild-type controls (**Fig. 3**).

To test whether these p53-dependent differences in proliferative activity also occur in vivo, liver regeneration was induced by administering a necrogenic dose of carbon tetrachloride. This produced a sharply defined wave of DNA synthesis in residual hepatocytes, as assessed by pulse labeling with BrdU, peaking 60 h after administration of $CCl_4$. As shown in **Fig. 4**, p53 null livers had a synchronous but significantly greater peak of DNA synthetic activity when compared with wild type, concordant with in vitro data above.

We next compared the hyperplastic responses of wild-type and p53 null livers to a xenobiotic mitogen. Such agents are well described as tumor promoters in experimental hepatocarcinogenesis. A single dose of a nongenotoxic mitogen, 1,4 bis 2-(3,5-dichloropyridyloxybenzene) (TCPOBOP) (16), induced a surge of hepatocyte DNA synthesis and mitotic activity at 3 days. This was accompanied by an increased liver:body weight ratio that was maintained at 23 days despite the return of BrdU incorporation and mitotic indices to baseline levels (**Table 1**). Both the mitogen-induced DNA synthe-

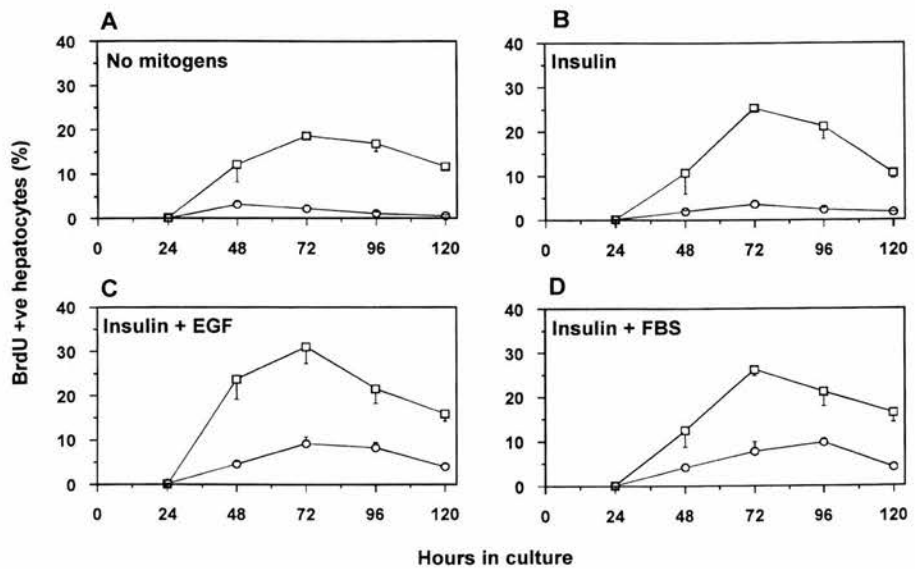



**Figure 2.** Effect of p53 genotype on hepatocyte DNA synthesis in primary culture. Hepatocytes were isolated from age-matched male mice (18) by two-step retrograde perfusion (41) and Percoll purification (42), then plated onto chamber slides (Lab Tek). Cultures were pulse-exposed to 40 μM BrdU (3 h) before ethanol fixation. BrdU incorporation (% positive of 500 cells) was assessed by immunocytochemistry using monoclonal rat anti-BrdU $IgG_2a$ (Sera Lab). Cells were cultured in serum-free Chee's medium with 30 nM dexamethasone (Sigma) plus no mitogen (*A*); 300 nM insulin (*B*) (Life Technologies); 300 nM insulin + 25 ng/ml epidermal growth factor (*C*) (EGF, Sigma, mouse submaxillary gland); 300 nM insulin + 1% fetal bovine serum (FBS) (*D*). Wild type (○); p53 null (□). Mean ± SEM for duplicate cultures from three mice of each genotype (n = 6).

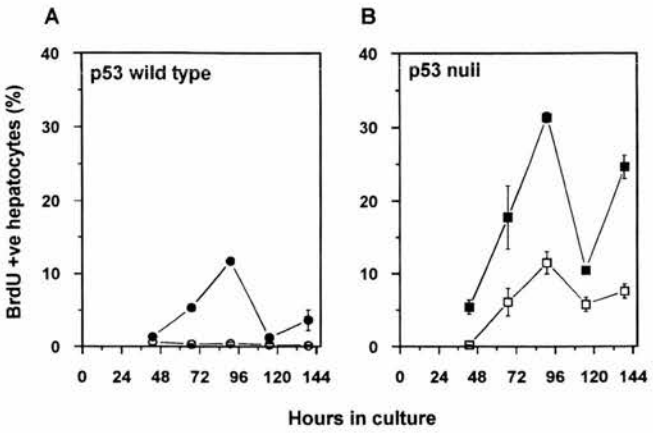



**Figure 3.** Inhibition of hepatocyte DNA synthesis by TGFβ, assessed by BrdU immunocytochemistry (see Fig 2). Cells were cultured in Chee's medium with 100 nM dexamethasone, 1.5 μM insulin, and 25 ng/ml EGF to which 160 pM TGFβ was added after 20 h. Negative controls omitted TGFβ. *A*) Wild type; *B*) p53 null. Open symbols, TGFβ-treated; filled symbols, controls. Mean ± SEM.

sis and the ultimate increase in liver:body weight ratio were greater in p53 null livers compared with wild type, showing an amplified response of the p53-deficient hepatocytes to the TCPOBOP (Table 1). No dysplasia or foci of persistent proliferation were apparent in any treated livers.

### p53 regulates hepatocyte dependence on survival factors in primary culture

p53-deficient hepatocytes had lower apoptotic indices in culture than wild type (**Fig. 5**). The lower apoptotic indices were apparent before BrdU uptake began and thus were not directly related to either DNA synthesis or cell proliferation. The survival benefit conferred by p53 deficiency was greatest when trophic support in the culture medium was least. p53 deficiency therefore reduces dependency on survival factors, but confers little survival advantage when these factors are not limiting and wild-type apoptotic rates are very low anyway.

### Hepatocyte responses to DNA damage

Two well-characterized genotoxic stimuli were tested: γ-irradiation and UV-c (254 nm) (17).

Gamma irradiation readily induces p53-dependent apoptosis in some cell types (2, 18), but doses of up to 15 Gy did not induce hepatocyte apoptosis or accumulation of immunoreactive p53, either in vivo or in proliferating primary cultures (data not shown), confirming previous observations (19). Since γ-irradiation induces a p53-dependent growth arrest in other cell types (1) and inhibits liver regeneration (20), the effects of irradiation on regenerating wild-type and p53-deficient livers were compared. Livers were γ-irradiated (15 Gy) 48 h after $CCl_4$ administration, when most hepatocytes were in $G_1$, just before entry to S phase (**Fig. 6*A, B***). It is evident from Fig. 6*A and C* that irradiation of wild-type mice suppressed both entry to S phase ($G_1$/S arrest) and the subsequent passage through mitosis at 72 h after $CCl_4$. By contrast, irradiation of p53 null livers reduced by less and did not delay the subsequent S phase peak of BrdU positivity (Fig. 6*B*), indicating a much reduced $G_1$/S arrest. The reduced peak and prolonged fall in BrdU positivity may be due to slowed progression through the S phase, well described after γ-irradiation (21). This was followed by a striking rise in the mitotic index 24 h after irradiation (mitotic counts of 103, 77, and 205 compared with 15, 13, and 8 in unirradiated mice) (Fig. 6*D*). Clearly, as well as defective $G_1$/S arrest, there was no significant $G_2$ arrest. Many of the mitotic figures were abnormal, with aberrant forms, isolated chromosomes, and chromosomal bridges (**Fig. 7**). It is therefore likely that this mitotic peak is due to damaged hepatocytes proceeding to and arresting within mitosis. The excess in mitotic figures was not evident at later time points, suggesting that the cells had undergone either catastrophe and elimination or delayed and possibly aberrant completion of mitosis. Even a slight increase in the normally brief duration of mitosis (30–45 min) would cause the observed mitotic index to rise considerably, as cells accumulated in this phase. $G_2$ arrest by wild-type livers cannot be demonstrated in the present study because release from the $G_1$ arrest was not observed. However, $G_2$ arrest by regenerating rat liver after γ-irradiation has been reported (22, 23). Therefore, these experiments show for the first time that in liver, $G_1$ and $G_2$ checkpoint arrests after γ-irradiation are p53-dependent.

UV irradiation of wild-type hepatocyte cultures produced both p53 protein accumulation and dose-dependent apoptosis (**Fig. 8**). However, unlike other cell types (1, 2, 18), apoptosis was p53-independent (Fig. 8). UV-induced apoptosis was reduced by survival factors (insulin, serum, dexamethasone) independently of p53 genotype (data not shown). Like γ-irradiation in vivo, UV blocked entry of cultured hepatocytes to S phase in a p53-dependent manner (data not shown), confirming a previous report using antisense p53 (24) and together demonstrating that p53 in hepatocytes mediates growth arrest but not apoptosis after DNA damage.

## DISCUSSION

### p53 sensitizes hepatocytes to cytokine regulators of proliferation and apoptosis

There was no phenotype for p53 deficiency in unstimulated livers. Differences in both proliferation

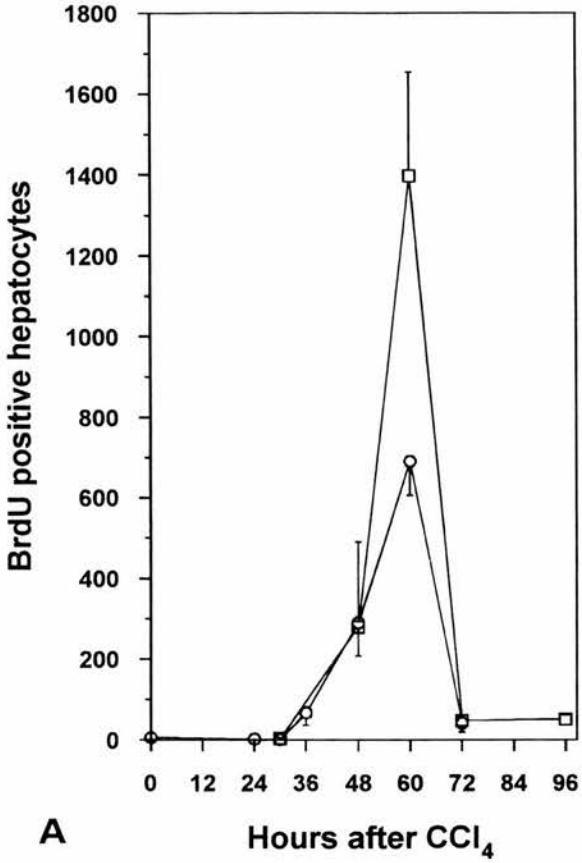
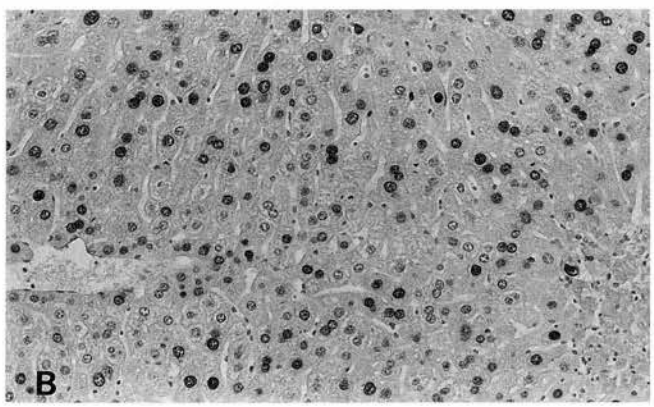
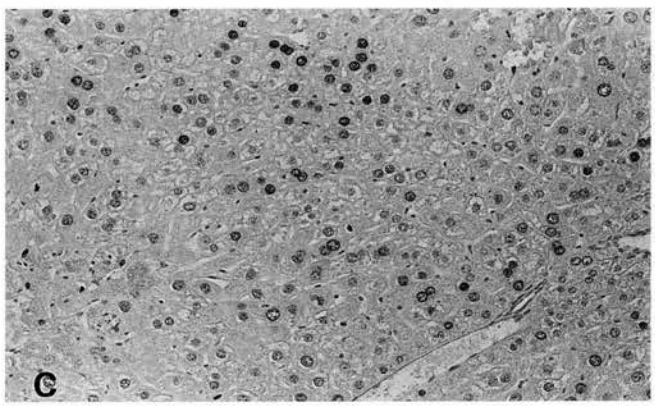



**Figure 4.** Liver regeneration by wild-type and p53 null mice after administration of carbon tetrachloride (0.5 ml $CCl_4$/kg i.p. 5% v/v in corn oil). Two h before killing, mice received an i.p. bolus of BrdU labeling agent (Amersham). *A*) Hepatocyte BrdU incorporation assessed by immunohistochemistry (see Fig. 2) (positive cells per 20 high-power fields) at the indicated times after $CCl_4$ injury. Mean ± SEM (three to six mice per observation). Wild type (○); p53 null (□). *B, C*) Liver BrdU immunopositivity (dark-stained nuclei) 60 h after $CCl_4$ injury to p53 null (*B*) and wild-type (*C*) mice. Hematoxylin counterstain.




and cell death became apparent only after hepatocytes were released from quiescence ($G_0$) by culture, xenobiotic (TCPOBOP), or $CCl_4$-induced liver necrosis. In cells thus stimulated, p53 deficiency increased the proportion of hepatocytes that proceeded through S phase. Moreover, the increase in cycling fraction was shown in culture to be relatively independent of and unaffected by mitogens. In parallel, loss of p53 reduced dependence on cytokine support for survival. Thus, cells with no p53 were able to survive and proliferate under conditions that caused wild-type cells to undergo growth arrest and apoptosis. Therefore, there is no evidence that p53 is necessary for replicative quiescence; indeed, it does not appear to have a role in normal liver. However, p53 becomes important once hepatocytes are released from $G_0$, sensitizing them to mitogen and cytokine regulators of cell cycle progression and apoptosis.

This model is consistent with data that normal rat hepatocytes stimulated by isolation and primary culture (or by partial hepatectomy) progress apparently autonomously from $G_0$ to mid-$G_1$, but are critically dependent on mitogens for further progression through the cell cycle (25). The passage from $G_0$ to $G_1$ has been defined in hepatocytes by the sequential expression of certain oncogenes, ending with p53 expression in mid-late $G_1$ (26). Although no significance has been previously ascribed to this physiological elevation of p53 during liver regeneration, the

TABLE 1. *Effect of p53 genotype on hepatic responses to a xenobiotic mitogen (TCPOBOP)*[a]

| | | 3 days after treatment | | 23 days after treatment | |
|---|---|---|---|---|---|
| | | Corn oil | TCPOBOP | Corn oil | TCPOBOP |
| BrdU index (% of 700 cells counted) | Wild type | 0.32 (0.13) | 8.25 (0.13) | 0.23 (0.19) | 0.07 (0.04) |
| | p53 null | 0.57 (0.24) | 14.7 (2.91)* | 0.13 (0.06) | 0.04 (0.04) |
| Liver/body wt (%) | Wild type | 5.51 (0.18) | 8.82 (0.51) | 5.52 (0.12) | 9.80 (0.43) |
| | p53 null | 5.41 (0.40) | 8.61 (0.49) | 5.65 (0.37) | 11.17 (0.70) |

[a] Adult male mice were given an i.p. bolus of 3 mg/kg 1,4 bis 2-(3,5 dichloropyridyloxybenzene) (TCPOBOP) in corn oil. BrdU labeling agent (Amersham) was administered i.p. 18 h before mice were killed. BrdU immunopositivity (% of 700 hepatocytes) was assessed on methanol-fixed tissue sections (see Fig 2. legend). Mean (SEM), for 3–5 mice per observation. * Significantly greater than wild type ($P = 0.04$, Mann Whitney test).

present results suggest that p53 is a critical effector of the mitogen restriction point. Absence of p53 disables the restriction, allowing appropriately stimulated hepatocytes to cycle independently of mitogens.

The reduced ratio between peak labeling and mitotic indices in p53 null mice compared with wild type suggests a prolonged S phase and/or $G_2$ phase in the regenerating p53-deficient hepatocytes. Although this is not a well-recognized feature of p53 deficiency, we have also observed a prolongation of S phase in p53-deficient embryonic stem cells compared with wild type (S. Prost et al., unpublished results).

After liver regeneration, cytokines are believed to be important in order to stop hepatocyte proliferation, and TGFβ is perhaps the most potent (27). However, although loss of p53 made cultured hepatocytes less responsive to TGFβ mitoinhibition, regenerating p53-deficient livers in vivo did not show abnormally persistent proliferation. Therefore, mechanisms other than TGFβ are important to terminate proliferation in vivo. p53 probably sensitizes hepatocytes to cell cycle inhibition by TGFβ through cooperative activation of p21 (28). The potential for p21 to inhibit liver growth has been shown in mice with a liver-specific p21 transgene (29).

### In hepatocytes, p53 couples DNA damage to growth arrest but not apoptosis

p53-coupled genotoxic injury of hepatocytes to growth arrest and the finding of abnormal mitoses after γ-irradiation of regenerating p53 null livers demonstrated circumstances where the loss of cell cycle checkpoints may generate abnormal ploidy. By contrast, there was no simple relationship between p53 and apoptosis after DNA damage, since UV irradiation led to p53-independent apoptosis even though p53 was stabilized. The induction in hepatocytes of the DNA repair enzyme $O^6$-alkyl guanyl transferase by γ-irradiation has been shown to be p53-

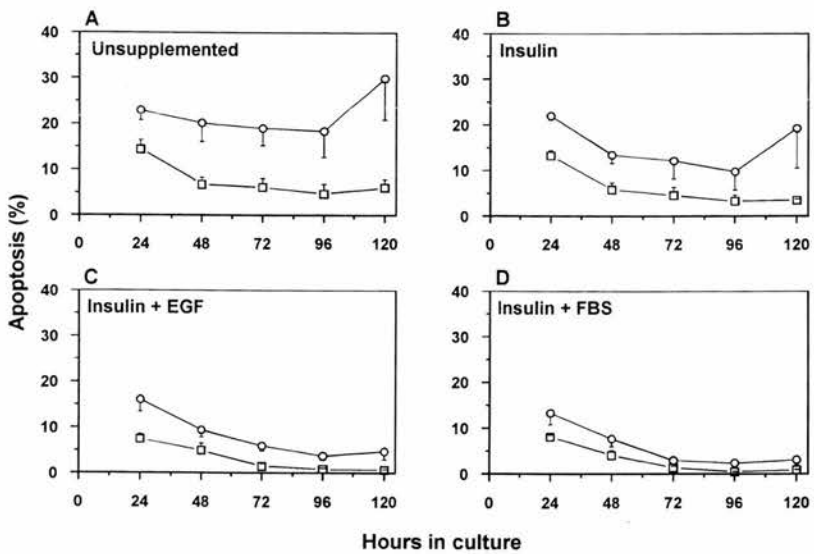



**Figure 5.** Hepatocyte apoptosis in culture (% of 500 cells), assessed on Feulgen-stained hepatocyte monolayers. Cells were cultured in Chee's medium with 30 nM dexamethasone plus unsupplemented (*A*); 300 nM insulin (*B*); 300 nM insulin + 25 ng/ml EGF (*C*); 300 nM insulin + 1% FBS (*D*). Mean + SEM for duplicate cultures from three mice of each genotype (n = 6). Key: wild type (○); p53 null (□).

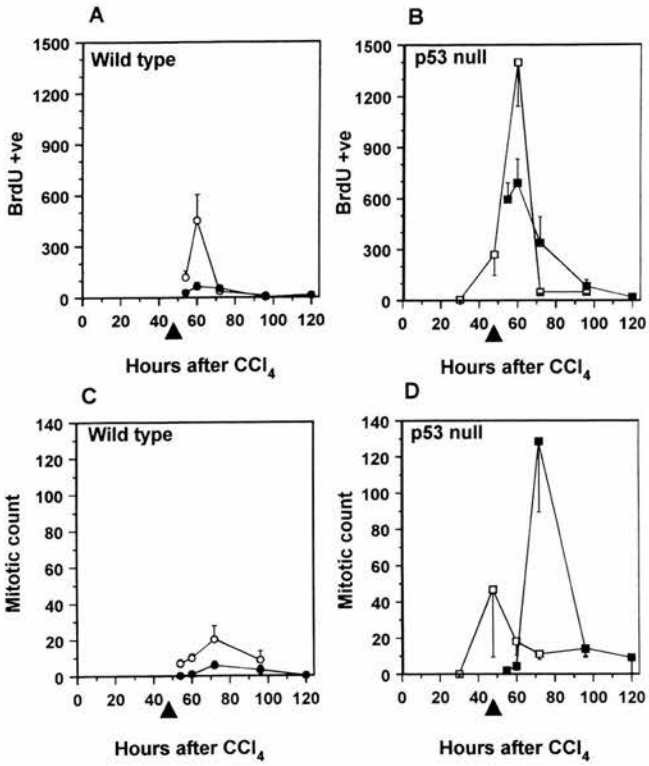



**Figure 6.** Effects of 15 Gy γ-irradiation on DNA synthesis (*A, B*) and mitotic activity (*C, D*) by regenerating wild-type and p53 null livers. Mice given $CCl_4$ at time zero (see Fig. 4) were γ-irradiated or mock-irradiated 48 h later. Mitotic activity (per 20 high-power fields) and BrdU incorporation (see Fig. 4) were assessed on hematoxylin-stained tissue sections; mean ± SEM (three to five) mice per observation). The large error bar for the mitotic count of unirradiated null mice at 48 h is due to a single exceptional animal and is unexplained. Other animals at this time point had mitotic counts of 10 or below. Wild-type unirradiated (○); irradiated (●); p53 null unirradiated (□); irradiated (■).

dependent (30); taken together, these data suggest that in hepatocytes p53 facilitates cellular rehabilitation rather than deletion after DNA injury, in contrast with cell types such as intestinal crypt epithelium (2), thymocytes (18), and splenocytes (19). These observations provide further evidence that apoptosis and cycle arrest mediated by p53 are independent pathways, and illustrate the tissue specificity of different p53-dependent responses. Moreover, the finding that hepatocyte apoptosis after DNA damage is not regulated by p53 suggests the existence of apoptotic pathways under different genetic control, but may also have relevance to tumor suppression and represent better targets for novel cancer chemotherapeutic strategies. The observation that cytokines reduced apoptosis induced by DNA damage also has implications for genotoxic drug therapy of liver neoplasms, suggesting a utility for cytokines to either manipulate tumor cell chemosensitivity or promote normal cell survival. Similar observations have been made in other systems (31, 32).

Thus, p53 assists hepatocytes sustaining DNA damage to recover from the genotoxic injury through growth arrest if they are proliferating, and perhaps by enhancing repair activity, but other factors (including the type of injury, the cytokine environment, and perhaps other genes) regulate cell death.

## Control of liver ploidy

Polyploidization is a feature of normal postnatal liver development (33), but neither the purpose nor the regulation of this irreversible process is well understood (34). The finding that p53 genotype did not affect the progression from diploid to polyploid in the aging liver is in keeping with the model presented that p53 has no role in unstimulated hepatocytes, but does not exclude a role for a p53-dependent pathway in determining altered polyploidization after DNA damage. Indeed, hepatocyte polyploidization increases after γ-irradiation (35), and ERCC1-deficient mice that are defective in DNA repair show persistent p53 accumulation in hepatocytes and develop abnormal liver polyploidy (36). The relationship of polyploidy to aneuploidy is not well defined, but present findings that p53 deficiency might allow generation of abnormal hepatocyte ploidy after DNA injury suggest it will be of interest to compare polyploidization responses to DNA damage of wild-type and p53-deficient livers.

## Implications for hepatocarcinogenesis

In a permissive environment p53-deficiency allows sustained survival, increased proliferation, and impaired damage responses that could accelerate carcinogenesis. Hepatitis B X protein and aflatoxin interact directly with the p53 protein and gene, respectively, suggesting a role for p53 dysfunction in

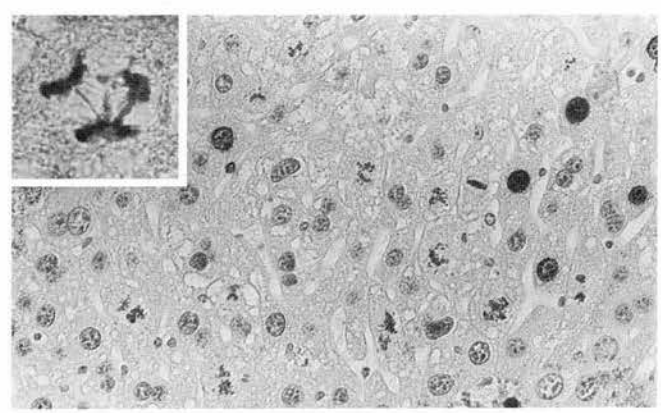


**Figure 7.** Mitotic activity of regenerating p53 null liver after γ-irradiation at 72 h after $CCl_4$ (24 h after 15 Gy γ-irradiation). Note the large number of mitotic figures, many of which are abnormal. Dark staining nuclei are BrdU-immunopositive. Inset: abnormal mitotic figure, with chromosomal bridges visible. Hematoxylin counterstain.

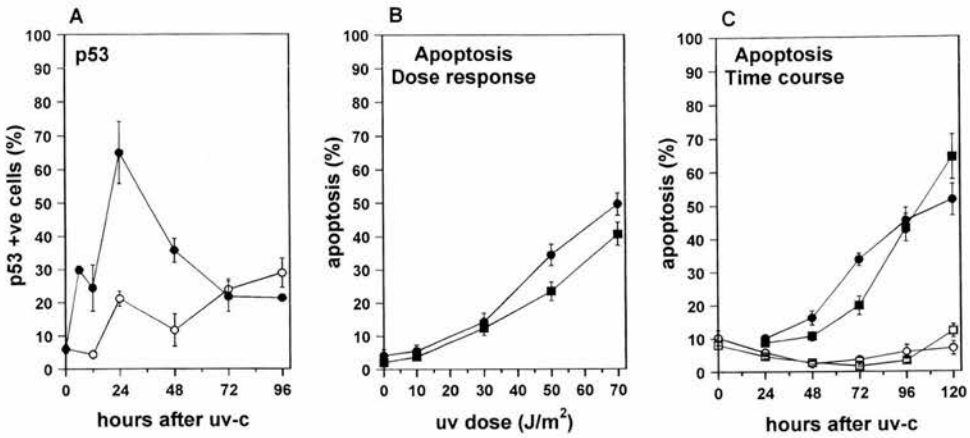



**Figure 8.** UV irradiation of primary hepatocyte cultures. *A*) p53 immunopositivity after 10J/m$^2$ UV-c. Immunocytochemistry was performed on acetone/methanol-fixed cultures using pAb421 (Oncogene Science). *B*) Dose response of apoptosis evaluated 72 h after UV irradiation. *C*) Time course of apoptosis after 50J/m$^2$ UV-c. Cells were cultured in Chee's medium with 100 nM dexamethasone, 1.5 μM insulin, 2% FBS. Wild-type unirradiated open circle; irradiated (○); p53 null unirradiated (□); irradiated (■).

early human hepatocarcinogenesis that this study has sought to identify. The results show that p53 has an important modulatory role on both proliferation and cell death, but only when the hepatocyte is released from $G_0$ or stressed (for example, by reduction of trophic support). Hence, p53 is likely to become important under disease conditions of chronic stimulation to proliferate and reduced trophic support wherein p53-deficient hepatocytes are likely to survive and outgrow p53-competent neighbors. These observations are of particular relevance to human hepatocarcinogenesis, which almost always occurs on a background of chronic liver damage and compensatory regeneration, and could explain selection for p53 dysfunction in clones of hepatocytes in an unfavorable environment within inflamed liver.

Perhaps it is not surprising therefore that there is no increased frequency of hepatocellular carcinoma in untreated or single dose chemical carcinogen-treated p53 heterozygous and homozygous deficient mice (37, 38). Such models do not produce the chronically disturbed hepatic environment that precedes by decades virtually all human hepatocellular carcinomas and that is probably necessary to manifest the otherwise latent phenotype of p53 dysfunction described here. A more appropriate model might be to evaluate the effects of a cross between p53-deficient and hepatitis B surface antigen transgenic mice. The latter develop a chronic necroinflammatory hepatitis, with regenerative hyperplasia in high producer lineages that leads eventually to development of hepatocellular carcinoma (39). FJ

C.O.C.B. is a Cancer Research Campaign Gordon Hamilton Fairley Clinical Research Fellow. A.R.C. is a Royal Society University Research Fellow.

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*Received for publication March 3, 1997.*
*Accepted for publication April 22, 1997.*