The role of the retinoblastoma gene in liver regeneration, polyploidisation and hepatocarcinogenesis.

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Declaration

All of the written work herein is my own. Any contribution made by others to the experimental work is acknowledged in the text. This work has not previously been submitted for any other degree or qualification.

Stephen A. Boyce

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This thesis is dedicated to my grandmother, Irene Paget, who always paid close attention to the collection of letters by her grandchildren.

Abstract

The Retinoblastoma Gene (Rb) plays a pivotal role in the control of cell division and cancer development. Loss of normal Rb function is thought to be a prerequisite for the development of all cancers. The observation that viral hepatitis, a major risk factor for the development of liver cancer, may modulate the function of Rb, suggests a role for Rb in the early stages of hepatocarcinogenesis.

It is not possible to model Rb loss *in vivo* using conventional knockout mice as Rb deletion is embryonic lethal. Instead, it was necessary to employ Cre Lox technology in order to develop a conditional knockout system. Using an adenoviral delivery system, liver-specific deletion of the floxed Rb gene could be achieved at levels proportional to the viral titre used.

To determine the role of Rb in the regulation of hepatocyte replication, two models of liver regeneration were developed. The first involved the use of the liver-specific toxin, carbon tetrachloride, to cause necrosis. The second involved performing a partial hepatectomy (PH) – surgical resection of approximately 2/3 of the murine liver. When liver regeneration following either hepatectomy or necrosis occurred following Rb loss, the result was deregulated hepatocyte division, with acceleration of hepatocyte proliferation. Loss of Rb also reduced the normal process of polyploidisation that usually occurs during regeneration.

To determine the role of Rb in liver cancer, a model of hepatocarcinogenesis was developed using the DNA damaging agent, diethylnitrosamine, together with protracted administration of carbon tetrachloride to produce sustained necrosis and regeneration. Following this treatment, loss of Rb was associated with increased levels of apoptosis and the development of preneoplastic foci that are characteristic of the early stages of hepatocarcinogenesis. These foci showed increased staining for markers of proliferation as compared to surrounding liver tissue. Quantification of foci demonstrated Rb loss was associated with an increase in the frequency, size, and total area of preneoplastic tissue.

Considered together, these results demonstrate an important role for Rb in the regulation of liver regeneration and polyploidisation. When Rb loss occurs on a

background of DNA damage and sustained inflammation, analogous to chronic liver disease, there is an acceleration of the preneoplastic phase of hepatocarcinogenesis suggesting loss of Rb may be a critical factor in the development of liver cancer.

Abbreviations

AFB	aflatoxin B
ARF	alternative reading frame
ATM	ataxia telangiectasia-mutated protein
ATR	ataxia telangiectasia - related
Bcl-2	B-cell lymphoma
BrdU	5-bromodeoxy-uridine
c-abl	abelson murine leukemia oncogene 1
CAH	chronic active hepatitis
CAR	Cocksackie Adeno Receptor
cdk	cyclin dependent kinase
CIP/KIP	cdk inhibitory protein/kinase-inhibitory protein
CKI	cdk inhibitor
CNS	central nervous system
Cre	cyclization recombinase enzyme
CTL	Cytotoxic T lymphocyte
DEN	diethylnitrosamine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNMTs	DNA methyltransferases
DP	dimerisation protein
E2F	early region factor 2
EAF	enzyme altered foci
EGF	epidermal growth factor
ERCC	excision repair cross-complementing rodent repair deficiency complementation group 1
FAH	focus of altered hepatocytes
floxed	flanked by loxP sites
HAT	histone acetylase
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBX	hepatitis B X protein
HCC	Hepatocellular carcinoma
HEC1	highly conserved in cancer 1
HDM2	human double minute clone 2 oncoprotein
HCV	hepatitis C virus
HDAC	histone deacetylase
IGF	insulin like growth factor
IL	interleukin
INK4	inhibitor of kinase 4
LEC	long-Evans rat with a cinnamon-like color
LOH	loss of heterozygosity
LoxP	locus of crossover of P1
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute clone 2 oncoprotein
MEFs	mouse embryonic fibroblasts
mRNA	messenger ribonucleic acid
MSi	micro satellite instability
NES	nuclear export signal
NF-kB	nuclear factor kB

NLS	nuclear localisation signal
NS5A	hepatitis C virus nonstructural 5A
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pfu	plaque forming units
PH	partial hepatectomy
PNS	peripheral nervous system
pRb	retinoblastoma protein
Rb	retinoblastoma gene
RFLP	restriction fraction length polymorphisms
RNA	ribonucleic acid
STAT3	signal transducer and activator of transcription 3
Skp2	S-phase kinase-associated protein 2 (p45)
TGFα	transforming growth factor α
TGFβ	transforming growth factor ß
TNFR	tumour necrosis factor receptor
TNFα	tumour necrosis factor

Chapter 1 Introduction

Cell cycle control is integral to the strict regulation of hepatocyte proliferation seen in liver regeneration and during the response of the liver to injury. By extension, the failure of such strict control in an environment of chronic repair and proliferation will have clear implications for the development of populations of cells which show deregulated cell cycling, a prerequisite to the development of cancer.

This chapter aims to review current understanding of the processes that control the normal responses to injury which occur during liver regeneration and repair, in relation to how such control is disrupted in the process of hepatocarcinogenesis.

Section A

An overview of the pRb pathway

A Retinoblastoma is a rare tumour which presents in childhood as a consequence of an inherited mutation of the Retinoblastoma Gene (Rb). Development of the tumour is associated with the loss of the second allele. Rb was the first tumour suppressor gene to be identified and cloned (Friend et al. 1986). The pattern of inheritance, and development of Retinoblastoma, gave rise to the "two hit" theory of tumour suppressor inactivation as a mechanism for the development of cancer (Knudson, Jr. 1971). Subsequent investigation demonstrated that Rb was frequently lost in other more common cancers occurring in adulthood, including lung cancer (Harbour et al. 1988) and osteosarcoma (Friend et al. 1986). Further examination of the function of Rb demonstrated a key role in cell cycle regulation, such that loss of Rb function is now seen as an essential step in the development of cancer (Hahn et al. 2002;Hanahan et al. 2000;Sellers et al. 1997;Sherr 1996)

pRb is a member of the pocket protein family. Fitting with a fundamental role in the development of human cancer, the functions of pRb, and fellow members of the pocket protein family, encompass the integration of many regulatory cell cycle pathways including those of cell survival, replication and death.

Structure of the Retinoblastoma Protein

The retinoblastoma protein (pRb) is a large nuclear protein (928 amino acids with a molecular weight of 110 kD and has a long half-life (Chen et al. 1989) . pRb is ubiquitously expressed in all mammalian tissues (Lee et al. 1987). The protein has three distinctive domains: an N terminal domain, a C terminal domain, and a central A and B region. The A and B regions interact to form the characteristic A/B pocket. This pocket contains a number of important protein binding domains, including binding sites for the early region factor 2 (E2F) group of transcription factors (Lee et al. 1998). pRb lacks a DNA binding domain, but instead is attached to promoters via transcription factors such as the E2Fs. The importance of the pocket region was first demonstrated through its association with DNA virus oncoproteins, which were shown to bring about cell transformation *in vitro* by binding to the pocket region, and hence, inactivating pRb (Dyson et al. 1989;Hu et al. 1990). It is the A/B pocket that determines the biological activities of the protein, and correspondingly it is this region of the protein that is most frequently mutated in human tumours (Hu et al. 1990). The C terminal domain of pRb interacts with a number of cell cycle related proteins, including MDM2 (mouse double minute clone 2 oncoprotein), p53, and c-abl (abelson murine leukemia oncogene 1) (Welch et al. 1993;Xiao et al. 1995). In addition, the C terminal domain has a number of cdk phosphorylation sites. The function of the N terminal remains unclear. The pocket region is the common feature of the pocket protein family which, in addition to pRb, includes the proteins p107 and p130.

p107 and p130, fellow members of the pocket protein family

Although all three members of the pocket protein family share the same pocket protein region, p107 and p130 have more in common with each other than with pRb. p107 and p130 have different binding partners and bind different members of the E2F family of transcription factors (Figure 1) than pRb. Whereas pRb is expressed in both proliferating and non proliferating cells, p107 is expressed at a higher level in proliferating cells than quiescent cells, and p130 is expressed at a higher level in quiescent cells compared to proliferating cells. Thus, during the transition from quiescence to proliferation, the levels of pRb remain relatively stable, whilst there is a rapid rise in levels of p107, and a corresponding decrease in the levels of p130 (Figure 2, next page). (Classon et al. 2001;Grana et al. 1998). In terms of function, Rb, p107, and p130 can all arrest cells in G1 when over-expressed (Claudio et al. 1994;Zhu et al. 1993). There is increasing acknowledgement that a degree of functional over-lap exists in the tumour suppressor activity of the pocket proteins. However, they also exhibit varying levels of specificity during different phases of the cell cycle.

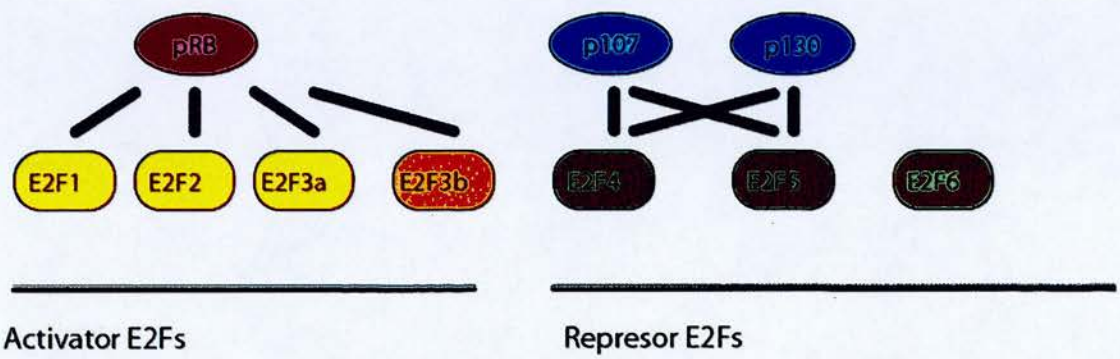


Figure 1 The pocket proteins have various binding affinities for different members of the E2F family.

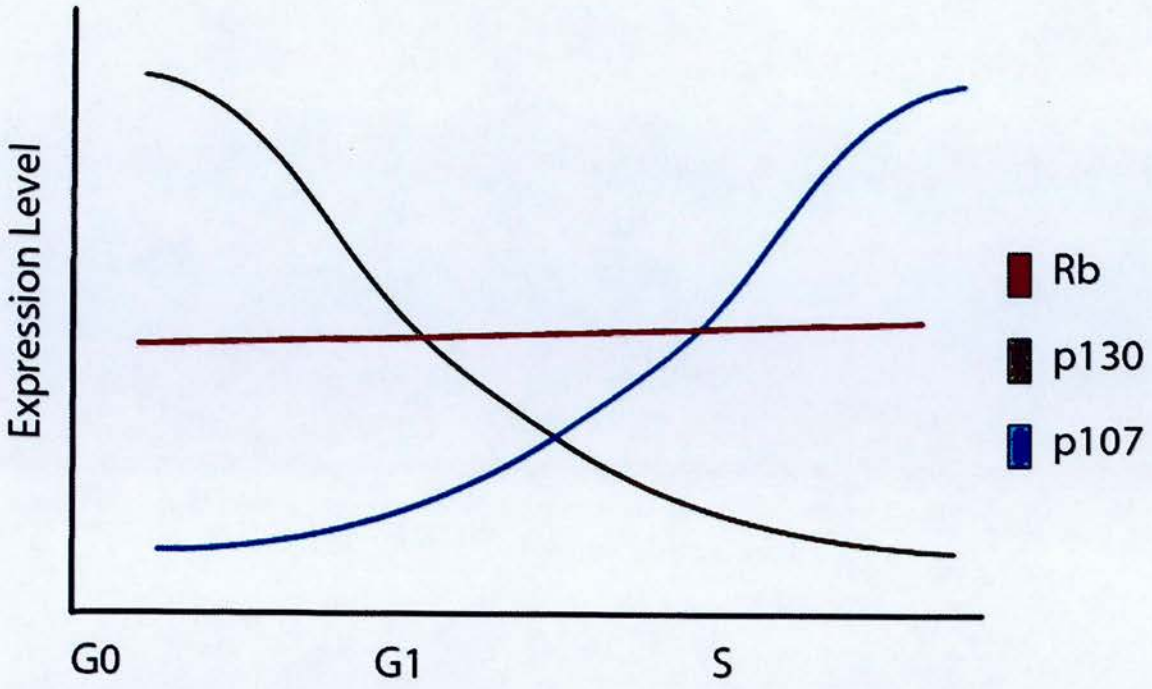


Figure 2 Schematic representation of the change in expression of pocket proteins during progression of the cell cycle.

Regulation of the Pocket Proteins

The activity of the pocket proteins is regulated by a number of different mechanisms which include:

- Phosphorylation by cyclin dependent kinases (CDK) during cell cycle progression
- Genetic inactivation as occurs in the development of cancers
- Functional inactivation by viral oncoproteins
- Caspase degradation during the process of apoptosis(Chau et al. 2003)

Pocket Protein Regulation during the cell cycle

The observation that multiple, differently phosphorylated forms of pRb existed *in vivo*, together with its long half-life; suggested Rb was predominantly regulated by post-translational phosphorylation. The phosphorylation of pRb and that of the other pocket proteins is now known to vary in a cell cycle-dependent manner (Beijersbergen et al. 1996;Grana et al. 1998). In G0, pRb is predominantly hypophosphorylated, whilst in G1, S, G2 and M phases of the cell cycle it is hyperphosphorylated. The hypophosphorylated form of Rb is the active form; therefore, phosphorylation of pRb, which occurs early in cell cycle progression, renders it inactive. Injection of hypophosphorylated Rb into cells brings about cell cycle arrest (Goodrich et al. 1991), and phosphorylation of pRb brings about ectopic cell cycle entry. Phosphorylation of Rb increases following serum stimulation, and decreases in response to anti-proliferative cytokines, such as transforming growth factor β, (TGFß) (Laiho et al. 1990). Phosphorylation of pRb causes a conformational change in the pocket region which results in reduced binding affinity for E2F transcription factors (Harbour et al. 1999). pRb remains inactivated until the end of mitosis when a combination of decreasing protein kinase activity, and increasing protein phosphatase activity, bring about the dephosphorylation and reactivation of pRb. (Yan et al. 1999)

An established, simplified working model for the function of pRb, proposes that in quiescence, active, hypo-phosphorylated pocket proteins prevent cell cycle entry. Suppression of cell cycle entry occurs predominantly through interaction with the E2F group of transcription factors. The repressive activity of pRb is determined by its phosphorylation status, which is dependent on the balance between protein kinases and protein kinase inhibitors. The balance of kinase activity is in turn determined by pro- and anti-proliferative signals. Mitogenic stimulation increases the activity of kinases, predominantly CDKs, and reduces the activity of cyclin kinase inhibitors (CKIs). Hence, the pocket proteins are phosphorylated and deactivated. Deactivation of the pocket proteins abrogates their repressive effects on proliferation

by releasing the E2F transcription factors that transcribe a host of genes required for cell cycle progression (Muller et al. 2001). Transcription of such genes therefore occurs and the cell consequently moves into S phase.

Regulation of pRb activity through phosphorylation by cyclin dependent kinases

Hyper-phosphorylation of pRb occurs at multiple sites on the protein and is mediated by the following cdks in a sequential fashion during progression of the cell cycle.

- Cyclin D-cdk4/6 in early G1
- Cyclin E-cdk2 at the end of G1
- Cyclin A-cdk2 during S phase.

Mitogenic signalling increases cyclin D levels (Matsushime et al. 1991), increases the synthesis and stability of cyclin D-dependent cdks, and reduces the association of cdks with cyclin kinase inhibitors (Sherr and Roberts 1999). Cyclin D1 has been shown to be required for cell cycle progression through G1(Baldin et al. 1993). The resulting phosphorylation of pRb by Cyclin D-cdk4/6 complexes increases E2F activity (Harbour et al. 1999). E2F then increases the activity of Cyclins E and A, which contribute further to the phosphorylation of pRb during S phase until the point of cell division at mitosis (reviewed (Sherr 2000). Ectopic expression of Cyclins D, E or A is able to abrogate the repressive effect of functional pRb.(Horton et al. 1995).

Regulation of pRb activity through inhibition of phosphorylation by Cyclin Kinase Inhibitors

The actions of cdks are opposed by two families of cdk inhibitors (CKIs), the CIP/KIP family, and the INK4 family.

1.The CIP/KIP family

The first family of CKIs is known as the Cdk-Inhibitory Protein Kinase –Inhibitory Protein family (CIP/KIP), and includes p27KIP1, p21WAF1/CIP1 and p57 KIP2. CIP/KIP proteins interact specifically with both cyclins and cdks. The most functionally important CIP/KIP is p21. p21 was first identified as a senescence-inducing protein (el Deiry et al. 1994), and is now known to mediate repression of cell cycle progression by two main mechanisms. Firstly, it associates with, and specifically inhibits, Cyclin E- and Cyclin A-dependent kinases, therefore maintaining pRb in the hypophosphorylated state (reviewed (Gartel et al. 2005)). During G1, increasing levels of Cyclin D-cdk 4/6 help to sequester p21 from Cyclin E-CDK2, thus increasing its activity late in G1. Secondly, p21 has a direct inhibitory effect on cell cycle progression through its action on the DNA synthetic machinery, including proliferating cell nuclear antigen (PCNA) (Waga et al. 1994).

The activity of the CIP/KIP family is regulated at a number of different levels. TGFß, which generally inhibits cell cycle progression, increases levels of both p21 and p15 (a member of the INK4 family) (Reynisdottir et al. 1995). DNA damage stabilises the tumour suppressor protein p53 which increases levels of p21, leading to pRb inactivation and cell cycle arrest (Agarwal et al. 1995)

2.The INK4 family

The second family of CKIs is the inhibitor of cdk4 family (INK4), whose members include p16 INK4a, p15 INK4b, p18 INK4c and p19 INK4d. Each member specifically binds and inhibits Cyclin D-dependent kinases by blocking the kinase

active site, thus preventing association with cyclins. In the absence of p16 protein, cdk4 binds Cyclin D and phosphorylates pRb, therefore stimulating entry into S phase.(reviewed (Roussel 1999). In contrast to the CIP/KIP family, mutations such as methylation and homozygous deletion in the INK4 family, particularly p16 INK4a, are commonly implicated in the aetiology of human cancer (Ortega et al. 2002).

p16 INK4a shares a chromosomal locus with p14 alternative reading frame (p14 ARF); equivalent to the mouse protein, p19 ARF. p14ARF acts as a tumour suppressor through specific activation of the p53 pathway. p14ARF stabilises p53 by inhibiting mouse double minute clone 2 oncoprotein (MDM2), which is the ubiquitin ligase responsible for the degradation of p53. p14ARF acts to sense the mitogenic current through the cell and is activated in response to oncogenic signals, such as c-myc and ras, leading to p53 stabilisation (reviewed (Serrano et al. 1996;Serrano 2000). Deletions of the INK4a/ARF locus result in the loss of two distinct tumour suppressor activities, and such deletions have been identified in a variety of human cancers (Ruas et al. 1998)

Deregulation of pocket protein function through viral inactivation in carcinogenesis

The normal regulation, and hence activity, of pRb is disrupted in a number of pathological states. Viral oncoproteins inhibit the binding of pRb to E2F (Chellappan et al. 1992). Study of the interaction between viral oncoproteins and pRb aided elucidation of the oncogenic mechanisms of such tumour suppressor proteins, and provided important observations which identified pRb as a key regulator of the cell cycle.

The deregulation of pRb in oncogenesis occurs through a number of different mechanisms that may involve both the loss of expression, or function, of pRb itself

(through deletion (Knudson, Jr. et al. 1976), mutation (Horowitz et al. 1989) or methylation (Ohtani-Fujita et al. 1993). The function of Rb can also be affected by either the loss of function, or gain of function, of other components of the Rb pathway, such as the cyclin kinase inhibitors or cyclins.

Loss of pRb function is considered to be an obligatory step in the development of cancer (Hanahan et al. 2000), though there is considerable variation between tumours as to which is the predominant dysfunctional element of the Rb pathway. Loss of one element of the pathway tends to be predominant in any particular tumour, mutually excluding the loss of another element. For example, the loss of function of p16INK4a can be demonstrated throughout the spectrum of human tumours, but, whereas loss of function of p16INK4a occurs in the majority of pancreatic cancers, (Rozenblum et al. 1997), it is an uncommon finding in small lung cancers.(Wistuba et al. 2001). Similarly, over expression of Cyclin D1 is very common in Mantle cell lymphoma (Dreyling et al. 1997), but is unusual in small cell lung cancer. The spectrum of perturbations which occur in the Rb/E2F pathway during the development of liver cancer are discussed in Section D "Liver Cancer"of this chapter.

Rb is regulated by apoptotic pathways

In addition to regulation through phosphorylation, pRb is also regulated as a consequence of activation of the apoptotic cascade and is degraded by caspases. (Chau et al. 2003)

In vivo models of Rb Loss

Examination of the consequences of Rb loss *in vivo* has helped elucidate the role of Rb in development, cell cycle regulation, and tumorigenesis (reviewed (Classon et al. 2002). *In vivo* models have employed murine knockouts, both conventional and conditional. Drosophila *melanogasta* have also proved a powerful experimental tool

in the study of Rb function, particularly because there are a smaller number of both pocket proteins and E2F family members in this species (Stevaux et al. 2002). Such knockouts have demonstrated the importance of Rb in the development of the whole organism, as well as in the development of tumours in a number of different organs. In addition to demonstrating that Rb loss may be a critical event in the development of various tumours, such studies have also demonstrated the differences that exist between humans and mice, particularly regarding functional redundancy in the pocket proteins, which is apparent in mice but not humans.

Rb appears to be essential for normal intrauterine development. Homozygous Rb null mice die in mid gestation (Clarke et al. 1992;Jacks et al. 1992). The reasons for this are not entirely clear. Initially, it was assumed Rb loss was directly responsible for the deranged phenotype seen in these ill fated embryos, which died at 13½ days exhibiting gross defects in the haematopoietic and nervous systems, as well as developmental abnormalities in the lens and skeletal muscle. Since then, it has been suggested that both the abnormalities and intrauterine death may be secondary to placental abnormalities, for which Rb loss is directly responsible (Wu et al. 2003).

Examination of Rb null embryos suggested a number of biological functions that might be regulated by Rb. These include ectopic cell cycle activity and increases in the level of apoptosis. The role of Rb in apoptosis is discussed later in this chapter. Germ line Rb loss is associated with increased E2F activity (Almasan et al. 1995). The phenotype resulting from Rb loss may, at least in part, be mediated through deregulated E2F activity. Evidence for this suggestion comes from the observation that the phenotype typical of Rb loss – one of increased apoptosis and deregulated proliferation, can be partly relieved by concomitant E2F deletion (Maehara et al. 2005;Yamasaki et al. 1998). Interestingly, in Rb null chimeras where Rb null cells contribute to all tissues, the mice survive and exhibit only very mild phenotypic alterations (Williams et al. 1994c) suggesting Rb may have an additional non autonomous role in development (Whyatt et al. 2002).

Children with Rb haplo-insufficency not only develop Retinoblastoma, but are at a much higher risk of developing further tumours in later life, particularly bone and soft tissue sarcomas and melanomas (Moll et al. 1997). Similarly, mice which have been engineered to carry a single germ line mutation of Rb develop a spectrum of tumours with complete penetrance. In these mice, tumours tend to be in the pituitary and thyroid glands, and exhibit loss of heterozygosity at the Rb locus (Harrison et al. 1995;Jacks 1996). However, Retinoblastoma does not develop in mice heterozygous for Rb, nor does Retinoblastoma develop in bi-allelic conditional knockouts in mouse photoreceptor cells (Vooijs et al. 2002). The development of Retinoblastoma in the mouse requires the additional loss of p107 (Robanus-Maandag et al. 1998). This functional overlap between Rb and p107 in the mouse has also been demonstrated in the phenotype of deregulated cell cycling following Rb loss. In mouse embryonic fibroblasts (MEFs), conditional acute Rb deletion leads to deregulated cell cycling, but when Rb loss is engineered at the germ line level, there is a compensatory increase in p107 and alleviation of deregulated phenotype (Sage et al. 2003). Similar findings have been demonstrated in a conditional liver-specific Rb knockout (Mayhew et al. 2005). In addition, mice with a conditional Rb deletion in combination with p107 loss are particularly susceptible to cancer (Dannenberg et al. 2004). This functional overlap between Rb and p107 in mice may be because p107 is a downstream transcriptional target of E2F (Muller et al. 2001) i.e. loss of Rb leads to increased E2F activity and p107 is up regulated. In humans, Rb deletion does not result in increased p107 activity. For example, p107 activity is not increased following Rb loss in human retinal progenitor cells (Dyer et al. 2005). Such a functional overlap occurring in mice, but not humans may explain why inactivation of Rb alone is sufficient for retinoblastoma development in humans but not mice.

Mice deficient in p107 or p130 develop normally and exhibit only very mild phenotypic alterations which tend to be strain-dependent (Cobrinik et al. 1996;Lee et al. 1996b;Lee et al. 1996a). Double knockout of p107 and p130 results in some developmental abnormalities, including abnormal bone development suggesting a functional overlap in the contribution of p107 and p130 to development (Cobrinik et

al. 1996). Mice homozygous or heterozygous for deletions of either p107 or p130, or both, do not develop tumours. Similarly, mutation of p107 or p130 is a rare event in human cancer (Mulligan et al. 1998)

E2F family of transcription factors

Rb mediates its repressive effects on cell cycle proliferation predominantly through its interactions with the E2F family of transcription factors. The E2F family are responsible for the transcription of a host of genes required for cell proliferation, including those required for DNA synthesis (such as PCNA), for cdk activity, and for recognition and utilization of replication origins (reviewed (Dyson 1998).

E2F was first demonstrated as a biochemical factor through which adenoviral infection could bring about expression of cell cycle genes (Nevins 1992). It was subsequently demonstrated that this transcriptional activity was restrained by the binding of Rb to E2F, and that the viral oncoprotein E1A could disrupt this association, thereby causing DNA synthesis and potentially contributing to oncogenic transformation (reviewed (Nevins 1992).

Early studies demonstrated that hypophosphorylated pRb binds to, and inhibits, the E2F transactivation domain, thus preventing cell cycle progression (reviewed (Berk 1986). Since then it has been demonstrated that, in addition to the regulation of replication, the E2F family of transcription factors exhibit a wide range of functions with different family members playing different, often contrasting roles, depending on the context (Attwooll et al. 2004). In addition to their predominant role in cell proliferation and differentiation, E2F family members also act on a wide and diverse range of transcriptional targets in order to regulate a number of different biological processes including apoptosis, development, and tumorigenesis (reviewed (Cam et al. 2003;DeGregori 2002).

Mammalian E2F Family

Eight members of the mammalian E2F family have so far been identified and these interact variably with the pocket proteins. Members of E2F family form functional heterodimers with the dimerization protein (DP) family, which includes DP1 and DP2 (Krek et al. 1993;Wu et al. 1995). These heterodimers associate with the pocket proteins and recruit further regulatory proteins to form complexes in order to regulate the transcriptional machinery. Members of the E2F family can be segregated on the basis of their binding affinities for the various pocket proteins and their predominant effect on transcription into either activators or repressors of transcription (reviewed (Trimarchi et al. 2002). Initially there was little evidence to suggest that individual E2Fs showed specificity for particular E2F responsive promoters (Flemington et al. 1993;Takahashi et al. 2000;Wells et al. 2000). However, there is now increasing evidence pointing to some degree of specificity, and this might explain the different effect of loss of individual E2F members in specific E2F knockouts (Attwooll et al. 2004).

Most of what is known of the function of the E2F family is confined to the first 5 E2F members to be identified (E2F1 E2F2, E2F3a, E2F4 and E2F5).

E2F1,2,3a: Activators of Transcription

E2F1, E2F2 and E2F3a are the predominant transcriptional activators of the E2F family. Each can form a heterodimer with either DP1 or DP2 to give functional transcriptional activity, and these complexes preferentially bind Rb rather than p107 or p130.These three E2F proteins show high levels of homology in their structure, DNA binding and transactivation properties. Over-expression of activator E2Fs drives quiescent cells into S phase (Johnson et al. 1993;Vigo et al. 1999), can override inhibitory growth arrest signals, including TGFß and CKI activity, (DeGregori et al. 1995b;Mann et al. 1996), and can lead to the transformation of primary cells (Johnson et al. 1994;Xu et al. 1995). Alternatively, antibody mediated

inhibition of E2F3 causes cell cycle arrest (Leone et al. 1998a), and E2F3 deficient MEFs have reduced rates of proliferation (Humbert et al. 2000). Inhibition of all three E2Fs can completely block cell proliferation (Wu et al. 2001). Levels of activator E2F appear to vary temporally, being highest early in S phase, and generally absent, or expressed at low levels, during quiescence (Aguilar et al. 1994;Dyson 1998).

The transcriptional activity of the activator E2Fs is tightly regulated through association with the pocket proteins, particularly pRb. Phosphorylation of the pocket protein region of pRb abolishes this association and results in increased E2F activity. Functional inactivation of pRb causes deregulated E2F activity and unscheduled proliferation (Sage et al. 2003). Loss of either E2F1 or E2F3 is sufficient to reduce not only the abnormal proliferative phenotype resulting from Rb inactivation, but also suppress the p53-dependent and p53 independent apoptosis that is characteristic of deregulated E2F activity (Sage et al. 2000;Tsai et al. 1998). Furthermore, loss of E2F-1 reduces tumour development in mice heterozygous for Rb and increases the life span of such animals (Yamasaki et al. 1996a).

E2F4,5: Repressors of Transcription

E2F4 and E2F5 are the predominant repressors of transcription and differ significantly in their structure from E2F1-3b. Importantly, members of this E2F repressor group contain a nuclear export signal (NES) (Boveri T 1914;Lindeman et al. 1997). (Figure 3, page 26). This export signal is absent in the activator group, which has instead a nuclear localisation signal (Muller et al. 1997b). In addition, the repressor E2F members show different pocket protein binding affinities compared to the activator E2F group; E2F4 can interact with all three pocket proteins (pRb, p107 and p130) at different points through the cell cycle, E2F5 binds only to p130. Although high levels of expression of E2F4 and E2F5 can bring about slight increases in cell cycle gene expression, they are poor transcriptional activators

(DeGregori et al. 1997). E2F4 and 5 appear to repress transcription by blocking interaction of the E2F activators with their target genes through formation of repressor complexes with p107 and p130. Consistent with their role as transcriptional repressors, E2F 4 and 5 appear to be required for exit from the cell cycle and terminal differentiation insomuch as cells lacking repressor E2Fs fail to respond to cell cycle arrest signals (Gaubatz et al. 2000;Mann et al. 1996). Mutation of E2F binding sites within E2F responsive gene promoters produces insensitivity to growth arrest signals and results in increased transcription. This demonstrates the importance of these repressor complexes in the negative regulation of the cell cycle (Li et al. 1997). Importantly, chromosome immunoprecipitation (ChIP) studies have demonstrated the association of such repressor complexes with many E2F responsive promoters during quiescence. In these studies, repressor E2Fs are replaced by the activator E2Fs as cells progress from G0/G1 toward S phase; a change that correlates with the induction of E2F dependent gene expression (Takahashi et al. 2000).

The division of E2F1-5 on the basis of their activity into activators and repressors is dependent on their preferential association with different pocket proteins, and also on their sub cellular localisation. When the pocket proteins are phosphorylated by rising cdk activity, the free E2Fs are directed in different directions. The activators are localized to the nucleus where they can effect transcription by virtue of their nuclear localisation signal, whereas the repressors are directed towards the cytoplasm (Muller et al. 1997a;Verona et al. 1997).

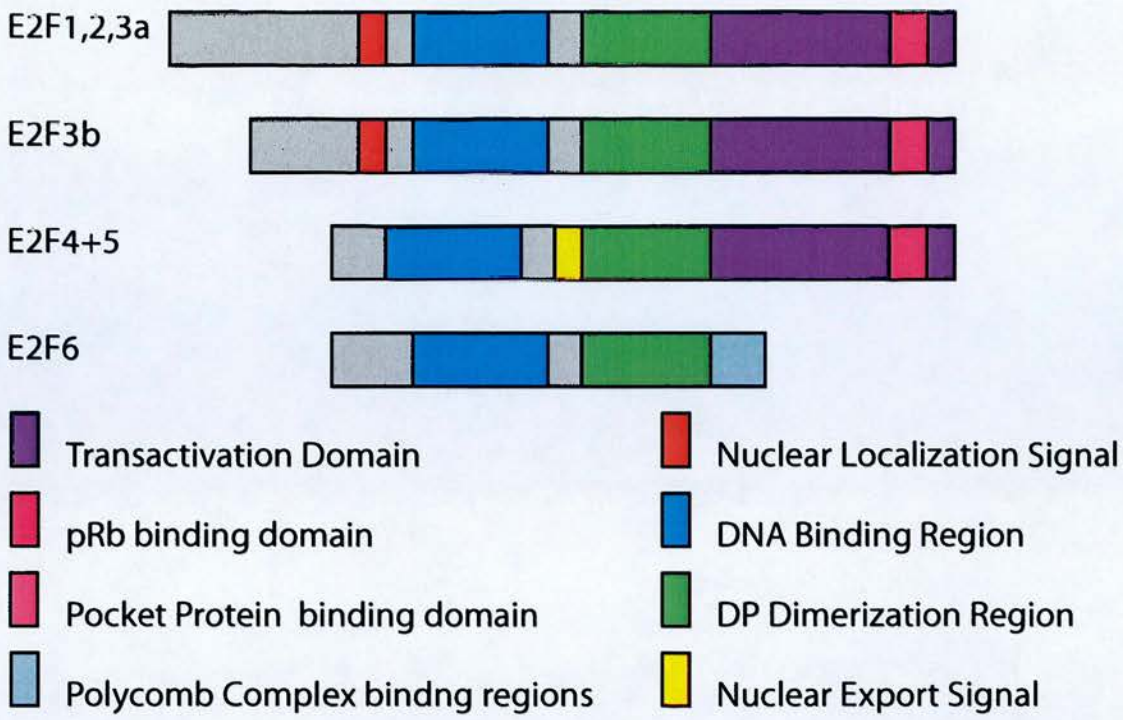


Figure 3 All of the E2F members have specific domains for DNA binding and dimerization with the DP proteins. The Activator E2Fs have a Nuclear Localization Signal, the Repressor E2Fs 4 and 5 have a Nuclear Export Signal. All E2Fs have a transactivation domain with the exception of E2F6 which shares little homology with the rest of the group and has a unique region for the binding of the Polycomb Complex. (Trimarchi and Lees 2002)

Latest members of the E2F family; E2F3b, E2F6 and E2F7.

The newest members of the E2F family to be identified include E2F3b, E2F6 and E2F7. E2F3b is transcribed from a distinct locus of the E2F3 gene, and in complex with pRb acts predominantly as a repressor of transcription during quiescence (Leone et al. 2000). E2F6 and E2F7 do not bind to the pocket regions of the pocket proteins, nor do they possess transactivation domains, yet nonetheless they do repress transcription (Gaubatz et al. 1998;Leone et al. 2000). E2F6 mediates this repression through association with the Polycomb proteins (PcG), a group of proteins which

repress transcription through modification of chromatin structure (Trimarchi et al. 2001). How E2F7 mediates transcriptional repression is not clear.

Mechanisms of Transcriptional regulation by Rb/E2F

The complex of Rb and E2Fs mediate transcriptional regulation through a number of different mechanisms.

Firstly, the pocket proteins bind to and physically block the transactivation domain of the E2F transcription factors (Flemington et al. 1993;Helin et al. 1993), inhibiting the ability of the E2F members to induce the transcriptional machinery.

Secondly, the pocket protein/E2F complex recruits chromatin remodelling enzymes to form "repressor complexes". A large body of work has demonstrated E2F mediated transcription is coincident with significant chromatin structural changes that are known to influence the transcriptional machinery (reviewed (Frolov et al. 2004;Harbour et al. 2000;Zhang et al. 2001). Rb, p107 and p130 all possess additional binding sites, distinct from their E2F binding sites, which can associate with chromatin remodelling enzymes. When bound to E2F, these chromatin remodelling complexes are recruited to the E2F responsive promoters. The modification of histones, which constitute a structural element of chromatin, is known to regulate transcription via conformational change of the DNA. Potential histone modifications include the addition or removal of acetyl groups, phosphorylation and methylation. The pocket proteins can associate with histone deacetylase (HDAC) enzymes, which can remove acetyl groups from histones resulting in the condensation of nucleosomes into chromatin. This inhibits transcription by hindering the access of transcription factors to the promoters. Conversely, hyperacetylation by histone acetyltransferases (HAT) is thought to cause conformational changes to the chromatin which might stimulate transcription. In mid to late G1 the replacement of the repressor complex p107/E2F4 by pRb/E2F

activator complexes is associated with the appearance of HAT and concomitant histone hyperacetylation (Rayman et al. 2002). Whether these modifications are simply coincident with the switch of repressor and activator binding, or an important mechanism of pocket protein mediated transcriptional repression is still unclear.

Rb/E2F and Regulation of apoptosis

In addition to ectopic cell cycling, increased E2F activity resulting from either over-expression of E2F or loss of Rb, results in increased apoptosis in a number of different experimental systems (Almasan et al. 1995;DeGregori et al. 1997;Pan et al. 1998;Shan et al. 1994;Symonds et al. 1994;Tsai et al. 1998). Initially, the ability to induce apoptosis was believed to be a unique property of E2F1 (Kowalik et al. 1998), but pro-apoptotic properties have since been demonstrated for both E2F2 and E2F3 (Leone et al. 1998b). Loss of E2F suppresses both unscheduled proliferation and apoptosis in Rb null mice (Takahashi et al. 2000;Tsai et al. 1998). In the central nervous system (CNS) of developing Rb null mice, loss of both E2F1 and E2F3, but not E2F2, inhibits apoptosis (Saavedra et al. 2002).

E2F can mediate apoptosis through a number of different pathways, both p53-dependent and p53-independent, which appear to vary according to cellular context (reviewed (Bell et al. 2004;Ginsberg 2002;Trimarchi et al. 2002). One of the earliest suggested pathways of p53 stabilisation by E2F was through the transcriptional activation of p14ARF, which is an acknowledged transcriptional target of E2F1 (Bates et al. 1998;DeGregori et al. 1997). p14ARF sequesters the protein HDM2 (MDM2 in the mouse) which normally binds to, and targets, p53 for ubiquitination and degradation. Thus, increased p14ARF levels result in increased levels of active p53. It has also been demonstrated that p53-dependent E2F mediated apoptosis can occur in the absence of p14ARF (Tolbert et al. 2002;Tsai et al. 2002). Alternative, less well characterised pathways of p14ARF-independent E2F mediated stabilisation

of p53 might include a direct pathway whereby the Cyclin A binding domain of E2F1 binds directly to, and stabilises, p53 following DNA damage (Hsieh et al. 2002). A second important alternative pathway is that of increased activity of the DNA damage inducible kinases, such as the ataxia telangiectasia-mutated protein (ATM) (Powers et al. 2004).

Potential p53-independent pathways of E2F mediated apoptosis include activation of the p53 homolog, p73 (Irwin et al. 2000). E2F1 increases transcription of p73 which directly links to the apoptotic machinery, ultimately leading to apoptosis. E2F1 also directly increases the expression of apoptosis protease activating factor 1 (Apaf1), which can lead to activation of caspases that are the ultimate downstream effectors of apoptosis (Furukawa et al. 2002;Nahle et al. 2002).

E2F1 may also induce apoptosis through inhibition of anti-apoptotic signalling pathways, such as disruption of the tumour necrosis factor (TNF) survival pathway (Phillips et al. 1999).

The characteristic apoptosis seen in Rb deleted systems suggests an active role for Rb in the suppression of apoptosis. The significance of these findings remains unclear. Apoptosis is not a natural consequence of the de-activation of pRb and consequent increase in E2F activity which occurs during the normal cell cycle. This has lead to the suggestion that apoptosis seen in experimental systems functions as an escape pathway for de-regulated cell cycle activity. Loss of this escape mechanism through, for example, loss of p53, increases tumorigenicity in Rb chimaeras (Harvey et al. 1995;Williams et al. 1994b). However, apoptosis is not an obligatory feature of Rb knockout systems and does not, for example, occur following conditional Rb deletion in the CNS (MacPherson et al. 2003). The occurrence of both unscheduled proliferation and increased apoptosis in these experimental systems suggests E2F could function as both a tumour suppressor and an oncogene. Fitting with its role as an oncogene, over-expression of E2F promotes cell cycle entry in quiescent cells

(Attwooll et al. 2004), and loss of E2F delays the development of pituitary tumours in Rb chimaeras (Yamasaki et al. 1998). Conversely, E2F null mice develop tumours (Yamasaki et al. 1996b), and loss of E2F suppresses the apoptosis seen in mice heterozygous for Rb (Field et al. 1996). These observations suggest a role for E2F as a tumour suppressor. Different models have been proposed in an effort to explain this apparent paradox. In the "conflict model", apoptosis may result from the conflict between the differentiation and unscheduled proliferation that occurs in Rb null embryos, with apoptosis as the default escape pathway (Chau et al. 2003). A modification of this model suggests a "threshold" of proliferation above which unscheduled proliferation triggers apoptosis (Trimarchi et al. 2002). Therefore, during the normal cell cycle Rb inactivation and E2F activity do not trigger apoptosis because this level of proliferation is below the required "threshold". A further, alternative explanation is that the inhibition of apoptosis is one of the prime functions of pRb, and that the apoptosis seen in Rb deleted systems is not primarily a response to unscheduled proliferation, but rather a reflection of the loss of the anti-apoptotic activity of pRb. Expansion of this idea has lead to the suggestion that pRb may function as a predominant regulator of both apoptosis and proliferation via differential activities at E2F-responsive apoptotic and proliferative genes. However, such a dual role for pRb has yet to be determined conclusively.

Section B

Liver Regeneration

The liver possesses a unique ability to regulate its size in order to meet the physiological requirements of the body. Although hepatocytes appear to be terminally differentiated, highly specialised cells with an extremely slow rate of cell turnover, these cells can, when required, proliferate rapidly to balance metabolic demand against liver function. This is demonstrated most dramatically in the rapid regenerative response that occurs following acute hepatic insufficiency, exemplified in the surgical model of partial hepatectomy.

Models of regeneration.

1.Hepatectomy

The experimental model of partial hepatectomy (PH) was first described in the 1930's by Higgins and Anderson when they demonstrated that following removal of 70% of the rat liver, restoration of liver tissue mass occurred within 14 days (Higgins et al. 1931b). Consequently, investigators demonstrated the rapidity with which apparently quiescent hepatocytes divide following PH, and showed that the number of dividing cells was proportional to the amount of liver resected (BUCHER et al. 1964).

Hepatectomy produces a compensatory hyperplasia, with hepatocytes in the remaining liver dividing to produce an anatomically different organ. This contrasts with the regeneration seen in cytotoxic liver damage, where repair brings about restoration of the original liver anatomy. Post hepatectomy, replication begins in the hepatocytes surrounding the portal triad, with waves of replication sweeping outwards towards the pericentral regions (Michalopoulos et al. 1997). Hepatocyte

replicative activity, as estimated by DNA synthesis, reaches its peak in the rat at 24 hours post surgery, and in the mouse at 36 hours. In larger animals the maximal rate of proliferation occurs later; 72-96 hours in canines and 7-10 days in primates (Koniaris et al. 2003a). It is estimated the majority of hepatocytes divide one to two times. The replication of hepatocytes is followed sequentially by that of biliary duct cells, Kupfer cells, Stellate cells, and finally, endothelial cells.

Although each hepatocyte replicates only once or twice, this belies the true replicative potential of the mature hepatocyte. This potential has been neatly demonstrated by a series of rescue transplantations of labelled normal hepatocytes into mice with engineered liver failure. The transplanted hepatocytes repopulate over 90% of the diseased recipient liver. The donor hepatocytes can be identified and serially transplanted more than 8 times; equivalent to a 7.3×10^{20} clonal expansion of the original population. (Overturf et al. 1997)

Such replicative potential raised doubts as to whether a mature differentiated cell could behave in such a way, and it was proposed there must be, within this population of replicating cells, actual stem cell progenitors responsible for such expansion (Sell 1990b). Such cells have certainly been identified and termed "oval cells", according to their morphology. Where controversy has reigned is the different roles these cells have been ascribed by various investigators in terms of their contribution to both replication in the short term, and hepatocarcinogenesis in the longer term (Fausto et al. 2003;Fausto 2004b;Sell 2001). There is little doubt that in particular experimental models, such as following the administration of retrosine, inhibition of mature hepatocyte proliferation occurs, emphasising the role of a progenitor population (Thorgeirsson et al. 2003), but the importance of such a cell subpopulation in liver disease remains controversial.

2. Liver repair following acute injury

Where the model of hepatectomy potentially provides a "clean" method for unravelling the intricacies of regeneration, many other toxin-induced methods of inducing regeneration exist (Koniaris et al. 2003a). One of the most established is the administration of carbon tetrachloride (CCL4). CCL4 is a hepatotoxin which, when injected into the peritoneal cavity, causes maximal necrosis in the central regions of liver lobules (zone 3) at around 24 hours post treatment. In this case, the proliferative response is more correctly termed regeneration because the repair response results in restoration of the previous anatomical structure. This involves an orchestrated inflammatory response, with removal of necrotic tissue and replication of neighbouring hepatocytes. The regenerative response following CCL4 treatment is similar to that following PH, although the cell cycle entry of hepatocytes is not as synchronous, and the peak of DNA synthesis occurs a little later.

Chronic liver injury has been modelled using either chronic toxin administration (Constandinou et al. 2005), or by the generation of transgenic mouse models (Sell 2003b).

Phases of regeneration

The regenerative events which follow hepatic resection or acute hepatic necrosis have been divided into different phases, allowing sequential consideration of those factors which are important in the restoration of functional liver mass. A number of key factors orchestrate the regenerative response including cytokines, growth factors and enzymes which modify the extracellular matrix. The interplay of such factors brings about a cascade of transcriptional activity with different banks of genes transcribed at different points following hepatectomy. (Fausto 2000a;Koniaris et al. 2003b;Taub 2003;Taub 2004).

The elusive start signal for liver regeneration

In the adult liver, hepatocytes are quiescent and the rate of hepatocyte replication is extremely low, with only 1 in 20,000 hepatocytes undergoing mitosis at any given time (Fausto et al. 1994). Neither the factors which keep hepatocytes in quiescence, nor those which initiate replication have been determined. It may be that the start signal for regeneration is insufficiency of liver function which occurs following loss of functional liver mass. How such physiological insufficiency initiates liver regeneration is not yet established.

Priming.

Priming is a reversible and non-committal period immediately following hepatectomy during which hepatocytes move from G0 to G1. During priming, a change in the sensitivity of hepatocytes to growth factor stimulation occurs. Priming can be induced experimentally through non-specific exogenous stimuli including sham surgery, dietary protein deprivation, or collagenase perfusion (Liu et al. 1994).The key events occuring in this period include remodelling of the extracelular matrix (ECM), which may be important in the early release of hepatocyte growth factors (reviewed (Mangnall et al. 2003), and the transcription of a bank of so called "immediate early genes". Factors which are thought to be important during the priming phase of liver regeneration are shown in Figure 4, on page 38.

Immediately following hepatectomy there is an increase in gut-derived factors, such as lipopolysaccharide (LPS), which reach the liver via the portal blood supply. LPS activates various cell types in the liver including endothelial cells, Kupfer cells and Stellate cells, which in turn produce a number of soluble mediators that render hepatocytes responsive to subsequent growth factor stimulation. Such factors include the cytokines tumour necrosis factor α (TNFα) (Yamada et al. 1997;Yamada et al. 1998) and interleukin 6 (IL-6) (Li et al. 2001). The increase in levels of TNFα and

IL-6 results in activation of pre-existing transcription factors including nuclear transcription factor κB (NF-kB) (FitzGerald et al. 1995), and STAT 3 (Cressman et al. 1994). Along with the immediate targets of NF-kB and signal transducer and activator of transcription 3 (STAT 3), there is a bank of immediate early genes which are transcribed in the first hour following hepatectomy. More than one hundred such immediate early genes have been identified by gene array experiments (Arai et al. 2003;Su et al. 2002).

The immediate early genes contain many transcription factors, including families of proto oncogenic transcription factors such as c-fos, c-myc and c-jun. These transcription factors then induce the expression of the delayed early genes and consequently amplify a cascade of transcription to bring about progression from G1 to S phase.

Progression Phase and Growth Factor Stimulation

Following priming, hepatocytes are responsive to, and indeed reliant upon, mitogenic stimulation to progress into S phase. Such cytokines including hepatocyte growth factor (HGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGFα), are termed complete mitogens because they bring about DNA synthesis in cells *in vitro*. Of these, HGF is arguably the most potent. HGF levels increase rapidly and remain elevated for 3 days following hepatectomy (Lindroos et al. 1991;Zarnegar et al. 1995). There are a number of potential sources of HGF including liver Stellate cells (Schirmacher et al. 1992), the ECM of the liver (Kim et al. 1997;Masumoto et al. 1993), and non-hepatic sources including the lung.(Yanagita et al. 1992) HGF stimulates hepatocyte proliferation and has pleiotropic effects on a number of mitogenic signalling pathways. The regenerative response to CCL4-induced liver injury can be blocked using antibodies against HGF(Burr et al. 1998). Constitutive over-expression of HGF in mice leads to

increased basal rates of hepatocyte proliferation and a two fold increase in the proliferative rate in response to hepatectomy (Shiota et al. 1998). Although it should be noted that these mice do not develop liver tumours, over-expression of TGFα produces mice with enlarged livers that demonstrate increased rates of hepatocyte proliferation and, as a consequence, develop liver tumours (Webber et al. 1994). Antibodies against TGFα reduce DNA synthesis following hepatectomy (Tomiya et al. 2000). TGF null mice develop normally (Mann et al. 1993), presumably because of a functional overlap with EGF. In the absence of a priming stimulus, these mitogens do not induce any real increase in hepatocyte proliferation when administered *in vivo* (Roos et al. 1995).

Those genes up-regulated during the progression phase, the so called "delayed early genes", include anti-apoptotic genes such as bcl-2 and many of the cell cycle genes including Cyclin D1, p53, p21 and p19 (Fausto 2000b). These proteins of the cell cycle orchestrate the now committed path from the restriction point to DNA synthesis and mitosis

Termination of Liver Regeneration

Hepatocytes move rapidly from quiescence to active replication and, upon restoration of functional liver mass, cell division ceases almost as quickly. The signals which terminate cell replication are not yet well understood. It may be that cessation of replication does not require a "stop" signal as such, but simply represents a return to a stable state (Bellamy 1997).There are situations, however, when an involution of the liver occurs. This is seen when over-sized orthotopic liver transplants undergo apoptosis in order to meet the physiological demands of the host (Francavilla et al. 1988) and suggests an active, rather than passive, method of halting hepatocyte replication.

The prime candidate for the cessation of hepatocyte division following hepatectomy is TGFß and related family members, including activin. TGFß is produced mainly by Stellate cells. Though the role of TGFß is complex, there is little doubt it has potent growth inhibitory effects on hepatocytes *in vitro* and *in vivo*. TGFß levels are normally very low in quiescent liver, increase steadily following hepatectomy, and peak 2-3 days post surgery (Bissell et al. 1995). Exogenous TGFß reduces rates of DNA synthesis following hepatectomy (Russell et al. 1988). But this occurs only when administered during late G1 and the effect is short lived. In this context, TGFß probably acts through p21 and p27 to inhibit G1 Cyclin/cdk activity and prevents cell cycle progression by blocking the phosphorylation of pRb. The over-expression of TGFß in transgenic mice leads to increased apoptosis, liver cirrhosis and, as a result, hepatocyte replication (Sanderson et al. 1995). Following hepatectomy, mice with conditional liver-specific deletion of TGFß receptor 2 demonstrate increased levels of proliferation and increased liver mass:body weight ratio compared to control animals (Romero-Gallo et al. 2005). However, an intact TGFß signalling pathway is not required for termination of regeneration (Oe et al. 2004). Therefore, although there is evidence to demonstrate TGFß has an inhibitory role to play in the regulation of regeneration, its role in the return to quiescence following regeneration remains unclear.

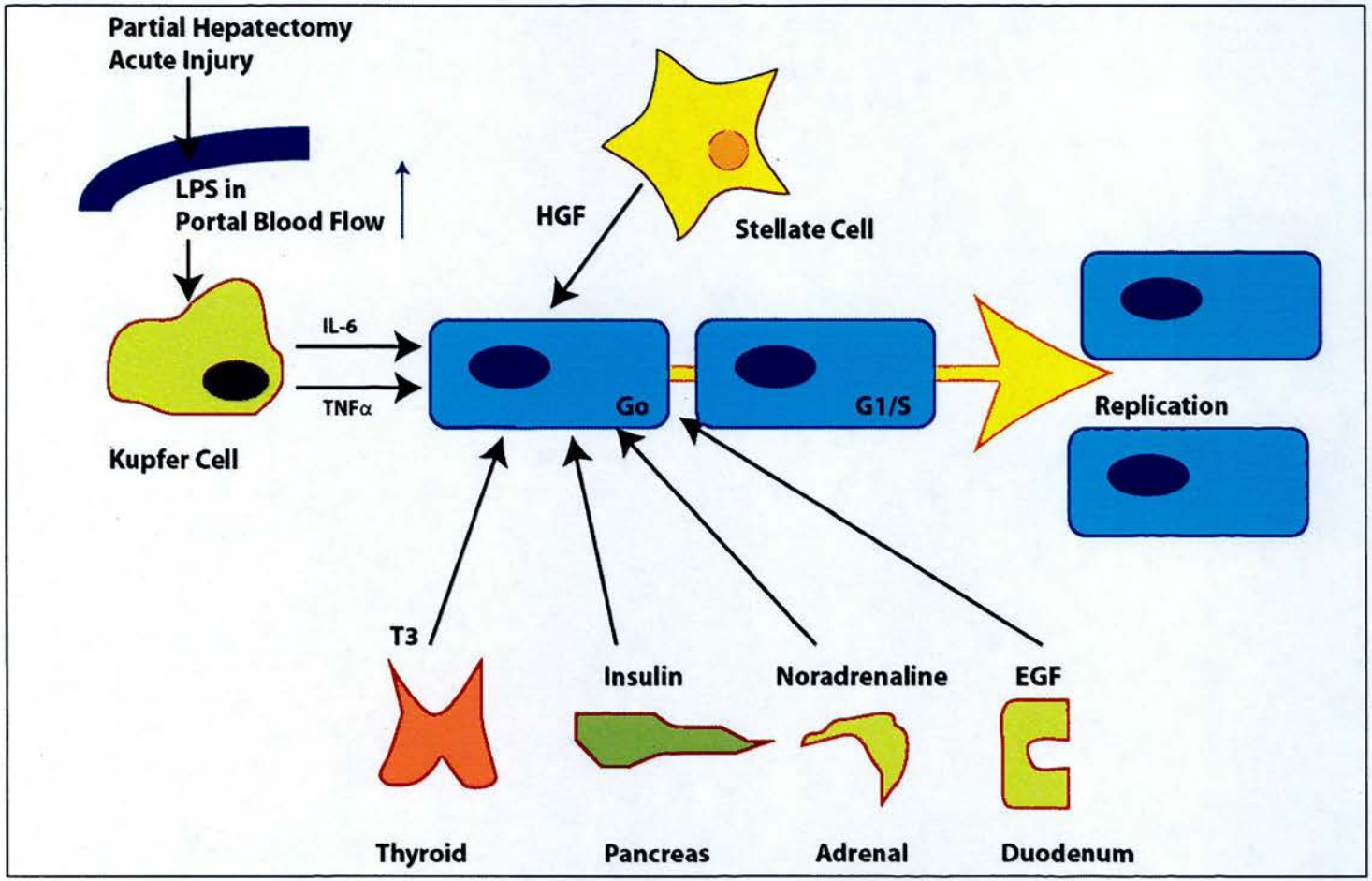


Figure 4. Schematic representation of those factors important in triggering hepatocyte replication following partial hepatectomy.(Taub 2004)

Apoptosis in Liver Regeneration

Apoptosis allows the elimination of defective hepatocytes which are either unable to go through replication, or whose replication has gone awry during regeneration. Apoptosis can be effected by ligation of cell-surface death receptors or be initiated by mitochondria degradation. The Bcl-2 family of proteins is amongst the most important of the upstream regulators of mitochondrial-induced apoptosis. The family, which contains both pro- and anti-apoptotic genes, controls the down stream effectors of apoptosis, leading ultimately to digestion of the nucleus and destruction of the cellular DNA. The prevalence of apoptosis is normally reduced following PH (Helvering et al. 1993) due to an upregulation of a number of anti-apoptotic members of the Bcl-2 family, most notably Bcl-2 and Bcl-XL (Bergelson et al. 1997). This up-regulation of anti-apoptotic Bcl-2 members may be mediated by pro-proliferative factors, such as IL-6. IL-6 null mice exhibit elevated expression of pro-apoptotic signals resulting in hepatocyte death and increased mortality (Kovalovich et al. 2001). p53 is normally expressed at very low levels in quiescent hepatocytes. p53 mRNA and protein levels increase at around 16 hours following hepatectomy, and decrease to basal levels by 18-24 hours (Thompson et al. 1986). p53 null mice show faster rates of proliferation following hepatectomy compared to wild type controls (Sell 2003a). Higher rates of proliferation in the absence of elevated levels of apoptosis result in an increase in both the number and density of cells in the regenerated liver compared to controls. This seems to imply a role for p53 as a negative regulator of regeneration (Yin et al. 1998). Levels of D cyclins and cdks 2 and 4 are also increased in p53 null mice (Yin et al. 1998).

Abnormal apoptotic activity is believed to be an important mediator of cell loss in a variety of liver disease states, including steatosis and cirrhosis, and to be a mediator of liver damage following transplantation (Guicciardi et al. 2005).

Maintenance of liver function during liver regeneration

Liver function is essential for life, and how the liver maintains homeostasis whilst hepatocytes are dividing is an important unanswered question. Among the bank of immediate early genes that are upregulated following hepatectomy are those which regulate gluconeogenesis. The remaining liver remnant increases its gluconeogenic activity to compensate for loss of liver mass, thus maintaining glucose homeostasis (Haber et al. 1995). The ability of the liver to maintain such function relies on an interplay between those genes which are normally expressed during quiescence, and those which are upregulated during regeneration (Leu et al. 2001).

Rb pathway in liver regeneration

A number of components of the Rb pathway have been studied in liver regeneration, during which levels of cell cycle mediators change rapidly. What remains unclear is how each of these components controls regeneration, and to what extent they contribute to the maintenance of quiescence.

Levels of Cyclin D and Cyclin D/cdk 4 activity rise rapidly following hepatectomy. In mice, the level of Cyclin D1 mRNA (the predominant murine form) increases significantly 12 hours following hepatectomy, with maximum levels occurring at 48-72 hours. This is followed by rapid increases in Cyclin D1/cdk4 complex formation and kinase activity. (Albrecht et al. 1998a). Transient over-expression of Cyclin D1 using an adenoviral delivery system is sufficient to produce DNA synthesis, mitosis and liver enlargement in mice.(Nelsen et al. 2001c).

Levels of both p21 and p27 are low in the quiescent liver and are upregulated by post transcriptional mechanisms following hepatectomy (Albrecht et al. 1997;Albrecht et

al. 1998b). Increased levels of p21 are also found in human liver disease, where they correlate with markers of proliferation (Crary et al. 1998). Over-expression of p21 in transgenic mice leads to inhibition of postnatal hepatocyte proliferation with abnormal liver development and a blunted regenerative response to hepatectomy (Wu et al. 1996). Although homozygous p21 null mice show normal liver development, they exhibit an increased rate of mitosis during the proliferative response to hepatectomy (Albrecht et al. 1998b;Dyson et al. 1989;Hu et al. 1990). However, this is not accompanied by increased tumorigenesis. A potential role for p21 in the maintenance of quiescence has not yet been proven.

During quiescence hypophosphorylated p107 and pRb are expressed at low levels, whilst p130, which is found in both hypophosphorylated and phosphorylated forms, is expressed at higher levels (Garriga et al. 1998). During liver regeneration, levels of hyperphosphorylated p107 and p130 increase with concomitant dissociation of the E2F/p130 complex. Levels of hyper-phosphorylated Rb increase following hepatectomy, and are at their greatest levels during S phase. (Fan et al. 1995). Over-expression of E2F-1 results in higher basal rates of hepatocyte proliferation and sees the early development of preneoplastic lesions in mice. However, increased E2F-1 expression has no effect on rates of proliferation following hepatectomy (Conner et al. 2000). Homozygous E2F-1 null mice exhibit normal proliferation following hepatectomy (Lukas et al. 1999).

Section C.

Polyploidy in the Liver

Polyploidy is a feature of all mammalian tissues, but along with binuclearity, is a particularly common feature of the adult liver. The proportion of polyploidy cells in rodents increases from birth. In the neonatal rat hepatocytes are exclusively diploid (2n) (Ravid et al. 2002b), with polyploidisation beginning after weaning (Sigal et al. 1999), and continuing throughout life as a physiological feature of ageing. In rats the proportion of tetraploid cells may be as many as 70% and tends to be lower than in humans (Seglen 1997). Levels of polyploidisation in the mouse tend to be higher in males than in females. (Lu et al. 1993)

The normal physiological process by which polyploidisation occurs in hepatocytes was determined by Guidotti *et al* using time lapse video microscopy (Guidotti et al. 2003). The process involves first the generation of the binuclear cell, which occurs through mitosis without cell division (acytokinesis). Subsequent mitosis of the binuclear cell then leads to two 4n daughter cells. (Figure 5, page 45)

During liver regeneration following PH there is an increase in hepatocyte ploidy, with an increase in the number of octaploid cells and a fall in the percentage of diploid cells (Gandillet et al. 2003). There is also a concomitant fall in the percentage of binuclear cells, which presumably undergo acytokinesis to form cells of higher ploidy (Brodsky et al. 1977). Thus, following PH there is a shift in the nuclear content to that of a higher ploidy. This shift persists for months following surgery.

An interesting characteristic of those hepatocytes of higher ploidy found following PH, is that they appear to exhibit markers of cellular senescence, including ß-galactosidase expression, and show attenuated proliferative capacity *in vitro* (Sigal et al. 1999). *In vitro*, hepatocytes of higher ploidy also show lower rates of proliferation in response to mitogens, such as insulin and EGF, compared to diploid cells (Mossin

et al. 1994). In murine transgenics which express mitogens such as TGFα (Webber et al. 1994) or HGF (Shiota et al. 1994), the proportion of diploid cells is increased. This fits with the observation that diploid cells are more sensitive to proliferative signals than cells in the same liver of higher ploidy.

The cellular mechanisms which determine ploidy have yet to be determined. Acytokinesis is dependent on the uncoupling of S phase from mitosis. This has focussed the dissection of the potential polyploidisation pathway on proteins which regulate the cell cycle. p21, initially identified as a senescence factor can induce cell cycle arrest at the G2/M check point, so preventing mitosis (Wu et al. 1996). Over-expression of p21 increases levels of polyploidy following hepatectomy (Wu et al. 1996), and conversely, levels of ploidy are reduced following PH in p21 null mice. p21 is a downstream effector of stabilised p53, and mice homozygous for p53 deletion show decreased levels of polyploidy (Bellamy et al. 1997).

Levels of ploidy in the liver appear to be altered in disease states. In animal models of carcinogenesis, diethylnitrosamine (DEN) treatment leads to an expansion of the diploid cell population (Schwarze et al. 1984b). In humans there is a similar shift, with higher proportions of diploid cells found in preneoplastic nodules (Hoso et al. 1991) and in hepatocellular carcinomas (Saeter et al. 1988c) than in surrounding tissue. There is a similar shift following chronic hepatitis infection (Anti et al. 1994), although more recent studies have used different methods to demonstrate increased levels of ploidy in viral hepatitis (Toyoda et al. 2005).

The biological function of polyploidisation is not clear. It has been suggested that acytokinesis allows a cell to increase its size, mass and metabolic output, without expending the energy required for cell division (Ravid et al. 2002a). This may be of benefit to the rapidly dividing hepatocyte following hepatectomy. In addition, multiple gene copies may allow the polyploidy cell to resist the mutational effects of oxidative damage, to which the liver is particularly exposed.

An attractive theory is that polyploidisation is a biological defence mechanism. Although they remain metabolically active, high ploidy hepatocytes demonstrate a decreased ability to respond to mitogenic signals (Seglen 1997), an increased susceptibility to apoptosis (Oren et al. 1999), and a resistance to genetic mutation due to multiple gene copies (Schwarze et al. 1984c). Those hepatocytes of high ploidy, whose fraction greatly increases following hepatectomy, exhibit markers of oxidative DNA damage (Gorla et al. 2001b). In a mutant model of Wilson's disease, the LEC rat, (Long-Evans rat with a cinnamon-like color), there is extensive oxidative liver injury accompanied by lipid peroxidation, which presumably occurs due to accumulation of copper. In LEC rats high levels of polyploidy preceed the development of cirrhosis and HCC (Terada et al. 1999). Transgenic mice deficient in pathways of DNA repair enzymes accumulate markers of DNA damage and show high levels of ploidy (Chipchase et al. 2003). Is polyploidisation a safety net for the aging, or damaged, hepatocyte? Is it a perturbation of the polyploidisation process, even an exhaustion of this process, which brings about, in the recurrently damaged and regenerating liver, the expansion of a diploid population from which potentially malignant clones may arise in an environment of chronic inflammation? The biological relevance of polyploidy in the liver remains to be determined.

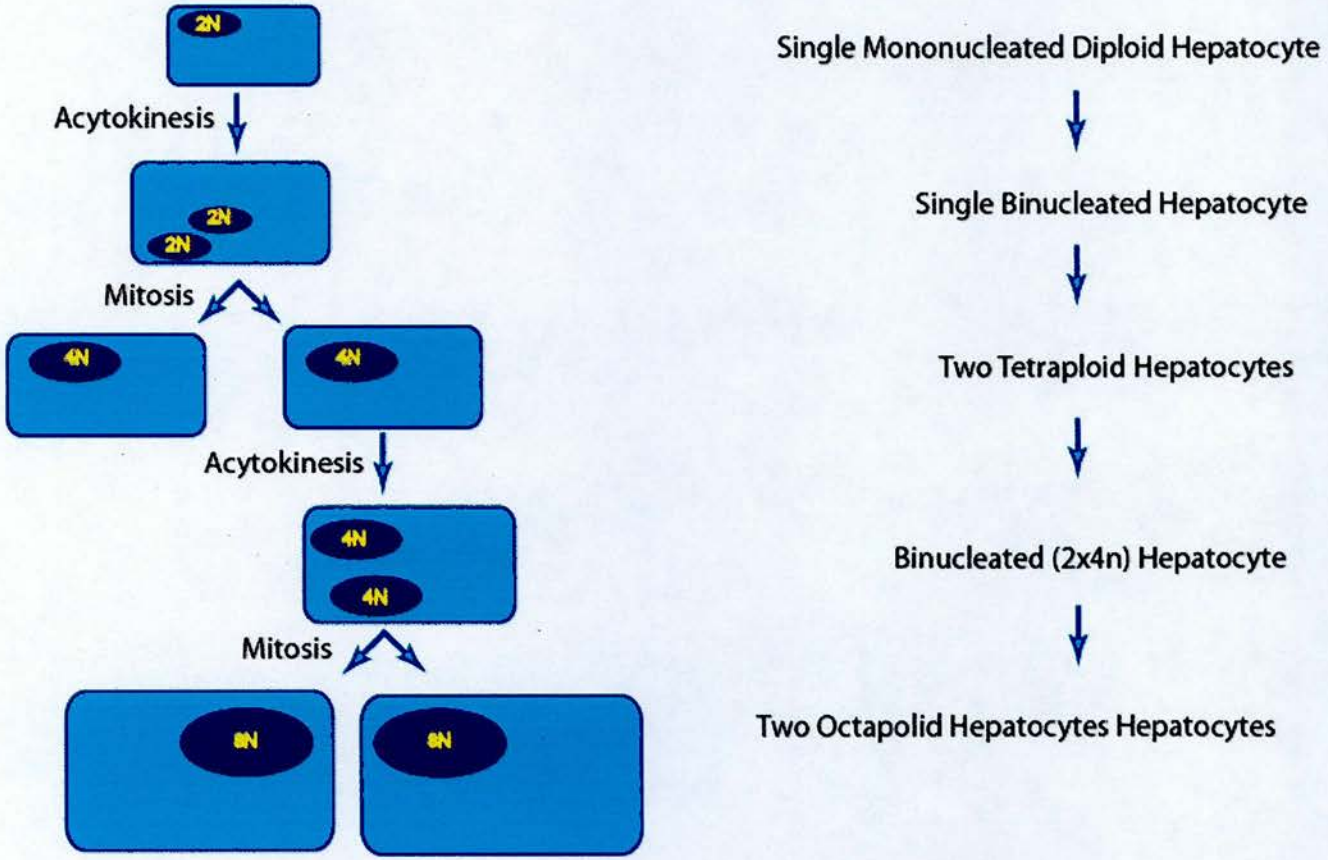


Figure 5. Schematic representation of polyploidisation during hepatocyte division. Rounds of acytokinetic mitoses increase ploidy followed by "normal" mitosis of polyploid hepatocytes to increase cell number

Section D

Liver cancer

Epidemiology and Aetiology

Hepatocellular carcinoma (HCC) is one of the most common forms of gastro intestinal malignancy worldwide. In 1990 there were approximately 437,000 new cases worldwide, rising to 564,000 new cases in 2000 (Parkin et al. 2001) with almost as many deaths. There is a significant geographic variation (Bosch et al. 1999b), with the incidence of hepatocellular carcinoma higher in developing, compared to developed, countries with the highest incidences found in Eastern Asia, middle Africa and West Africa. Annual age adjusted incidence rates may be as high as 48 per 100,000 in men in Western Africa. In the UK, the male age corrected incidence is currently between 4.5-5.4 cases per 100,000 and increasing (Office for National Statistics 2004). The stark differences in geographical incidence which occur not just between countries, but also within countries, reflect differences in exposure to known risk factors. Dominant among aetiological factors is chronic infection with viral Hepatitis B (HBV) or Hepatitis C (HCV). The combined effect of persistent HBV or HCV infection accounts for over 80% of HCC cases worldwide (Bosch et al. 1999b). HBV infection accounts for the majority of cases of HCC in Asia and Africa. In the West and Japan HCV infection is the main risk factor, in addition to other causes of cirrhosis including alcohol and haemachromatosis (Llovet et al. 2003). There is thought to exist a synergism between aetiological factors (Hassan et al. 2002). The disease is more common in men than in women, with a sex ratio varying between 1.5 and 3.0 (Llovet et al. 2003)

The incidence of HCC has been increasing slowly and there is an anticipated increase in the USA related to the current prevalence of HCV that is expected to last for the next two decades (El Serag et al. 1999;El Serag et al. 2003;Mizokami et al.

2004;Tanaka et al. 2002). HCC is now the leading cause of death in Europe amongst those with cirrhosis (Fattovich et al. 2004). This rising incidence has increased concern and interest in possible diagnostic, treatment, and prevention strategies for HCC (Hoofnagle 2004).

Treatment

The once dismal prognosis of HCC seems to be improving, certainly in developed countries where earlier diagnosis allows consideration of potentially curative treatments. Such treatments for "early" HCC include surgical resection (Inoue et al. 2004), liver transplantation (Llovet et al. 2000a;Suarez et al. 2000), and percutaneous treatments. This last group includes hepatic artery chemoembolisation (Llovet et al. 2000b) and ablative therapies such as alcohol injection and radiofrequency (Allgaier et al. 1999), which have been proven to be effective in certain patient groups. Outcome is determined by various factors including the severity of background liver disease, the treatment modality and the size, number, and behaviour of the tumour(s). Although the survival for this group of patients who are amenable to radical treatment is improving (Arii et al. 2000;Llovet et al. 1999;Mazzaferro et al. 1996), there is considerable variation in treatment outcomes and survival between different treatment groups, and this may be a reflection of the biological heterogeneity of these tumours. In the UK and USA survival rates following diagnosis of HCC are approximately 5-8%, 5 years following diagnosis of the disease (Ryder 2005)

Morphological changes in the development of HCC

There are common morphological characteristics seen during the development of HCC which include:

- Chronic liver injury with inflammation
- Cell death and regeneration

- Liver Cirrhosis
- Hepatocyte Dysplasia
- Hepatocellular Carcinoma

A simplified representation of when these observed morphological changes may be seen in the development of the disease is shown below (Figure 6**Error! Reference source not found.**.)

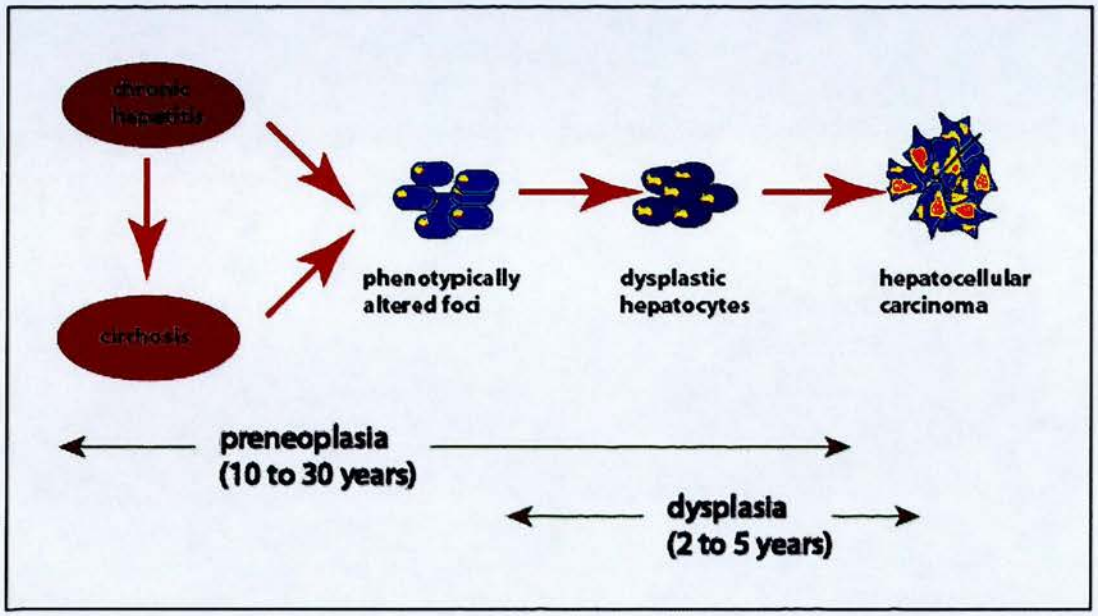


Figure 6 Schematic representation of the suggested chronological sequence of events in the development of HCC.

Chronic hepatitis is characterised by prolonged inflammation of the liver with inflammatory cell infiltrate, hepatocyte death and constant hepatocyte proliferation to replace dead hepatocytes. An inflammatory infiltrate of lymphocytes, plasma cells and macrophages permeate the liver parenchyma and bring about killing of damaged hepatocytes, predominantly through activation of death signalling pathways (Kagi et al. 1996;Yoong et al. 1998). Recruitment of these inflammatory cells is driven through increased expression of chemotactic cytokines and upregulation of appropriate cell adhesion molecules by the endothelial cells of the portal tracts (Yoong et al. 1998). These inflammatory cells in turn express multiple cytokines, including interferons and chemotactic cytokines, which recruit further populations of

inflammatory cells and increase endothelial cell expression of adhesion molecules and receptors for inflammatory cell ligands. The resulting balance of cytokines induces hepatocyte death via cytotoxic T cell-mediated killing and pro-inflammatory cytokines such as TNFα, which induce apoptosis. Hepatocytes divide and regenerate in response to growth factors in the inflammatory infiltrate and to replace dead hepatocytes in order to maintain liver function.

As a consequence of chronic inflammation the architecture of the liver is grossly altered. Inflammation and necrosis induce cirrhosis, which is a diffuse hepatic fibrosis characterised by deposition of a collagenous matrix whose septa segregate the normal liver parenchyma into liver nodules.(Anthony et al. 1978;Yoong et al. 1998). The development of cirrhosis is the greatest risk factor for the development of HCC in humans, although HCC can develop in non-cirrhotic livers (La Vecchia et al. 1998).

Regeneration in the context of chronic inflammation induces nodular aggregates of rapidly proliferating, often monoclonal, populations of hepatocytes. Such groups of hyperplastic hepatocytes often display abnormal phenotypic changes which are among the earliest morphological changes associated the development of HCC. These foci of altered hepatocytes (FAH) show metabolic abnormalities, such as increased production and storage of glycogen, producing characteristic clear cell FAH, or increased expression of RNA, which produces characteristic basophilic staining FAH. Such foci were first described in the liver adjacent to HCC (Schulte-Hermann et al. 1993). Clear cell foci are thought to represent hepatocytes harbouring the earliest of preneoplastic changes, mixed foci represent more advanced lesions, whilst hyperplastic or hypercellular foci exhibit small cell change, and are thought to be direct precursors of dysplastic nodules, which have a high probability of undergoing neoplastic transformation (Sugitani et al. 1998;Terasaki et al. 1998).

HCC are believed to develop from dysplastic hepatocytes that might be clustered in small foci, or develop within cirrhotic nodules. HCC have been categorised as early or late(Sakamoto et al. 1991). Early HCC may be very small, akin to a small focus from which malignant cells proliferate, whereas advanced HCC is characterised by rapid growth of malignant cells forming nodules which compress surrounding structures. Early HCC are usually well differentiated and become poorly differentiated as they invade locally and metastasize.

Behavioural characteristics of hepatocytes in chronic liver disease

As well as the characteristic cytological and histological changes that can be commonly identified in the progression of HCC, there are a number of common characteristics of chronically diseased liver which contribute to the emergence of malignant clones. These include increased hepatocyte proliferation, increased apoptosis and the development of monoclonal cell populations.

Increased Hepatocyte Turnover

Whereas hepatocytes normally proliferate slowly and are long-lived, chronic liver disease is characterised by high rates of both hepatocyte proliferation and apoptosis, which increase in parallel alongside morphological changes. In the disease-free state, the liver is often described as quiescent. Typically, rates of hepatocyte proliferation are extremely low with indices of 5-bromodeoxy-uridine (BrdU) incorporation of less than 0.5% (Tarao et al. 1989). A number of markers of proliferation including PCNA, Ki67 and BrdU, have been used to demonstrate a progressive increase in rates of proliferation from cirrhosis through the development of FAH, dysplasia and HCC (Coleman 2003;Grisham 2001a;Tarao et al. 1989). This increased proliferative index correlates with increased risk for development of HCC (Ng et al. 1994) and negatively correlates with patient survival (Borzio et al. 1998) .

Rates of apoptosis increase in parallel with increased rates of proliferation in cirrhosis and chronic inflammation (Tannapfel et al. 1999). Increased rates of apoptosis correlate with shorter disease-free survival in those with HCC (Ito et al. 1999a), although rates of proliferation persistently outstrip those of apoptosis (National Institute of Health 2002;Park et al. 2001).

Development of monoclonal Hepatocyte populations

The development of monoclonal cell populations permits the accumulation of genetic changes in continually proliferating cells. A highly sensitive method for determining the clonality of hepatocytes is the demonstration of identical sites of HBV DNA integration into the infected host hepatocyte. This method has been employed to demonstrate monoclonal populations among non-tumorous preneoplastic hepatocytes (Shafritz et al. 1981), cirrhotic nodules (Aoki et al. 1989), dysplastic nodules and in tumours (Yamamoto et al. 1999).

Genetic Mechanisms of Hepatocarcinogenesis

Individual HCCs show considerable biological diversity, and much of the variation in treatment outcomes and prognoses between individuals has been attributed to this heterogeneity. Much interest has focused on attempts to find a common genetic and mechanistic pathway linking HCCs that might provide a clearer insight into disease progression, and therefore identifiy potential therapeutic targets. The development of genetic profiling technology in the future may allow therapeutic strategies to be based on individual tumour characteristics (Kim et al. 2003;Lee et al. 2005).

However, underlying the common morphological changes which occur during the development of HCCs there is undoubtedly an extremely complex interplay of many different mechanisms that contribute to the development of the malignant phenotype (Feitelson et al. 2002b). The predominant mechanism may be influenced by the underlying aetiology, an obvious example being that of aflatoxin B1 exposure. However, even within tumours grouped together by aetiology, the contributory

influence of a particular mechanism may vary both between individuals and temporally within the same tumour.

Chronic liver disease is, as already mentioned, characterised by hepatocyte damage, necrosis and death. The monoclonal populations which emerge during the development of HCC exhibit a selective advantage over their neighbours. This results as a consequence of the random acquisition of genetic aberrations that, by chance, confer a proliferative advantage. Such alterations are demonstrable in preneoplastic lesions, with the severity of the lesions increasing in parallel with the morphological changes. As a result of the random nature of these aberrations, both genetic and epigenetic, clones which develop in diseased liver may share the common characteristics of a mutant phenotype, such as deregulated proliferation, but this common phenotype might result from alterations of very different pathways.

Genomic changes in preneoplastic liver

During the lengthy preneoplastic stage of the development of HCC (which may last for decades) changes in gene expression are brought about through epigenetic mechanisms, rather than the structural alterations of genes or chromosomes seen later in the development of this disease.

A characteristic of the preneoplastic phase of HCC development is the over-expression of liver mitogens, the most important of which include IGF-2, HGF and TGFα (Grisham 2001b). This increased expression is a consequence of a number of factors including chronic inflammation resulting from continued liver damage, as well as viral trans-activation and the regenerative response. Epigenetic mechanisms also make a contribution. Re-imprinting of the IGF-2 gene and altered methylation increase IGF-2 expression (Schwienbacher et al. 2000), resulting in the increased production of this growth factor in the diseased liver.

Further epigenetic changes associated with chronic hepatitis and cirrhosis include aberrant methylation of CpG groups (Kanai et al. 2000). The activity of DNA methyltransferases (DNMTs), which catalyse the methylation and demethylation of CpG groups, are increased in a proportion of cirrhotic and hepatitic livers (Saito et al. 2001), and greatly increased in HCCs (Lin et al. 2001;Tchou et al. 2000). Aberrant methylation has been demonstrated in a number of genes and chromosomes for example p16INK4A, where it has been shown to correlate with decreased expression in both HCC and cirrhosis (Kaneto et al. 2001). Aberrant methylation may not only contribute to epigenetic gene expression, but may also predispose to genetic instability through interaction of altered CpG sequences with chromatin, predisposing to chromosomal breaks (Wong et al. 2001)

Microsatellite instability (MSi), thought to represent a mismatch repair deficiency, has been demonstrated in some cases of chronic hepatitis and cirrhosis (Karachristos et al. 1999), as well as in non-tumorous tissue adjacent to HCC (Salvucci et al. 1999). MSi can be demonstrated in approximately 25% of HCC in a pooled analysis (Grisham 2001a).

Telomere shortening and increased telomerase activity have also been demonstrated in preneoplastic hepatic nodules and in HCCs, suggesting these processes may be important in the early development of the disease (Farazi et al. 2003;Kitamoto et al. 1999;Takaishi et al. 2000).

Further factors which may be of significance in the preneoplastic phase of HCC include the cis- and trans-activation of host genes. This results as a consequence of the direct integration of viral promoters into the host genome, as well as the interaction of viral proteins, such as the hepatitis B protein (HBx), in regulatory cell cycles. Such molecular events have the potential to alter the function of tumour suppressors and oncogenes at this early stage (considered later, see Section "Hepatitis Viruses in Liver Cancer")

The potential survival benefit which may accrue in cell clones harbouring gene mutations is amplified in an environment of continued proliferative pressure. Such pressure is brought about by both the persistence of epigenetic changes producing pro-proliferative growth signals, and the regenerative drive to replace dead hepatocytes.

Structural genetic alterations develop slowly in the early preneoplastic phase, but accelerate in dysplastic hepatocytes and HCCs. If multiple loci are examined, allelic deletions can be found in approximately 30-50% of livers with hepatitis, the majority of livers with dysplastic nodules, and all HCCs (Thorgeirsson et al. 2002b). Identical gene mutations have been demonstrated in dysplastic nodules and in adjacent HCCs, suggesting HCCs may arise from the clonal expansion of such nodules (reviewed (Grisham 2001a;Thorgeirsson et al. 2002a). Conversely, genetic aberrations in preneoplastic tissue may differ from adjacent HCCs, suggesting that within preneoplastic tissue there is a spectrum of mutations from which clones with a particular advantage may expand to form HCC (Grisham 2001a;Roncalli et al. 2000). The numbers of allelic deletions increase from a relatively low incidence in cirrhotic liver, to a higher incidence in dysplastic nodules, and are highest in HCCs.(Buendia 2000).

Genomic changes in HCC

Nearly all HCCs show structural alterations in multiple genes and loci. Restriction fraction length polymorphisms (RFLP) mapping has demonstrated recurrent allelic losses and losses on many chromosomal arms. Polymerase chain reaction (PCR) based studies have used microsatellites to determine loss of heterozygosity (LOH) in HCCs with much greater sensitivity. Chromosomal aberrations appear to be more common (occurring in >24% of HCC) at chromosome arms 1p, 1q, 4q, 5q, 6q, 8p,

9p, 13q, 16p, 16q and 17p (Coleman 2003). It is often accepted that loss of part of a chromosome implies it harbours a tumour suppressor gene, which could potentially lead to the identification of those genes that were critical in the progression of HCC. However, chromosomal alterations are not consistent between tumours and it is unusual to find common specific alterations which affect more than half of analysed HCCs (Thorgeirsson et al. 2002a). This variation persists even with strict categorization according to tumour morphology, based on tumour size or histological grade. Although some similarity exits when tumours are sorted according to aetiological agents, such as the characteristic Ser 249 mutation of p53 that occurs following AFB exposure, there remains a wide variation.

Hepatitis Viruses in Liver Cancer.

Infection with HBV and HCV is the commonest cause of liver cirrhosis and ultimately HCC. Chronic hepatitis infection causes hepatocyte death, necrosis, chronic inflammation and morphological, changes including cirrhosis. The relative contribution of a number of factors, including viral infection, the immune response, chronic inflammation, fibrosis and liver regeneration, to the development of HCC is extremely complex.

Hepatitis B

The World Health Organisation (WHO) estimate a third of the world's population have been infected with HBV (World Health Organization 1996). Hepatitis B accounts for 316,000 of the 530,000 cases of HCC each year.(Lai et al. 2003). Those with chronic HBV infection are 100 times more likely to develop HCC than uninfected individuals (Chu 2000). HBV is a partially double stranded hepadna virus. The HBV genome encodes for a number of proteins including those for a reverse transcriptase, an envelope protein and the HBx protein.

The mechanisms through which chronic Hepatitis B infection brings about the development of HCC are not yet fully understood. Certainly chronic infection seems to be linked to the perturbation of a myriad of cellular processes and regulatory networks. The mechanisms through which the virus effects these changes can be grouped under the following four headings.

- Chronic Inflammation
- Physical integration of the viral genome causing structural host genomic changes
- Functional integration of the viral genome causing aberrant gene expression
- Expression of the viral genome and transcription of viral proteins, predominantly HBx

Chronic Inflammation

The expression of viral proteins by infected hepatocytes induces a cytotoxic T lymphocyte (CTL) mediated immune response with immune mediated destruction of hepatocytes through apoptosis. The downstream inflammatory cascade results in inflammatory liver disease and liver necrosis. There is a pro inflammatory cytokine milieu, characterised by increased levels of cytokines such as TNFα and IL2. (Diao et al. 2001)) This pro-proliferative environment itself may be sufficient in a chronic setting to promote the development of HCC, through a combination of sustained mutagenesis and regeneration. This hypothesis has been supported by the finding that the immune response to transgenic expression of HBV surface antigen alone is sufficient to cause necro-inflammatory disease and HCC (Nakamoto et al. 1998). Attenuation of this immune response by inhibiting T cell mediated apoptosis through the neutralization of Fas ligand is sufficient to prevent the development of HCC in this model (Nakamoto et al. 2002).

Integration of the viral genome causing structural host genomic changes

Integration of the Hepatitis B genome is not required for the successful replication of the virus. This integration is random, and does not preserve the viral genome sequence. Therefore, the integrated sequence is insufficient to function as a template for viral replication. HBV DNA sequences have been identified in liver tissue both in cirrhotic patients with chronic hepatitis and in HCC (Brechot et al. 1980). Integration of the viral genome has been demonstrated early in the course of infection (Murakami et al. 2004) and can bring about structural chromosomal instability by inducing chromosomal deletions at the integration site, as well as translocations from one chromosome to another (Pineau et al. 1996). Such random chromosomal alteration could lead to the loss of function of genes essential to cell cycle control, differentiation or apoptosis (Staib et al. 2003b).

Functional Integration of the viral genome causing aberrant gene expression

The extent to which HBV genome integration can bring about transactivation of cellular genes is still debated. There are very few examples of the integration of HBV DNA within, or near, known human genes. In the experimental woodchuck model of hepatitis (WHV), insertion of the WHV genome has been frequently found in the c-myc oncogene (Jacob et al. 2004), though in human HCC integration of the HBV genome into known oncogenes is thought to be a rare event. There is sporadic evidence to suggest HBV integration can occur in genes important to the control of cell signalling, such as steroid receptors (de The et al. 1987), in genes controlling proliferation (Wang et al. 1990), and in genes contributing to chromosomal instability, such as those for telomerases (Brechot 2004a;Paterlini-Brechot et al. 2003). However these reports are exceptions and HBV DNA integration into known cellular genes appears to occur so infrequently it is unlikely to be a major factor in the development of HCC.

Expression of the viral genome and the transcription of viral proteins, predominantly HBx

Many of the cellular effects of chronic viral infection are thought to be mediated through the expression of the HBx protein. The HBx protein can act as a transcriptional transactivator of a number of different cellular genes associated with the control of proliferation. It is named the "X" gene because its role in terms of the life cycle of the virus was initially unclear. Loss of the HBx gene from the woodchuck hepatitis virus results in the virus being unable to sustain chronic infection in the host (Zoulim et al. 1994), therefore suggesting a role in the pathogenesis of chronic infection. The HBx viral genome is the most commonly found portion of the viral genome found in HCC (Paterlini et al. 1995), and expression of HBx protein increases with the development of cirrhosis and is very common in HCC (Wang et al. 1991). High levels of HBx expression in both cirrhotic patients and in HCC suggests this protein may play an active role in the pathogenesis of HCC.

The evidence from transgenic mouse studies is not straightforward. Although over-expression of HBx in a mouse model on a CD1 background is associated with the development of HCC, the results are not consistent between strains and appear to be dependent on both the level of HBx expression and the genetic background of the mice (Kim et al. 1991;Koike et al. 1994;Yu et al. 1999). HBx expression on backgrounds other than CD1 does not necessarily lead directly to HCC, but sensitises mice to DEN (Slagle et al. 1996) and c-myc induced malignancies (Terradillos et al. 1997).

HBx has no direct DNA binding activity, but has been demonstrated to modulate the expression of many target genes through protein-protein interactions (Brechot 2004b;Feitelson et al. 2005).

Microarray studies have shown HBx expression in hepatoma cells can up- or down-regulate the expression of a large raft of genes which could contribute to Hepatitis B pathogenesis (Ng et al. 2004). Potentially important, though still controversial, roles HBx may play in the pathogenesis of HCC include modulation of proteasome function, mitochondrial interactions, and modulation of calcium homeostasis.

The potential effects HBx may play on cell cycle regulation, apoptosis and hepatocarcinogenesis through its affects on the p53 and Rb pathways will be discussed in the sections "Disruption of the Rb/E2F pathway in Hepatocellular Carcinoma" and "Disruption of the ARF-MDMD2-p53 pathway in Hepatocellular Carcinoma".

Hepatitis C Virus

Hepatitis C infection is now the leading cause of viral hepatitis, liver cirrhosis and HCC worldwide, and its incidence, particularly in the west, is increasing (National Institute of Health 2002). HCV is a single stranded RNA flavivirus whose infection of the liver is associated with greater levels of cirrhosis compared to those of HBV infection (70% vs. 50%), and of these cirrhotics a greater fraction will develop HCC (75% vs. 29%) (Ikeda et al. 1993). The HCV RNA genome encodes a polyprotein which is processed by viral and cellular proteases into a core protein, two envelope proteins, E1 and E2, and several non-structural proteins. Constitutive expression of the HCV core protein produces HCC in mice, particularly on a background of chronic inflammation (Kato et al. 2003). HCV core protein has been demonstrated to interact with a number of host regulatory pathways including cell signalling, transcriptional modulation, transformation, and translational regulation (Tellinghuisen et al. 2002). However, the relevance of these interactions to human disease has not been convincingly demonstrated.

Aflatoxin B1 in Liver Cancer

Aflatoxin is a fungal metabolite produced by *Aspergillus flavus*. Metabolites of aflatoxin B1 (AFB1) can cause DNA damage. The base guanine (G) is particularly susceptible to AFB1-induced damage, and this characteristically results in a guanine: cystine to thymidine: adenine (GC→TA) transversion. HCCs which develop in individuals with a high exposure to AFB1 (such as southern Africa and the Qigong area of China) exhibit this specific mutation at codon 249 of the p53 gene. This specific mutation of p53 is rare in areas where the exposure to AFB1 is low, suggesting this mutation may be unique to AFB1-induced HCCs. Epidemiological evidence has been supported by experiments demonstrating AFB1 exposure can produce this characteristic mutation both *in vitro* and *in vivo* (reviewed (Smela et al. 2001). The intake of AFB1 is proportional to the p53 mutational load and aflatoxin exposure concomitant with hepatitis B infection increases the risk of HCC, suggesting a synergism between these two aetiological factors (Ming et al. 2002;Sun et al. 1999).

Disruption of the Rb/E2F pathway in Hepatocellular Carcinoma

As already discussed, disruption of the Rb pathway is deemed essential for the development of cancer (Hanahan et al. 2000). Which component of the pathway is disrupted varies somewhat between tumours, depending on their biological heterogeneity, but it would appear that where the composite members of the Rb pathway are examined in individual HCCs, it is possible to demonstrate the loss of function of one such member in the majority of cases (Azechi et al. 2001;Edamoto et al. 2003).

Cyclins D and E

Amplification of the Cyclin D1 gene and over-expression of Cyclin D1 protein is a common finding in HCCs, and is associated with a more aggressive tumour phenotype and shortened disease-free survival (Azechi et al. 2001;Edamoto et al. 2003;Ito et al. 1998;Ito et al. 1999a;Nishida et al. 1994).

Over-expression of Cyclin E has been demonstrated in both HCCs (Ito et al. 1999a), and in adjacent non-tumorous tissue. (Peng et al. 1998).

These finding of increased cyclin levels in human HCCs are in keeping with observations from animal models. Transient over-expression of Cyclin D1 in mice using an adenoviral delivery system is sufficient to induce DNA synthesis, mitosis and liver enlargement (Nelsen et al. 2001b). Constitutive over-expression of Cyclin D1 in transgenic mice results in dysplastic change in the liver, with adenomas appearing at 9 months and progression to HCC over the ensuing 6 months (Deane et al. 2001). Similarly, transfection of hepatocytes *in vivo* with Cyclin E in combination with skp2 causes hepatocyte replication and liver hyperplasia (Nelsen et al. 2001a).

The CIP/KIP family

Mutations of the CIP/KIP family are unusual in human tumours (Shiohara et al. 1994), although low expression of p27 is associated with a less favourable prognosis in breast tumours (Barnes et al. 2003). Down-regulation of p21 has been demonstrated in HCC, but this is generally in conjunction with, and thought to be a consequence of, p53 loss. (Hui et al. 1997)

Interestingly, HBx transcriptionally down-regulates p21expression through both p53-dependent (Wang et al. 1994) and p53-independent mechanisms(Ahn et al. 2001), resulting in increased hepatocyte proliferation. Similarly, the HCV non-structural protein, NS5A, suppresses the transcription of p21 in HepG2 (Human hepatocellular liver carcinoma cell line) cells (Ghosh et al. 1999). Co-expression of HBV and HCV proteins in a hepatocyte cell line results in co-repression of p21 activity (Han et al. 2002;Peng et al. 1998). Typical of the multiplicity of effects of HBx, its effect on p21 expression seems to be determined to a degree by the system in which it is examined, and by which natural HBx variant is examined (Kwun et al. 2004).

The INK4a-Family

In contrast to the CIP/KIP family, mutations in the INK4 family, particularly p16INK4a, are much more commonly implicated in the aetiology of human cancer (reviewed (Ortega et al. 2002). A number of studies have demonstrated complete loss of the p16INK4a protein in HCC (Hui et al. 1996;Jin et al. 2000). This loss of expression is associated with high rates of gene methylation (Matsuda et al. 1999;Ortega et al. 2002), although gene deletion has also been described (Biden et al. 1997;Liew et al. 1999). Interestingly, germ line mutations of the p16INK4a gene have been identified in a small percentage of HCC. (Chaubert et al. 1997) Those patients which carry the germ line mutation develop HCC at a relatively early age in the absence of cirrhosis.

pRb

Lack of pRb expression is a common finding in HCC, occurring in 54% in pooled studies (Grisham 2001a). Loss of expression correlates closely with allelic deletion. The frequency of LOH varies from 10% (Nakamura et al. 1991) to 73%(Ashida et al. 1997), and in accumulated studies in one exhaustive review (Grisham 2001a), represents 32% of sampled HCCs.

LOH of Rb has also been demonstrated in cirrhotic nodules adjacent to HCCs, with the same alleles lost in both cases. In the same study Rb was maintained in HCCs whose nearby cirrhotic nodules also retained Rb. Such a finding suggests HCC may develop from cirrhotic tissue in which Rb loss could be an early transforming event. Supportive evidence for a potential role for loss of Rb function as an early event in hepatocarcinogenesis, comes from *in vitro* observations that HBx can abrogate the repressive activity of pRb (Sirma et al. 1999a). However, such a conclusion is not supported by other studies that demonstrate Rb is retained in cirrhotic tissue surrounding HCCs where LOH of Rb can be demonstrated (Kuroki et al. 1995). Futhermore, allelic loss of Rb occurs only in advanced or poorly differentiated HCCs, rather than early, well differentiated tumours (Murakami et al. 1991;Zhang et al. 1994).

E2F

Neither mutation nor deletion of E2F1 have been identified in HCCs, nor indeed in a spectrum of other human tumours (Nakamura et al. 1996).

Disruption of the ARF-MDMD2-p53 pathway in Hepatocellular Carcinoma

p53

As mentioned already, acute Rb loss causes p53 stabilisation and increased apoptosis. This has important implications for incipient tumour cells which may have a deregulated Rb pathway. If the mechanisms for p53 stabilisation are intact, the cell will be directed towards cell cycle arrest or death. Evasion of apoptosis is an important hallmark of cancer, allowing the incipient cancer cell to escape death.

p53 is a homo-tetramer transcription factor which responds to a number of cellular stresses including irradiation, hypoxia, genotoxic activation and oncogene activation (Giaccia et al. 1998;Levine 1997). p53 may effect two quite different outcomes to such stimuli, either countering cell proliferation by inducing cell cycle arrest, or inducing cell death through apoptosis. Lesser members of the same family include p73 and p63 (reviewed (Sherr 2004).

Similarly to Rb, loss of p53 function is considered an obligatory step in tumour development, and it is one of the most frequently mutated genes in human cancer. As disruption of pRb function may be brought about by loss of function, or increased activity, of one part of the Rb pathway, similarly loss of function of p53 may be brought about through disruption of some part of the p53 pathway, primarily that of the ARF-MDM2-p53 axis.

ARF deficient mice exhibit a high tumour burden, particularly lymphomas and sarcomas, and die early in life, although they do not develop liver tumours (Kamijo et al. 1999). The ARF gene is methylated in a proportion of HCCs (Edamoto et al. 2003;Tannapfel et al. 2001), and interestingly, loss of ARF is inversely correlated with loss of p53, suggesting that loss of p53 and ARF are mutually exclusive events.

Increased expression of HDM2 is a commonly found feature of a large number of solid human tumours (reviewed (Rayburn et al. 2005). A high level MDM2 expression is a feature of preneoplastic foci in DEN-induced rodent models of liver cancer, where it is associated with reduced p53 function (Van Gijssel et al. 2000), and increased expression of HDM2 has been observed in regenerative nodules in cirrhotic liver (Schlott et al. 2002). Over-expression of MDM2 has also been demonstrated in a significant proportion of HCCs and correlates with reduced survival (Endo et al. 2000).

Mutation of p53 occurs in approximately a third of HCCs, although the frequency of mutations varies between different geographical regions. As already mentioned, exposure to the carcinogen, AFB1, produces a characteristic p53 mutation which accounts for the higher rates of p53 mutation seen in Africa and China compared to the West (Staib et al. 2003a). In addition to the characteristic mutation seen following AFB1 exposure, over 75 mutations have been demonstrated in the p53 gene in different HCCs (Staib et al. 2003a). The resultant p53 mutant proteins confer a proliferative advantage to hepatocytes.

Attempts to model the effect of p53 loss in the development of HCC in mouse models of the disease are confounded by the fact that mice homozygous for p53 deletion die from lymphomas within 6 months (Purdie et al. 1994) and so are unsuitable for long term hepatocarcinogenesis experiments. However, using mice which are heterozygous for p53, models have demonstrated that along with male sex, AFB1 exposure and hepatitis B surface antigen (HbsAg) exposure, p53 is an independent risk factor for the development of HCC in such models. Combinations of these factors are synergistic in the development of HCCs, which mirrors the synergy of risk factors seen in the human disease (Sell 2003b).

HBx and p53.

Studies investigating the pathobiological effect of the HBx protein have demonstrated that it binds to, and inactivates, p53 (Elmore et al. 1997;Ueda et al. 1995;Wang et al. 1994;Wang et al. 1995). This deregulates cell cycle check point controls and blocks p53 mediated apoptosis (Feitelson et al. 2005). This amelioration of the apoptotic abilities of p53 may provide a selective advantage for incipient neoplastic hepatocytes. Transfection of primary hepatocytes with HBx using an adenoviral vector followed by subsequent array analysis reveals that a host of transcripts are up-regulated in transfected cells compared to control cells, suggesting that HBx may effect gross changes in a large array of cellular pathways either through, or independently of, its effects on p53 (Feitelson et al. 2005;Wu et al. 2002).

HCV and p53

Much less is known of the mechanisms of action of HCV proteins compared to HBx, although in specific reference to p53, some evidence suggests the HCV protein, NS3, binds to and transcriptionally represses the p53 promoter (Ray et al. 1997). There is a comparatively much lower rate of mutation of p53 in HCV compared to HBV infection, suggesting that in HCV functional inactivation may be more important than selection for mutation in escape from apoptotic control.

Section E. Conditional Models of Gene Deletion

Conditional gene targeting allows the temporal and tissue-specific inactivation of genes whose deletion through conventional gene targeting strategies leads to embryonic lethality. The more commonly used of the available systems is that of Cre/Lox technology. Cyclization recombination (Cre) is an Escherichia coli bacteriophage P1 enzyme expressed during virus replication. It recognises a palindromic sequence on the P1 genome known as locus of crossover of P1(LoxP). The Lox P sequence comprises two identical 13 base pair (bp) regions either side of a non-palindromic core that confers directionality. Each 13 bp sequence binds a single Cre molecule. Depending on the orientation of LoxP sites, Cre can either invert or excise the portion of interposing DNA.

The Cre/Lox system has been used extensively in many tissues to bring about conditional gene targeting. Such tissue specific knockouts have been used to examine the effect of Rb loss *in vivo* in the lung (Meuwissen et al. 2003), pituitary gland (Vooijs et al. 2002), ovary (Flesken-Nikitin et al. 2003) and mid brain (Marino et al. 2000).

Summary of Introduction

In the healthy liver hepatocytes are quiescent, and replicate only very slowly. However, when the liver is damaged liver regeneration results in the rapid restoration of liver mass through a strictly regulated proliferative response. This is associated with an increase in polyploidisation in the liver, the significance of which remains unknown. In chronic injury, as seen in Hepatitis infection, there is on going hepatocyte death and regeneration which precedes the development of liver cancer. The Retinoblastoma gene, a prototypic tumour suppressor, has been ascribed a fundamental role in the control of cell replication and the suppression of

carcinogenesis in all organs. Current evidence suggests the function of Rb may be affected both directly and indirectly by Hepatitis infection, and that loss of function of the Rb pathway occurs in the development of liver tumours. The role of Rb has not been previously demonstrated in either the regulation of liver regeneration and polyploidisation, nor has the loss of function of Rb in the development of liver cancer been examined.

Chapter 2 Hypothesis and Aims

Hypothesis

Loss of Rb function will de-regulate the processes of liver regeration and polyploidisation, predisposing to the development of liver cancer.

Aims

1. To establish *in vivo* recombination of a floxed Rb gene using an adenoviral Cre delivery system.
2. To establish two models of liver regeneration, surgical (PH) and toxic (CCL4).
3. To determine the role of Rb in the processes of hepatocyte proliferation and polyploidisation by using a system of conditional Rb loss in the two models of regeneration
4. To determine the effect of Rb loss on the preneoplastic development of HCC using the system of conditional Rb loss in a chemical model of hepatocarcinogenesis (DEN).
5. To determine the effect of the loss of p53 in combination with that of Rb loss in a carcinogenesis (CCL4/DEN) model

Chapter 3 Methods

Immunohistochemistry

BrdU Immunohistochemistry

Bromodeoxyuridine (BrdU; Amersham Biosciences RPN 201) was injected into the peritoneal cavity at a dose of 10 $\mu l.g^{-1}$ 2 hours prior to culling. At culling, the liver was removed and fixed in cold, freshly prepared methocarn (60% methanol; 30% chloroform; 10% acetic acid). The tissue was fixed (4 °C; overnight) prior to processing and mounting in paraffin. Tissue sections of 4 μM were cut onto BDH SuperFrost® Plus glass slides (Cat no 406/0179/00) and dried (37 °C; overnight). The sections were hydrated by washing in xylene (5 min) and graded alcohols through to double distilled water (ddH_20). The slides were then incubated in hydrochloric acid (HCl; 5M) for 45 min, before repeated washing in PBS (5mins; x3). Following this, the slides were incubated (10 min) in H_2O_2 (1%) to block endogenous peroxidase activity, and then washed twice in PBS (5 mins). Slides were then incubated (10 mins; room temperature) in blocking solution (PBS containing normal rat serum (20%) plus Tween 20(0.05%)). Slides were then drained and incubated (1 hr; room temperature) in rat anti-BrdU antibody (Oxford Biotechnology cat no OBT 0030) diluted 1:100 in blocking solution. After repeated washing in PBS (5 min; x3) the slides were incubated (30 min; room temperature) with rabbit anti-rat, horse radish peroxidase conjugate (Sigma product no A5795) diluted 1:100 in blocking solution. After three further washes the slides were incubated (15 mins; room temperature) in diaminobenzidine (DAKO cat no K3467) and washed in ddH20. Sections were counterstained with Harris haematoxylin, and then dehydrated through graded alcohols to xylene. The slides were mounted using pertex.

PCNA Immunohistochemistry

Tissue sections were prepared as above. The sections were hydrated after washing in xylene (5 min) and graded alcohols through to ddH20. The slides were then microwaved (10 mins) at full power in Vector Antigen Unmasking Solution (cat no H-3300), and then allowed to cool to room temperature. The slides were repeatedly washed in ddH20 (x3) and then washed once in PBS. The slides were then incubated (10 min; room temperature) in H_2O_2 (3%) to block any endogenous peroxidase activity and then repeatedly washed in PBS (x3). Slides were then incubated (10 min) in Vector Avidin block solution, followed by repeated washing in PBS (x3), and incubation (10 mins) with Biotin Block solution (both from the Vector Avidin Biotin Blocking Kit cat no SP-2001). The slides were then incubated (10 min) in DAKO Protein Free Serum Block (cat no X0909). The primary mouse anti-PCNA biotin conjugated antibody (Ebiosciences cat no 13-9910) was diluted to 1:100 in DAKO Ready-to-use diluent (cat no S0809) and incubated with the slides (1 hr; room temperature). The slides were then washed repeatedly in PBS (x3) and incubated (15 mins) with Vector ABC Stain Kit (Kit PK-4000). Finally, the slides were washed thoroughly in water, counterstained (1 min) with Harris Haematoxylin and then dehydrated through graded alcohols to xylene. The slides were mounted using pertex.

Ki67 Immunohistochemistry

Tissue sections were prepared as above and antigen retrieval was performed as described for the PCNA immunohistochemistry. Following incubation in Protein Free Serum Block (DAKO), the monoclonal primary rat anti-mouse Ki67 antibody (DAKO Ki67 Clone TEC-3, Code no M7249) was diluted to 1:25 in DAKO Ready-to-use diluent (cat no S0809) and incubated with the slides for 2 hours at room temperature. The slides were then washed in PBS (x3) and incubated with rabbit anti rat secondary antibody rabbit anti-rat, horse radish peroxidase conjugate (Sigma)

diluted 1:100 in ChemMate (DAKO) for 2 hours at room temperature. After washing (PBS, x3), slides were incubated (15 min)with diaminobenzidine (DAKO), then washed in ddH20. Sections were then counterstained with Harris haematoxylin, prior to dehydratation through graded alcohols to xylene. The slides were mounted using pertex.

Flow Cytometry

Using a 1ml syringe and a 23G needle, a gentle fine needle aspirate (FNA) was extracted from fresh mouse liver. The aspirate was then gently flushed into a sterile 1.5 ml eppendorf, immediately snap frozen in liquid nitrogen, and stored (-80°C) until analysis. Analysis was carried out as soon as possible (typically within 1 month) as there was notable sample deterioration following prolonged storage, even at -80°C (Vindelov et al. 1983a). To prepare the aspirate for determination of nuclear ploidy, a standard Vindelov preparation was used as described below (Vindelov et al. 1983b). Composition of the buffers used is given in the Appendix. Samples were defrosted at room temperature before microcentrifugation (4000 rpm; 4 mins). The citrate buffer supernatant was carefully aspirated and 450 µl of Vindelov A solution added to the cell pellet, which was re-suspended by tapping the tube gently on the bench top. The cell pellet was then incubated with the Vindelov A solution for 10 minutes on a rocker at room temperature. Following this 10 minute incubation, 325 µl of Vindelov B was added to the tube before a second incubation of 10 minutes on a rocker at room temperature. Following this 10 minute incubation, 250 µl of Vindelov C solution was added to the tube and the sample incubated for 10 minutes on a rocker at room temperature, protected from light. Following this final incubation, the sample was placed on ice and analysed by flow cytometry as soon as possible (typically within 1 hour). Flow cytometric analysis was performed using a Coulter®Epics®XL Flow Cytometer (Beckman Coulter Electronics). A maximum of 10 samples were analysed on each occasion. Immediately prior to flow cytometric analysis, the sample was passed through a 100 µm cell strainer (Becton Dickson) to remove any potentially undigested material. Propidium iodide (PI) was excited in 488 nm argon laser light and the emitted fluorescence was detected at 620 nm. The

stochiometric binding of PI to DNA means nuclear fluorescence is directly proportional to DNA content. The percentage of 2n, 4n, 8n and 16n nuclei were expressed as a percentage of the sum of the total gated events.

On each occasion, prior to analysis of each batch of mouse liver samples by flow cytometry, a control sample of adult chicken cells (TCS Biosciences Ltd Ref FB010) was analysed to act as a reference point for the relationship between DNA content and absolute fluorescence. 45 µl of adult chicken cells were added to 95 µl of PBS. The sample was then processed and analysed exactly as described above for the cellular aspirates.

Animal Procedures

Partial Hepatectomy

Partial hepatectomy (PH) in the rat was first described by Higgins and Anderson in 1931 (Higgins et al. 1931a). The technique for PH in the rat has been well described, both in the original Higgins manuscript and in addition specialised texts (Waynforth et al. 1992) (Figure 7, below). Although the procedure of PH in the mouse appears relatively frequently in the literature, the actual technique used is rarely accurately described. The anatomy of the rat liver varies considerably from that of the mouse; specifically in its peritoneal attachments and anatomy of the biliary tree (the mouse has a gallbladder whilst the rat does not). Therefore, it was necessary to adapt the technique of PH described for the rat in order to be able to perform it safely in the mouse.

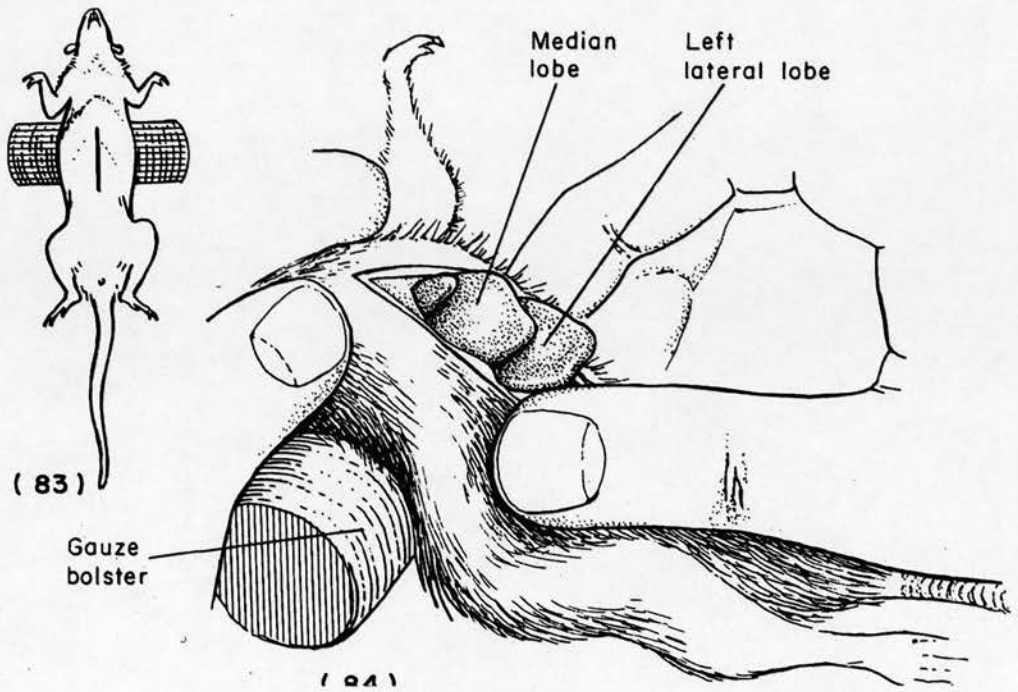
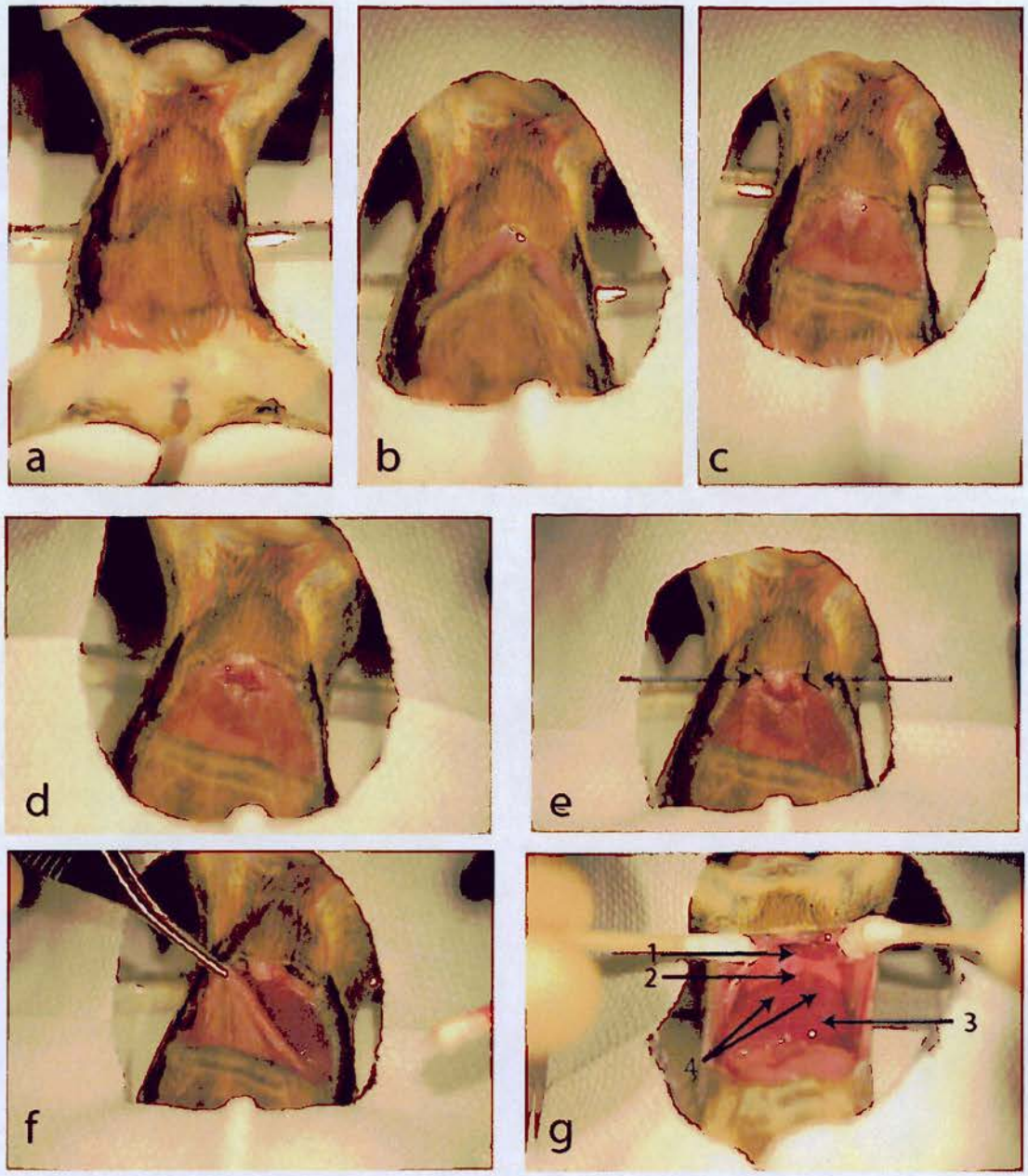


Figure 7. Established surgical approach to PH in the rat showing the lobes of the liver prolapsed through a midline incision. (Waynforth et al. 1992)

Surgical Approach

As shown in Figure 7, the established surgical approach to the rat liver is via a midline abdominal incision. Gentle abdominal pressure is sufficient to deliver the lobes of the liver through this wound. The desired lobes of the liver can then be picked up, ligated and excised. This technique is not appropriate for the mouse for a number of reasons; the abdominal wall in the mouse is much thinner and although pressure on the abdomen will deliver the lobes of the liver through the wound, there is insufficient tension around the edges of the wound to hold the prolapsed liver lobes out of the peritoneal cavity. In addition, the mouse liver is extremely delicate and traction on the peritoneal attachments of the liver is sufficient to cause bleeding from the liver capsule, which can be irretrievable. Although this technique has been employed for PH in the mouse by some groups in the UK, it was felt necessary to make a number of adaptations in an effort to overcome some of the limitations described above. The technique of PH is illustrated in Figure 8a-r from page 77. In terms of the surgical approach, a bilateral sub-costal "roof top" abdominal incision was adopted because it gives excellent access to, and exposure of, the liver, particularly when the mouse is positioned over a bolster. One potential drawback of this approach is bleeding from the anterior abdominal wall, which looks trivial but can be significant given the average blood volume of a mouse is 2 ml. In order to avoid this problem, the superior epigastric vessels were routinely ligated before incising the abdominal musculature (Figure 8e). The excellent exposure afforded by the bilateral subcostal approach allowed mobilisation of each of the liver lobes by division of their peritoneal attachments. This was important because it allowed the ligatures to be placed close to the pedicle of each lobe. Additionaly, it reduced the possibility of such ligaments causing bleeding at their attachments to the liver during retraction. The rat liver does not contain a gallbladder whilst in the mouse the gallbladder is located at the sulcus between the left and right portions of the median lobe. It was therefore necessary to excise the median lobe of the liver in two sections, thus leaving the gallbladder in it native position. The closure of the subcostal wound is straightforward and the skin heals very well (Figure 8r). Skin clips can be removed

10 days post surgery. Figure 9 (page 80) demonstrates the hyperplasia which occurs in the residual liver lobes following 2/3 PH.



Technique of partial hepatectomy in the mouse

a. The mouse is positioned in the supine position with a 1 ml syringe providing a bolster to produce extension. The limbs are secured such that the mouse inclined with the head higher than the tail

b, c The xiphisternum is easily palpable A transverse incision is made in the skin just beneath the xiphisternum and this extended inferiorly and laterally to produce a bilateral subcostal incision

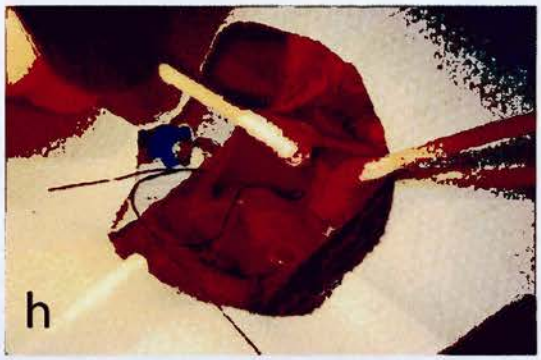
d. The musculature of the anterior abdominal wall is incised in the midline to create a pneumoperitoneum

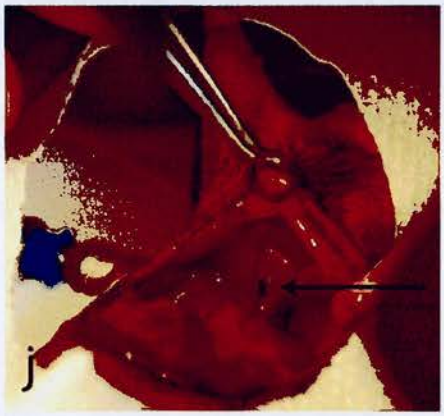
e. The superior epigastric vessels are ligated in continuity using a 5/0 silk suture (arrows)

f, g The incision is then extended laterally on each side. The inferior flap of the wound tends to fall away to give excellent views of the liver

1.Falciform ligament
2.Gall bladder
3.Left lobe of the liver
4.Two parts of the median lobe of the liver

Figure 8





h. The peritoneal attachments of the left lobe are divided and the lobe is gently retracted to the left.

A loop of silk suture is manoeuvred around the lobe and tied at its narrow pedicle.

i. Upon tying the lobe immediately becomes dusky, it is then excised, leaving just a small remnant (j)

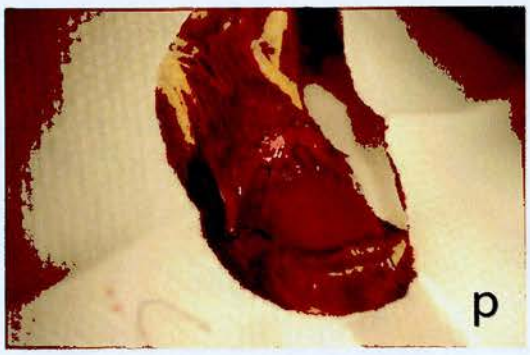
k. A second ligature is manoeuvred around the right part of the median lobe.

Care is taken to avoid both the gall bladder and the common bile duct.

l, m The ligature is carefully tightened, taking care not to include the right lobe which sits inferiorly and the lobe is excised.

Figure 8 (cont)

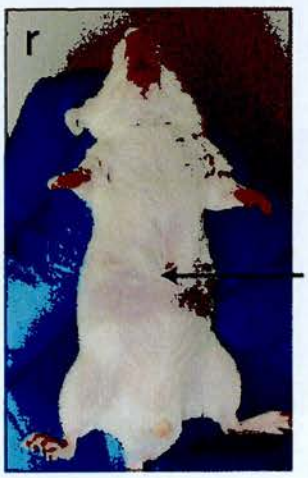
Figure 8 (cont)



n A third ligature is placed around the left portion of the median lobe, tied and the lobe is excised (o)

p The abdominal wall is closed using continous 5/0 silk suture. The skin is closed using skin clips.

q The mouse is allowed to recover until it is mobile on the warming operating pad then returned to a warming cabinet



r 10 days following the procedure wound clips can be removed, the wound heals very well

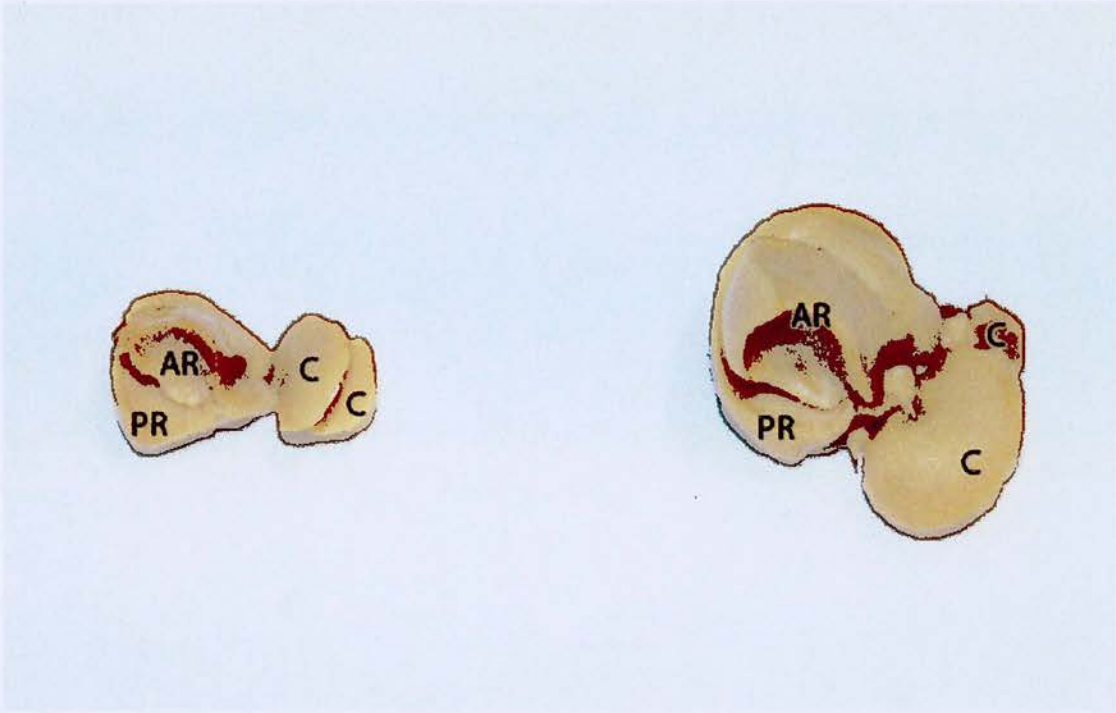


Figure 9. Photograph demonstrating the hyperplasia of the residual lobes of the liver which occurs following PH. The liver on the left of the photograph from a non operated animal shows the anterior (AR) and posterior (PR) lobes of the right liver lobe along with caudate (C) lobes, which make up the remnant of the liver following PH. The liver on the right of the photograph shows the same lobes of the liver from a littermate of the same size, 10 days following a 2/3 PH. In each case the liver has been fixed in methocarn.

Anaesthesia for Surgery

Isofluorane inhalational anaesthesia, as opposed to injectable anaesthesic agents which are often used in rodents, was found to be preferable for a number of reasons:

1. Inhalational anaesthesia allows the depth of anaesthesia to be varied throughout the procedure. This is important as it became apparent that the depth of anaesthesia increased with the length of the procedure. This may be

because of slight decreases in the mouse core temperature and hence, decreases in the metabolism of the anaesthetic agent.

2. Upon completion of the procedure, anaesthesia can immediately be withdrawn and the animal recovered, avoiding the potential complications of prolonged anaesthesia, such as hypovolaemia and hypothermia.
3. Injectable anaesthetic agents are often metabolised in the liver and following partial hepatectomy there is the obvious concern of reduced liver function.

Isofluorane was used rather than halothane, which can be hepatotoxic in the mouse as well as the human.

Analgesia, Fluid Replacement and Temperature Homeostasis

The opiate, buprenorphine, was chosen for peri operative analgesia – a non-steroidal agent would be contraindicated in the presence of liver insufficiency. It was found preferable to give buprenorphine subcutaneously at the beginning of the procedure because giving analgesia at this point meant that on recovery the opiate was already in the circulation, removing the potential for the mouse to experience pain during recovery before analgesia can be given. Buprenorphine was given at a dose of 0.05 – 1 $mg.kg^{-1}$ subcutaneously.

Hypoglycaemia is a common complication of total hepatectomy in the rat, though it would not be expected to be a problem following resection of 2/3 of the liver mass because the gluconeogenic activity of the liver remnant is up-regulated rapidly following surgery. However, recovery following surgery appeared to be improved when mice were given a bolus of warmed dextrose saline, as opposed to normal physiological saline solution. A bolus of approximately 0.75 – 1.0 ml of dextrose saline (NaCl 0.18%, dextrose 4%) was given both at induction, and at completion, of the procedure.

In the post-operative period animals were observed very closely in the hours following surgery and routinely on the following days. If animals did not quickly

return to normal activities, further doses of of analgesia and/or subcutaneous fluids were administered.

During the procedure, the animals' core temperature was maintained using a warming blanket (Harvard Apparatus). These specialized devices automatically adjust the heat output of the blanket in response to the animal's temperature, which is measured using a rectal thermometer. Following recovery, animals were all routinely kept overnight in individual, humidified warming chambers maintained at 28 °C, with free access to water and mash. They were then returned to individual, standard caging on the first post-operative day.

Mortality Following Surgery

The mortality following 2/3 PH is shown in Figure 10. Mortality is expressed using cumulative summation methodology (CUSUM) (Bolsin et al. 2000). This allows the mortality for a procedure to be assessed against an acceptable standard, in this case 10%, which was the mortality limit agreed in the Home Office licensing for this project. From procedure 50 onwards, there is clearly a rapid improvement in mortality. The overall post operative mortality for the procedure was 6.7% (n=194). Mortality for the first 50 procedures was 18% and for the next 144 procedures was 2.7%. This probably reflects a number of factors including refinement of surgical techniques and equipment, improved peri operative care, and possibly improved overall health of the animals following their-derivation and re-housing of the colony. This process began at approximately procedure number 54.

Further factors that may have affected mortality are shown in Table 1. Interestingly, all of the mice that received viral treatment survived. This may be related to some sort of non-specific pre-conditioning effect, though as the percentage of deaths is relatively small, it is difficult to draw any firm conclusions from this observation.

Table 1. Contingency table illustrating the characteristics of those mice that survived or died following 2/3 PH.

	Total	Age (days)	Sex		Genotype				Virus Treatment		
			Male	Female	wt	p53 null	p21 null	p53/21 null	AdCre	DL70	No Virus
Numbers											
Survived	181	67	94	87	111	14	48	14	64	29	88
Dead	13	66	5	8	6	0	7	0	0	0	13
Percentages											
Survived	93.3%		94.9%	91.6%	94.9%	100.0%	87.3%	100.0%	100.0%	100.0%	87.1%
Dead	6.7%		5.1%	8.4%	5.1%	0.0%	12.7%	0.0%	0.0%	0.0%	12.9%

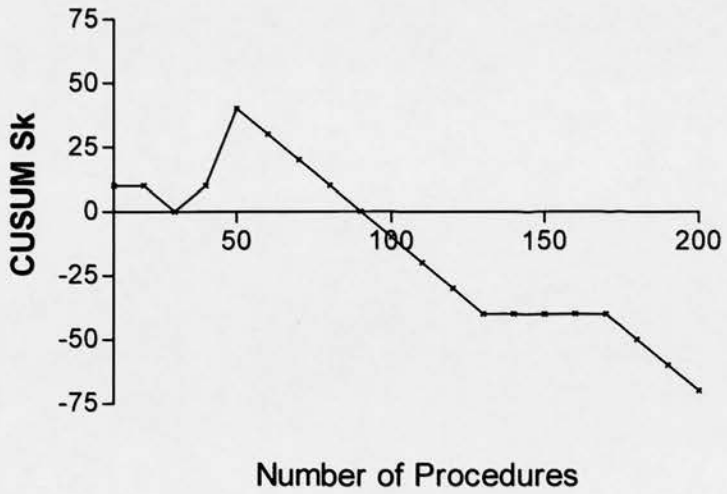


Figure 10. CUSUM survival chart showing change in operative mortality against procedure number for 2/3 PH. The formula for CUSUM is Sk = SUM(X_{BAR} - T) where X is the mean survival from each group and T is the target percentage survival.

Animal treatment with Diethylnitrosamine

14-day-old mouse pups were injected intra-peritoneally using a 50 µl glass gas chromatography syringe (Syringe SGE 004232; Fisher Scientific) with a single dose (50 $mg.kg^{-1}$) of N-Nitrosodiethylamine (DEN; Sigma Aldrich) freshly prepared by dilution in filter-sterilised PBS.

Treatment with Carbon Tetrachloride

Carbon Tetrachloride (CCl4; Sigma Aldrich) was diluted in freshly prepared filter-sterilised olive oil (Sigma) to a concentration of either 2.5% for chronic administration in the carcinogenesis protocol, or 10% for regeneration experiments. The solution was freshly prepared in glass and vortexed vigorously prior to each injection using a 500 µl glass gas chromatography luer lock syringe (Syringe SGE 005230; Fisher Scientific). A dose of 10 µl of the CCl4 solution was given per gram of animal body weight. Figures 11 and figure 12d (page 86) respectively show the histological and macroscopic appearance of the liver 24 hours following the injection of a single dose of CCl4 at a volume of 10 µl per gram body weight.

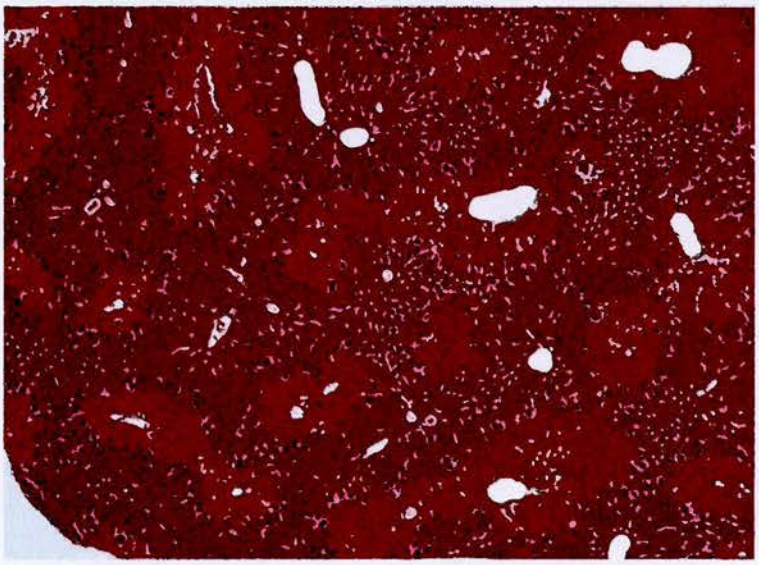


Figure 11. An H&E stained liver section showing typical centrilobular necrosis 24 hours following a single dose of CCl4. The vascular structures in this section have been filled with white for the purposes of quantifying the percentage of necrosis on the section. (Magnification x50)

Injection of Adenovirus

The desired amount of virus was prepared in sterile PBS to a volume of 100 µl. Prior to injection, each animal was warmed in a commercial warming device at 35 °C for no more than 10 minutes. Continuous careful inspection was essential to avoid causing distress or inadvertent dehydration to the animal. The mouse was then restrained in a commercially produced, small animal plunger-type restrainer (Braintree Scientific Inc., Braintree, Massachusetts) and injected via the lateral tail vein using a 29 gauge, 0.5 ml insulin syringe (Becton Dickson Micro Fine^{+}). Great care was taken to avoid inadvertently injecting air into the vein by fastidiously removing all air bubbles prior to injection. Successful injection can be determined by observation of general, rather than localised, blanching of the tail and the absence of resistance to depression of the plunger. Subcutaneous injection is quite apparent due to obvious local swelling and significant resistance to injection. The injection site was compressed for a few moments for the purposes of haemostasis before returning the animal to its cage. The technique for tail vein injection is shown in Figure 12. The AdenoCre used in these experiments was an E1-deleted, replication-deficient, serotype 5 adenovirus expressing Cre recombinase under control of the CMV promoter. An "empty" adenovirus of the same serotype without transgene was used as a control vector. Both viruses were propagated using standard techniques by Virapur (virapur@sbcglobal.net.).

Figure 12 Intravenous injection of adenovirus (a-c) and (d) the macroscopic appearance of murine liver 24 hours following administration of a single dose of CCl4.

a. The mouse is restrained in a specially designed commercial restaining device. b. The 29G needle is introduced into the lateral tail vein.

c. If the needle is correctly sited in the vein, the skin of the tail will blanch upon injection, without subcutaneous swelling.

d. (left). Typical appearance of the murine liver 24 nours after intraperitoneal carbon tetrachloride

Histological Quantification

Quantification of Preneoplastic Foci

Individual preneoplastic foci were identified and marked on H&E stained liver sections by a liver pathologist blinded to the experimental protocol. The area of each liver section was determined by capturing a digital image of the section using a low-powered microscope (x 2.5 magnifcation) with the section adjacent to a scale bar. The area of the section was then determined using Image-ProPlus image analysis software (Media Cybernetics, Silver Spring, Maryland), and the area of individual foci determined using PALM RoboSoftware (Zeiss).

Quantification of Apoptosis

Apoptotic cells were identified on H&E stained liver sections the basis of morphological criteria (cell shrinkage, chromatin condensation, margination, and apoptotic bodies). Results are expressed as the number of apoptotic bodies in 50 random high-powered fields (hpf).

Quantification of Necrosis

Necrosis was estimated on the basis of differential staining between necrotic tissue which stains predominantly pink and non necrotic tissue which with H&E staining appears blue. Using a macro designed within an image software analysis program (KS 400, Carl Zeiss MicroImaging, www.zeiss.de) the proportion of necrotic and non necrotic tissue in each view can be estimated. Four low-powered images (x5 magnification) were captured for each tissue section. Vascular structures were excluded from analysis using Adobe Photoshop (Seattle, Washington). The percentage area of each section occupied by necrotic tissue was estimated and averaged for the four sections.

Quantification of PCNA staining in preneoplastic foci.

The percentage of hepatocytes positively stained for PCNA within a total of 62 preneoplastic foci within 11 sections from AdCre treated male mice from the carcinogenesis protocol, was estimated using parallel H&E stained sections to help identify foci. The percentage of PCNA-positive hepatocytes within these foci was compared with the percentage of positive hepatocytes in a minimum of 500 hepatocytes outwith the preneoplastic tissue, or "non lesional" liver.

Laser Capture

Preneoplastic foci were dissected from paraffin fixed liver sections. To aid the identification of foci in liver sections, two adjacent sections were taken from each paraffin block of tissue – an 8 μm section and a 4 μm section. Laser capture of preneoplastic foci was performed on the 8 μm liver sections on a plain glass slide stained in H&E (without mounting medium or cover slips) using the P.A.L.M. Combi System (P.A.L.M. Microlaser Technologies, Germany). The adjacent 4μm section was stained in H&E, and mounted for light microscopy to aid identification of foci.

Extraction and purification of genomic DNA

DNA was extracted from murine liver using the Qiagen DNeasy ® Tissue Kit. (Qiagen). The principle of the system was the selective binding of DNA to a silica gel membrane following a proteinase K digest. The selectively bound DNA was retained on the membrane following centrifugation. Following two washing steps the DNA was eluted using a Tris based elution buffer.

PCR reactions

Polymerase Chain Reactions (PCR) were carried out on Thermo Fast® 96 Skirted Low Profile PCR plates (Abgene) and sealed using a Thermo-Mat™96 (Abgene cat no AB-0427).

.PCR reactions were performed using an Eppendorf Mastercycler. PCR products were diluted 5:1 with Invitrogen PCR loading dye and 10 µl of this mixture was run per well on a 1.5% agarose gel. All PCR reagents were obtained from Invitrogen.

PCR Primers

	Primer	Primer Sequence	Product Size
P53	Exon 6 Intron 7 Neo	GTGGTGGTACCTTATGAGCC CAAAGAGCGTTGGGCATGTG CATCGCCTTCTATCGCCTTC	Wt 642bp Deleted 510bp
Rb	Rb 18 Rb 212	GGCGTGTGCCATCAATG GAAAGGAAAGTCAGGGACATTGGG	

Master Mix Recipes

	P53	Rb
dH_20	17.25 µl	24.75 µl
10x PCR Buffer	5 µl	5 µl
W1 detergent	2.5 µl	0
DMSO	2.5 µl	0
Mg (50 mM)	2 µl	1.75 µl
dNTP (1.25 mM)	8 µl	8 µl
Primer (10 µM)	2.5 µl each	2.5 µl
Taq Polymerase	0.25 µl	0.5 µl
DNA	5 µl	5 µl

Thermocycler Protocols

	P53	Rb
Melting	94°C 5 mins	94°C 5 mins
Melting	94°C I min	94°C 30sec
Annealing	62°C I min	58°C 30sec
Extension	72°C I min	72°C 50sec
Cycles	35	35

Statistical Analysis

Graphs were prepared, and statistical analyses performed using GraphPad Prism © Version 3.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Chapter 4 Results

Section A

Rb floxed alleles can be deleted in the mouse liver using a Cre Lox system of conditional gene targeting.

Conditional gene targeting allows temporal and organ-specific control over gene deletion. One method of conditional gene targeting is CreLox technology. The mice used in these experiments have LoxP sequences flanking exon 19 in both Rb alleles (Vooijs et al. 1998). This arrangement is described as the allele being "floxed". The enzyme, Cre recombinase, catalyses a recombination between the two LoxP sites, resulting in excision of the interposing DNA. The resulting frameshift produces a truncated non functional Rb protein. The mutation resulting from Cre-mediated recombination is similar to that engineered through conventional means and is therefore equivalent to a null allele. (Clarke et al. 1992)

A requisite step in ensuring recombination of floxed sequences is the effective delivery of the Cre recombinase enzyme to the floxed sequence. Tissue-specific expression can be achieved using Cre expression driven by a tissue-specific promoter (Huelsken et al. 2001;Vooijs et al. 2002;Wagner et al. 1997) which may, or may not, be combined with a ligand based expression system giving temporal control (Tannour-Louet et al. 2002). An alternative is the use of an adenoviral delivery system. Human adenoviruses of serotype 2 and 5 are popular candidates in the development of gene therapy techniques. Such vectors are relatively stable *in vitro*, can be produced at high titres, and do not integrate their genomes into host cells. These characteristics make such viral delivery systems relatively safe (reviewed (Barnett et al. 2002). An additional advantage is that adenoviruses do not require host cells to be replicating for expression of the viral transgene. One of the biggest potential drawbacks of the use of these vectors in human gene therapy is their limited uptake by a number of epithelial tissues, limiting their usefulness in, for example, the targeted treatment of lung epithelium (Crystal et al. 1994). This tissue specificity is

due to the selective expression of the Cocksackie Adeno Receptor (CAR), the primary method of cellular uptake of these viruses in both humans and mice (Bergelson et al. 1997;Tomko et al. 1997). When designing these experiments it was possible to take advantage of the fact that the CAR receptor is preferentially expressed in the liver;when the adenovirus is given intravenously, more than 90% of the dose is delivered to the liver (Herz et al. 1993;Kass-Eisler et al. 1994).

In these experiments, intravenous delivery proved essential to achieving recombination; even when relatively large doses (up to $1x10^{10}$ plaque forming units (pfu)) of AdenoCre virus was administered via intra peritoneal injection there was no deletion in the liver. This is fitting with observations that efforts to use an adenoviral vector to deliver transgenes in order to treat malignant peritoneal disease produce variable expressions in the abdominal viscera (Saimura et al. 2002). However, when the AdenoCre virus was administered via tail vein injection, the levels of recombination achieved were proportional to the viral titre used. Figure 13 demonstrates in vivo recombination of floxed Rb sequences. Figure 14 demonstrates liver-specific recombination of floxed Rb sequences. High viral titres (10^{10} pfu) were found to be associated with increased mortality when combined with regenerative procedures such as PH, or CCl4 treatment, suggesting combined toxicity of the AdenoCre virus when given with a second insult. For this reason an AdenoCre viral titre of $1x10^{9}$ pfu, which did not quite achieve complete recombination at 24 hours but did produce levels of recombination comparable to that achieved *in vitro*, was chosen for the remainder of the *in vivo* experiments.

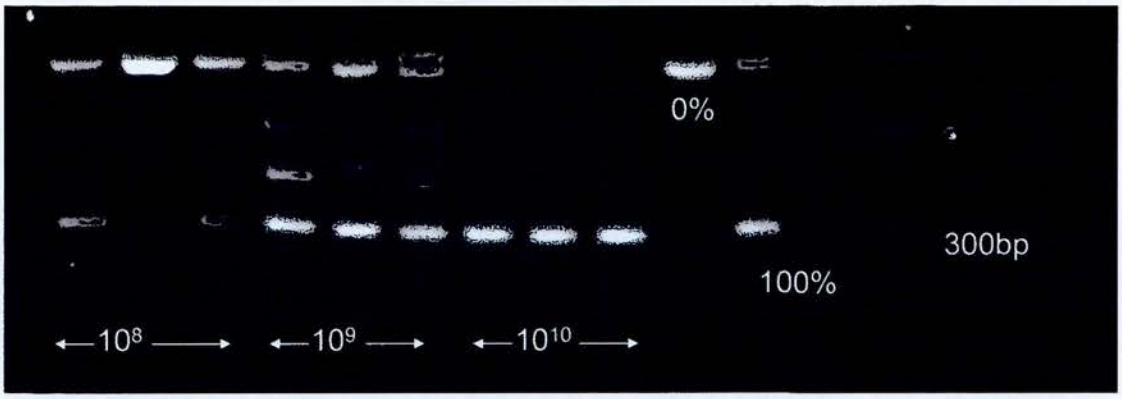


Figure 13. PCR detection of recombined Rb floxed alleles. Lanes 1-9 represent genomic DNA taken from the livers of mice 24 hours following injection with AdenoCre. Lanes 1-3 = dose of $1x10^8$ p.f.u. Laness 4-6 = dose of $1x10^9$ p.f.u. Lanes 7-9 = dose of $1x10^{10}$ p.f.u.. n=3 for each viral dose. Lane 10 is liver DNA from an untreated mouse (negative control). Lane 11 is DNA from hepatocytes treated in vitro (positive control). Lane 13 is molecular markers. 1.5% agarose/ethidium bromide gel.

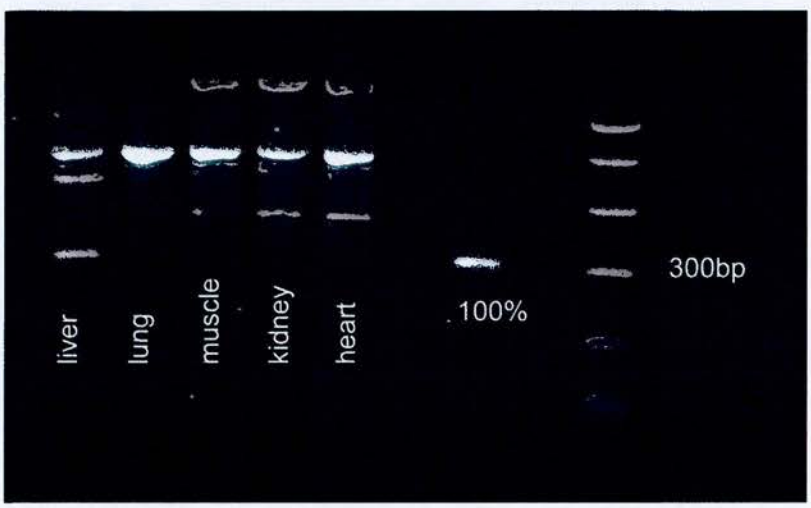


Figure 14. PCR detection of recombined Rb floxed alleles in various tissuses as indicated. Lanes 1-5 = genomic DNA samples extracted from different mouse organs 24 hours following intravenous injection of $1x10^8$ p.f.u. AdenoCre virus. Lane 7 = control DNA from in vitro experiments (positive control for recombination). Recombination could only be detected in the liver. 1.5% agarose/ethidium bromide gel

Section B

The effect of Rb loss on liver regeneration in two murine models

A central aim of this thesis was to test the hypothesis that Rb plays an integral role in the regulation of cellular proliferation in the liver. Normal rates of cell division in the liver are extremely low, with mitotic figures demonstrable in only 1 in 20, 000 hepatocytes (Tarao et al. 1989). Therefore, in order to examine the role of Rb in an *in vivo* system where hepatocytes are regenerating, it was necessary to develop a model of liver regeneration in the mouse. The first of these was liver hyperplasia induced by PH; the second was liver regeneration following a single toxic insult. The purpose of employing two different models was to allow the role of Rb to be determined in two differing environments. PH allows a simple method of inducing regeneration; the single factor believed to drive regeneration in this model is hepatic insufficiency. However, in terms of relevance to a clinical, or disease setting, PH has only limited application – the analogous situation in humans being liver resection. Thus, a second model was developed, in which liver regeneration occurs following acute cytotoxic injury, induced by carbon tetrachloride (CCl4) treatment. CCl4 induces acute liver necrosis and therefore, produces an altered environment in which liver regeneration takes place. This altered environment is characterised by an acute inflammatory infiltrate and hepatocyte cell death by necrosis. This type of injury, therefore, more accurately reflects the pattern of liver damage seen in human disease.

Partial Hepatectomy can be used to model liver regeneration in the mouse.

It was necessary to establish the model of PH prior to using this technique to determine the contribution of Rb to liver regeneration. These preliminary experiments were carried out using Rb floxed mice, which have a normal phenotype. Figure 15 demonstrates the regenerative response that follows PH. During the first 30 hours post surgery, rates of hepatocyte proliferation remain very low. After 30 hours, a rapid and synchronous entry into S phase occurs, with almost 50% of hepatocytes staining positively for BrdU by 36 hours post surgery. There is a subsequent second peak of regenerative activity at 72 hours. This biphasic response is similar to that described elsewhere (Fausto et al. 1994).

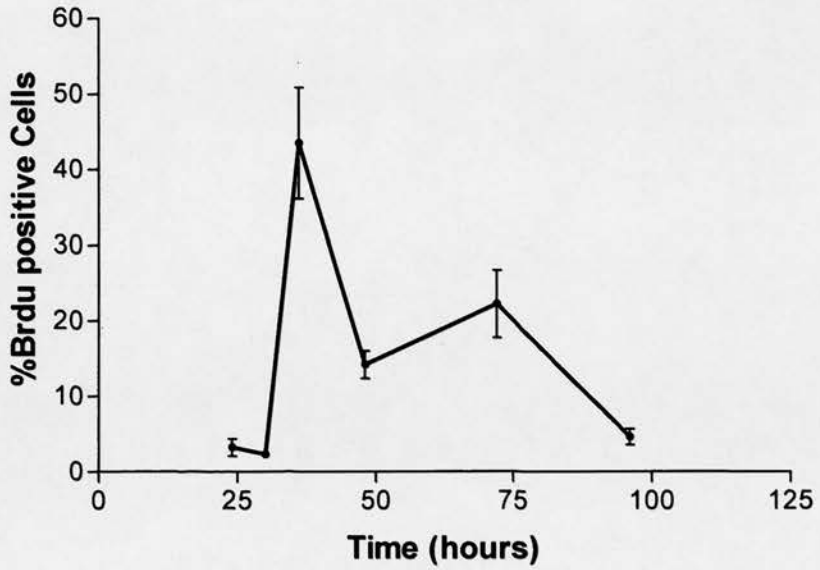


Figure 15. Liver regeneration occurring in Rb floxed mice following PH in the absence of adenoviral treatment. A pulse of BrdU was given 2 hours prior to culling. The percentage of BrdU positive hepatocytes is expressed against time post PH as mean ± SEM. (n=3-5 mice per observation)

Carbon Tetrachloride can be used to model liver regeneration in the mouse

Again, it was necessary to establish the model of acute toxic injury using CCl4 treatment prior to determining the contribution of Rb to liver regeneration in this model. Figure 16 demonstrates the regenerative response that follows a single dose of CCl4 in Rb floxed mice. When compared with PH, S phase entry is less synchronous, the peak of BrdU incorporation is much reduced and the period of regeneration appears extended. These differences may reflect the fact that liver damage, and so the drive for proliferation, evolves over a period of hours to days. Therefore, the signal for regeneration is less discrete, resulting in a slower and less synchronous regenerative response. Additionally, the acute inflammatory response that accompanies regeneration following CCl4 treatment may have an effect on hepatocyte replication.

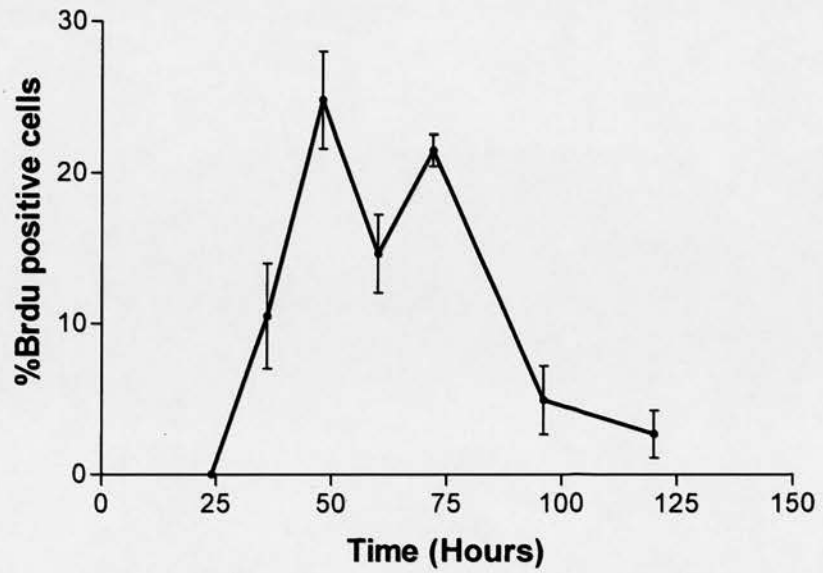


Figure 16. Liver regeneration occurring in Rb floxed mice following a single dose of CCl4 in the absence of adenoviral treatment. A pulse of BrdU was given 2 hours prior to culling. The percentage of BrdU positive hepatocytes is expressed against time post PH as mean ± SEM. (3-6 mice per observation)

AdenoCre has no effect on the levels of liver necrosis following carbon tetrachloride.

To ensure that the recombination event had no effect on the amount of necrosis produced by the single dose of CCl4, the percentage area of necrosis was estimated and compared between mice with, and without, adenoviral treatment prior to administration of CCl4 (Figure 17).

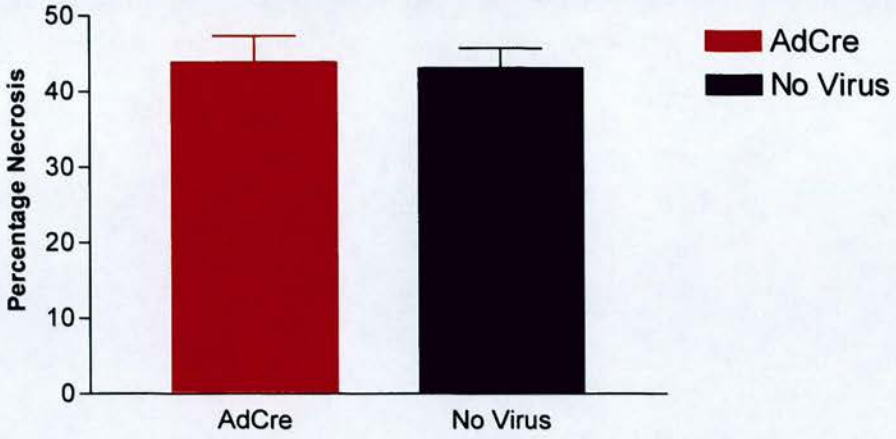
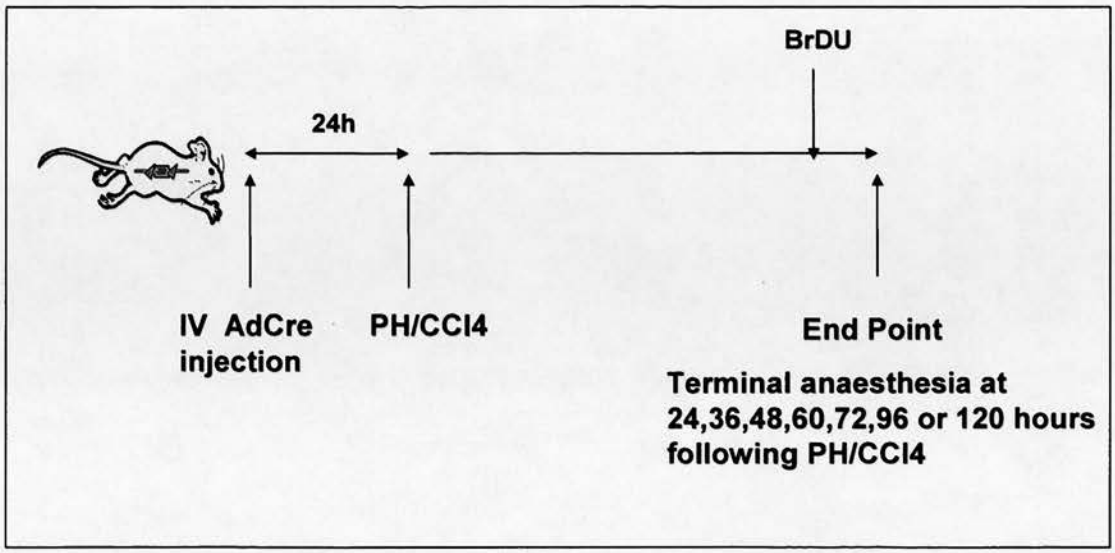


Figure 17 Percentage of necrosis 24 hours following a single dose of carbon tetrachloride in mice previously treated with AdCre (n=6) or no virus (n=4). Results are expressed as the average of each group+/-SEM.

Rb loss can be used in conjunction with models of regeneration to determine the effect of Rb loss on the regulation of hepatocyte replication.

To determine how acute Rb loss affects the process of regeneration, rates of liver regeneration were examined in the two different models following acute Rb loss. AdenoCre virus was used to produce recombination in the Rb flox/flox mice. BrdU incorporation during the regenerative response was then examined. The experimental protocol is shown in Figure 18. BrDU was given 2 hours prior to terminal anaesthesia.

Figure 18. Experimental Protocol to determine the role of Rb in liver regeneration.



Rb loss accelerates cell cycle entry and brings forward the peak of DNA synthesis of liver regeneration following partial hepatectomy.

As demonstrated in Figure 19 and Figure 20, when AdenoCre was given prior to PH, Rb deletion induced premature S phase entry compared to empty vector controls. Average rates of BrdU incorporation were 28.4 ± 6.1 % (SEM+/-6.14356%) in AdenoCre treated mice at 24 hours post PH *vs* 2.7 ± 1.3 % (SEM +/-1.260539%) in empty vector treated mice. The maximum rate of proliferation occurred at 30 hours post PH in the Rb deleted mice (AdenoCre treated mice) and 36 hours in the empty vector treated mice. The rate of DNA synthesis in the AdenoCre treated group was persistently higher for the duration of the time course compared to empty vector treated controls. Analysis by two-way ANOVA revealed Rb loss had a significant effect on BrdU incorporation following PH ($P<0.0001$).

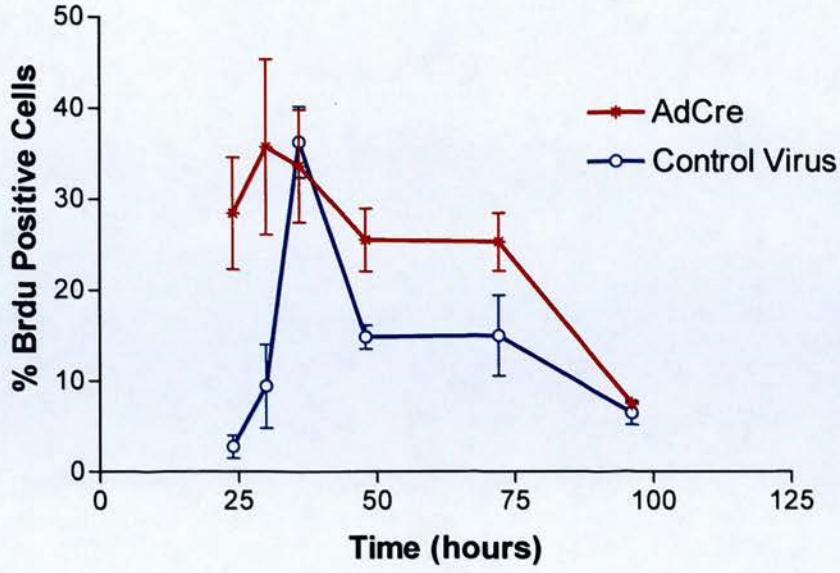


Figure 19. The effect of Rb deletion on liver regeneration following PH. The two curves represent Rb floxed mice treated with either AdenoCre or an empty adenovirus 24 hours prior to PH. A pulse of BrdU was given 2 hours prior to culling. The percentage of BrdU positive hepatocytes is expressed against time post PH as mean ± SEM. (n=3-5 mice per observation). Two-way ANOVA analysis of the two regenerative curves revealed Rb deletion had a significant effect on BrdU incorporation (P<0.0001)

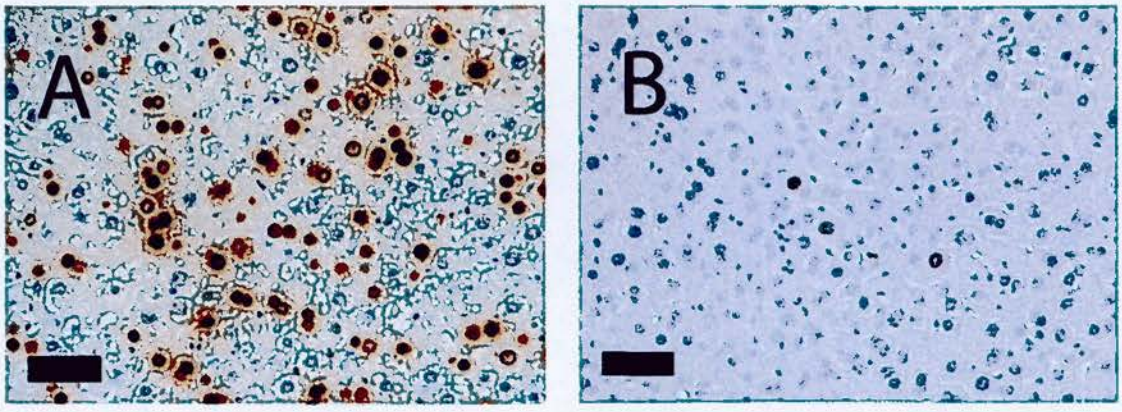


Figure 20. BrdU staining following PH in mice treated with AdenoCre (A) or empty vector control virus (B). Darkly stained nuclei are BrdU positive. Bar Scale represents 50 µm.

Rb loss accelerates cell cycle entry and brings forward the peak of DNA synthesis during liver regeneration following acute liver necrosis.

The effect of Rb loss in liver regeneration following CCl4 treatment was similar to that following PH (Figure 21 and Figure 22). When AdenoCre virus was administered 24 hours prior to CCl4 treatment, Rb deletion produced premature entry into S phase as assessed by BrdU incorporation. Rates of BrdU incorporation at 36 hours were 66.1 ± 3.9 % (SEM+/-3.9) in AdenoCre treated mice compared to 9.1 ± 4.4 % (SEM+/-4.4%) in mice treated with empty vector control virus. Maximum rates of BrdU incorporation occurred at 36 hours following Rb deletion (AdenoCre treatment) compared to 60 hours in the empty vector treated control group. Higher rates of DNA synthesis were maintained in the AdenoCre treated group compared to the empty vector treated group throughout the duration of the experiment. Interestingly, regeneration in the Rb deleted (AdenoCre treated) group showed a biphasic pattern of regeneration with a peak at 30 hours, followed by a second peak at 60 hours. This biphasic pattern contrasts with the single peak of regeneration seen in the empty vector treated group. Analysis by ANOVA revealed Rb deletion had a significant effect on the rate of BrdU incorporation compared with empty vector treatment ($P<0.0001$;Figure 21)

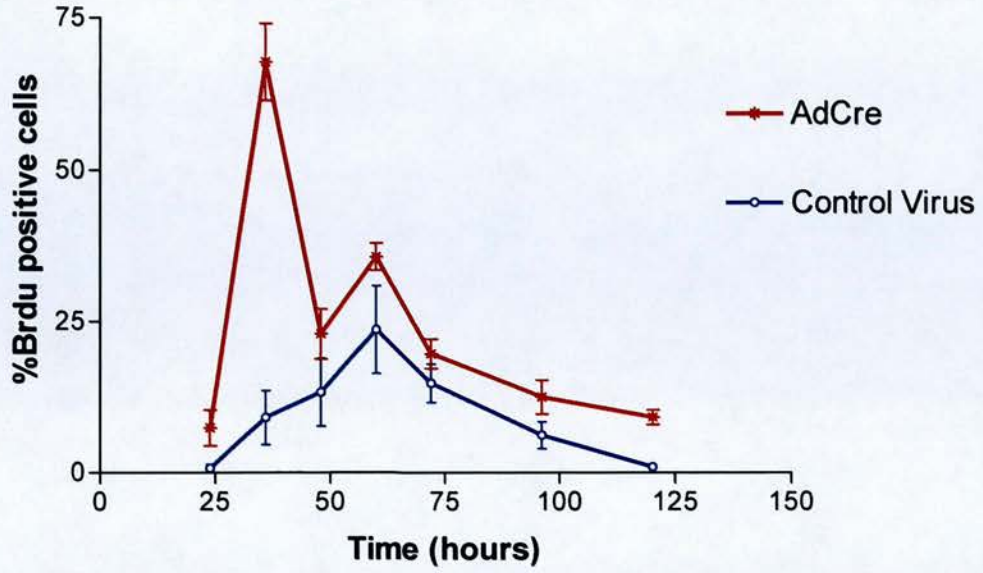


Figure 21. Liver regeneration in response to a single dose of CCl4. The two curves represent Rb floxed mice treated with either AdenoCre or an empty adenovirus 24 hours prior to CCl4 treatment. A pulse of BrdU was given 2 hours prior to culling. The percentage of BrdU positive hepatocytes is expressed against time from CCl4 injection as mean ± SEM. (n=3-5 mice per observation). Analysis of the two regenerative curves by two-way ANOVA revealed Rb deletion had a significant effect on BrdU incorporation (P<0.0001)

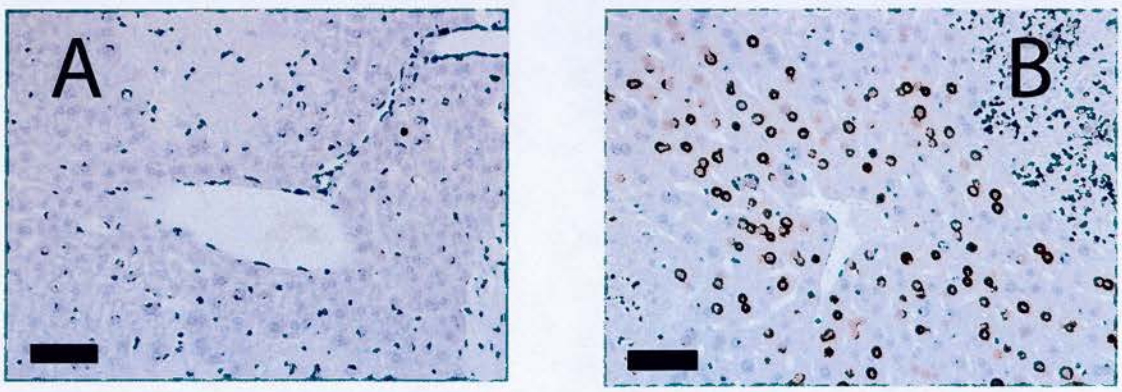


Figure 22. BrdU staining following CCl4 treatment in combination with empty vector control virus (A) or AdenoCre (B). Darkly stained nuclei are positive for BrdU. Bar Scale represents 50 µm.

Section C

The effect of Rb loss on liver polyploidisation in two murine models

Polyploidisation is a process of unknown significance, which occurs in parallel with normal liver growth. Levels of polyploidy normally increase during regeneration(Sigal et al. 1999). Because polyploidisation is associated with an increase in gene copy number, a reduced sensitivity to mitogens (Rajvanshi et al. 1998) and an increased sensitivity to apoptosis, it has been suggested that this process may be protective against carcinogenesis. Conversely, it has been suggested tetraploid cells may form an important intermediary in the development of aneuploidy, which is an important event during carcinogenesis (Fujiwara et al. 2005;Margolis 2005;Storchova et al. 2004). Both p53 and pRb have been implicated in the regulation of polyploidy through their involvement in centrosome duplication (Borel et al. 2002b) and regulation of a putative "tetraploidy checkpoint" (Margolis 2005;Storchova et al. 2004). In order to determine how loss of Rb may affect polyploidisation in the regeneration following both PH and acute necrosis, the percentage of nuclei exhibiting each polyploidy class during the regenerative response was analysed.

There is a polyploidizing pattern of growth in the regeneration that follows both PH and CCl4 administration.

Firstly, the normal changes in polyploidisation that occur during liver regeneration following PH and CCl4 were characterised using Rb floxed mice that had not been treated with either AdenoCre or empty vector virus (Figure 23 and Figure 24).

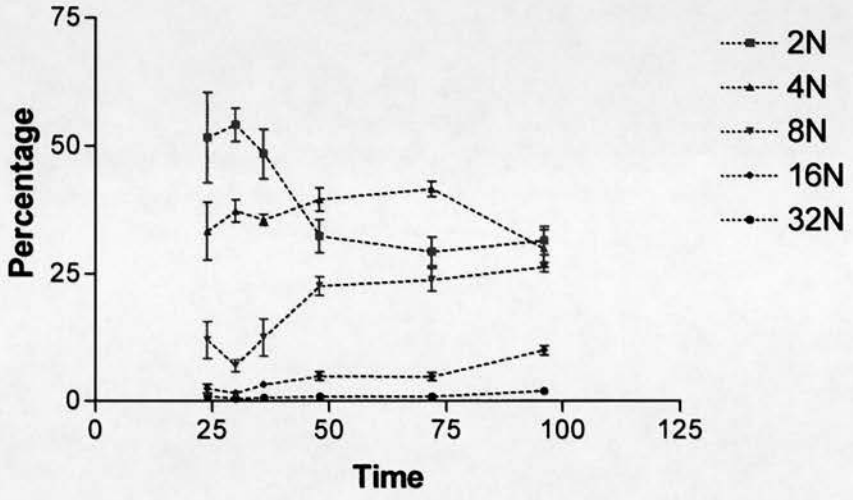


Figure 23. Changes in nuclear ploidy following PH in Rb floxed mice. The percentage nuclei of each class is expressed against time post PH as mean ± SEM. (n=3-6 mice per observation)

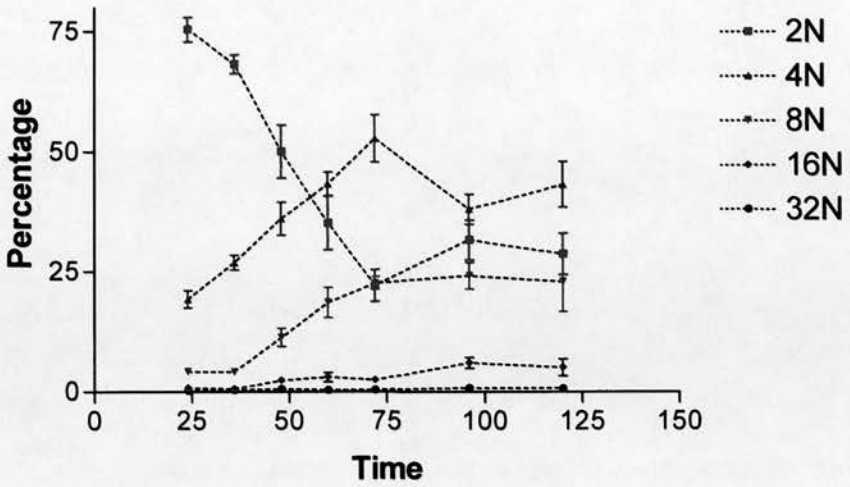


Figure 24. Changes in nuclear ploidy following a single dose of CCl4 in Rb floxed mice. The percentage nuclei of each class is expressed against time from CCl4 as mean ± SEM. (n=3-6 mice per observation)

Figure 23 and Figure 24 demonstrate that during regeneration in both models there is a fall in the number of diploid cells and an increase in the number of cells of higher polyploidy. This is particularly obvious in regard to the increase in the percentage of 8N nuclei. This polyploidising growth pattern is typical of liver regeneration(Sigal et al. 1999).

Rb deletion is associated with a loss of the normal process of polyploidisation that occurs during liver regeneration in two different models

To determine the effect of Rb on the normal polyploidizing growth pattern that occurs following both PH and CCl4 treatment, the changes that occurred in ploidy following treatment with either AdenoCre or empty vector control virus were analysed. For ease of comparison, the ploidy classes have been amalgamated to show only changes in diploid nuclei and in those nuclei whose DNA content is 4N or greater, termed "polyploid".

Effect of Rb deletion in polyploidisation following PH

Figure 26 and Figure 25 show the effect of Rb deletion on the polyploidisation that normally occurs following PH. Interestingly, loss of Rb was associated with an increase in the percentage of diploid nuclei, with a corresponding fall in the percentage of polyploid nuclei whose DNA content was 4N and greater. 96 hours post PH there was a significantly higher percentage of diploid nuclei in Rb deleted (AdenoCre treated) mice compared to empty vector treated control mice (43.6 ± 4.7%; *vs* 26.1 ± 1.0 %; n=5 for both treatments; P=0.006; student's t test) The very large nuclei typically seen during regeneration following empty vector treatment (Figure 27) were not seen following AdenoCre treatment.

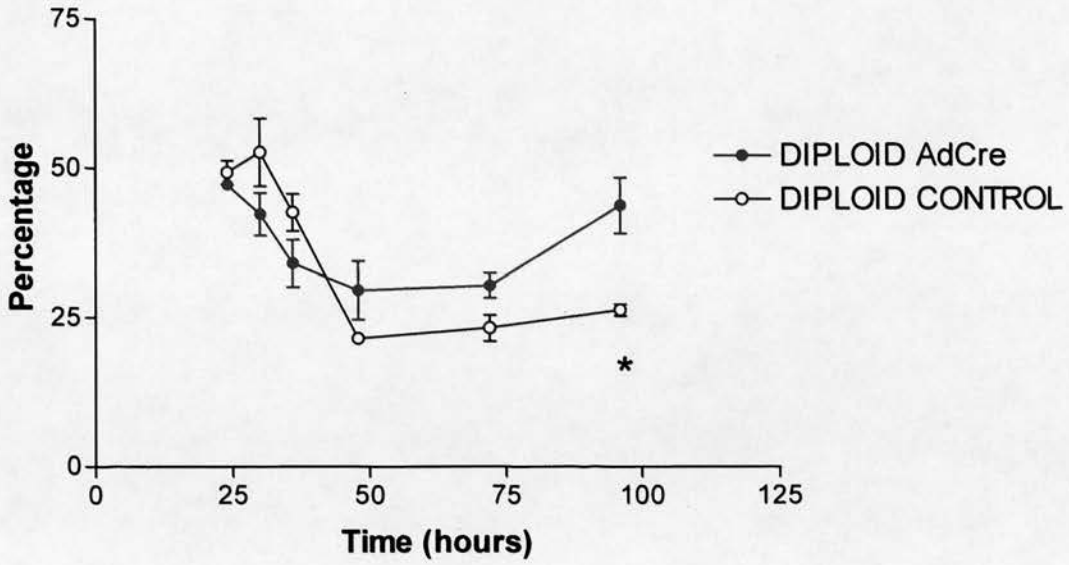


Figure 25. The effect of Rb deletion on the percentage of diploid cells following PH in Rb floxed mice. The percentage diploid nuclei is expressed against time post PH as mean ± SEM. (n=3-6 mice per observation)

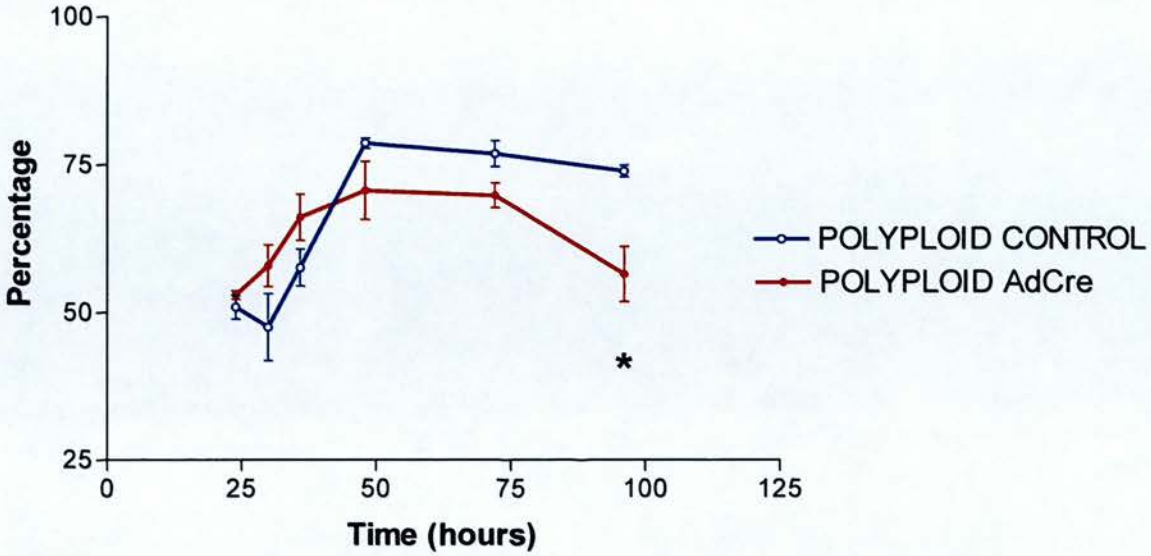


Figure 26. The effect of Rb deletion on the percentage of polyploid nuclei (4N and greater) following PH in Rb floxed mice. The percentage of polyploid nuclei is expressed against time post PH as mean ± +/-SEM. (n=3-6 mice per observation)

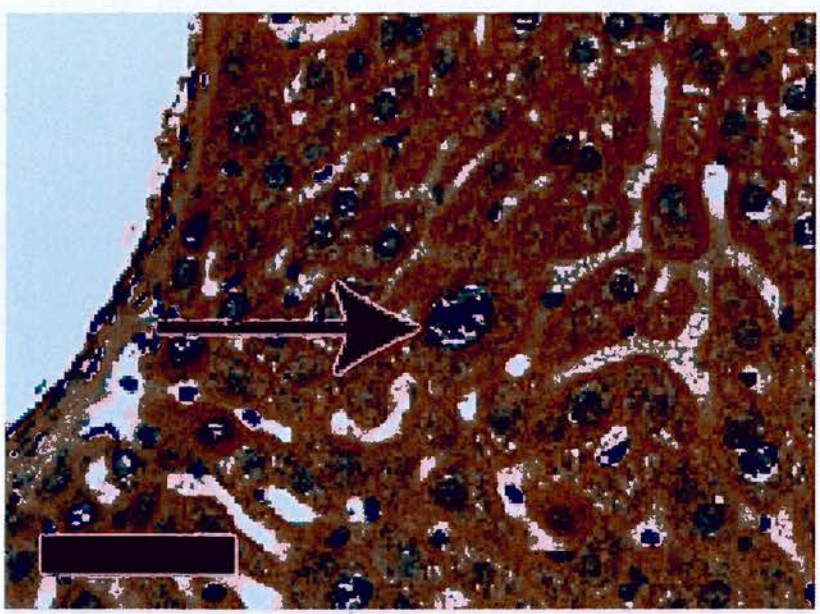


Figure 27. Typical large polyploid nuclei seen 120 hours following CCl4 treatment. Scale Bar represents 50 µm.

Effect of Rb deletion on polyploidisation following acute necrosis

Rb deletion had a similar effect on polyploidisation following CCl4 treatment as PH as shown in Figure 28. Again, there was not the same fall in the percentage of diploid nuclei, nor the same rise in the percentage of polyploid nuclei following Rb deletion (AdenoCre treated) compared to empty vector treated controls. 120 hours post CCl4 treatment, the percentage of diploid nuclei in the AdenoCre treated group tended be to greater than that in the empty vector treated control group (49.4 ± 3.9 % vs 37.0 ± 6.3%; n=4 for both treatments) but in this trend did not reach significance ($P > 0.05$)

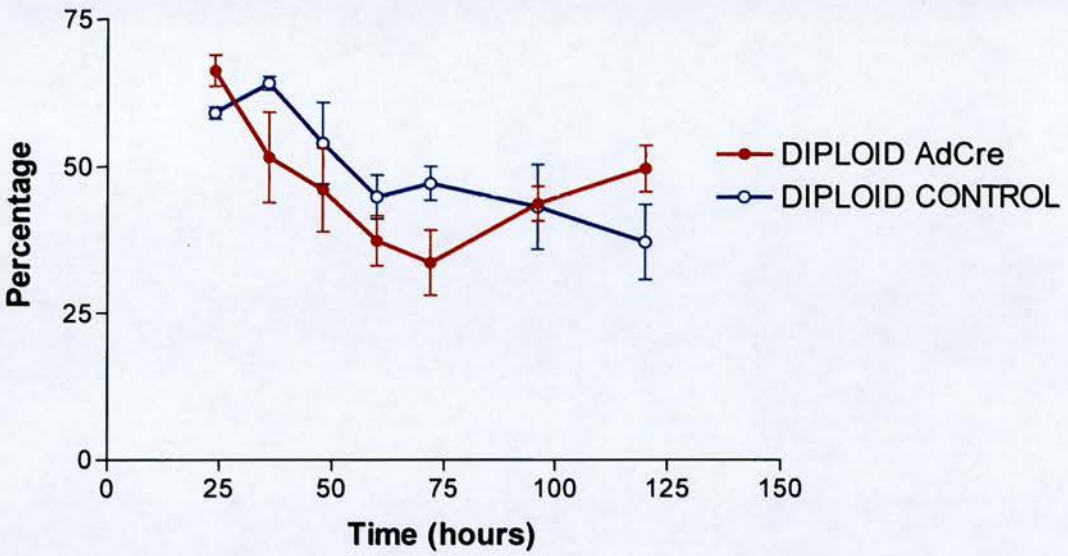


Figure 28. The effect of Rb deletion on the percentage of diploid nuclei following CCl4 treatment in Rb floxed mice. The percentage of diploid nuclei is expressed against time post PH as mean ± SEM. (n=3-6 mice per observation)

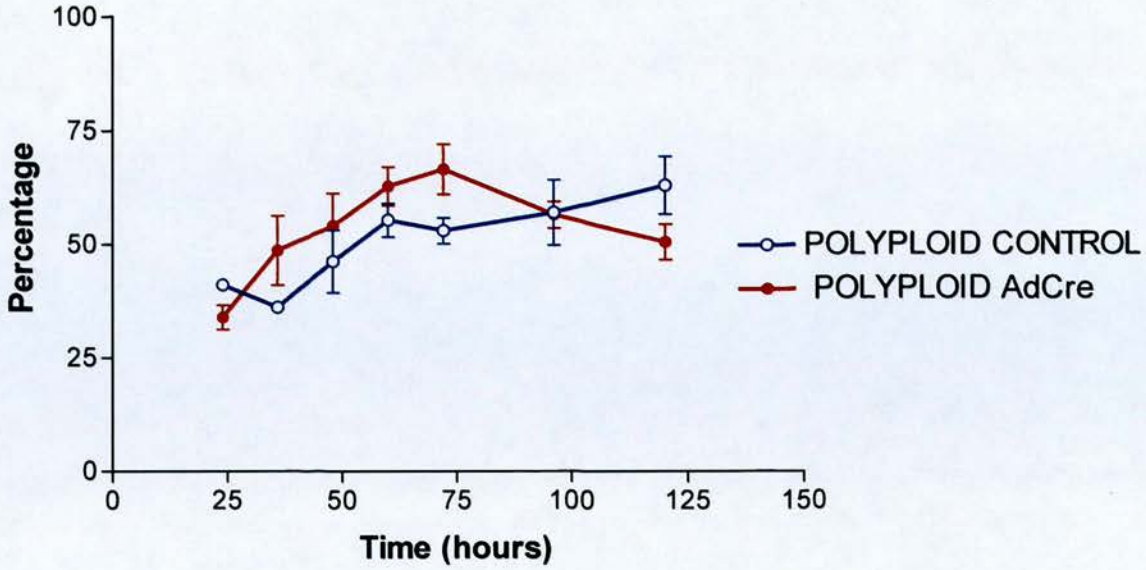


Figure 29. The effect of Rb deletion on the percentage of polyploid nuclei (4N and greater) following CCl4 treatment in Rb floxed mice. The percentage of polyploid nuclei is expressed against time post PH as mean ± SEM. (n=3-6 mice per observation)

These results suggest Rb deletion is associated with a reduction in the normal polyploidising growth pattern seen after both PH and acute necrosis. As already demonstrated, loss of Rb is associated with increased proliferative rates in both the PH and CCl4 models of liver regeneration. Increased proliferation might have been expected to increase polyploidisation, but surprisingly, the reverse was demonstrated. This is an unexpected and interesting result.

Section D

The effect of Rb loss in a Model of Carcinogenesis

After demonstrating that Rb deletion de-regulated the processes of liver regeneration and polyploidisation following both PH and acute necrosis, the longer term effects of Rb loss in an environment of sustained inflammation and DNA damage (as is the case in chronic liver disease in humans), were investigated. During the prolonged period of chronic inflammation that precedes the development of HCC in humans, a strongly pro-proliferative environment exists. Such an environment produces a selection pressure for those hepatocytes that have acquired advantageous mutations during this period (Thorgeirsson et al. 2002a). In an attempt to reproduce these elements of a mutagenic and pro-proliferative environment, diethylnitrosamine (DEN) treatment, a well-characterised mutagenic compound that produces DNA adducts, was combined with repeated administration of CCl4. The protocol for these experiments is summarised in Figure 30. These experiments were designed to test the hypothesis that loss of Rb can provide hepatocytes with a selective advantage in this environment. The protocol was necessarily short because the aim was to subsequently compare the additive effect of p53 deletion in combination with Rb loss. p53 deletion results in mice with a shortened life span (Donehower et al. 1992;Williams et al. 1994a).

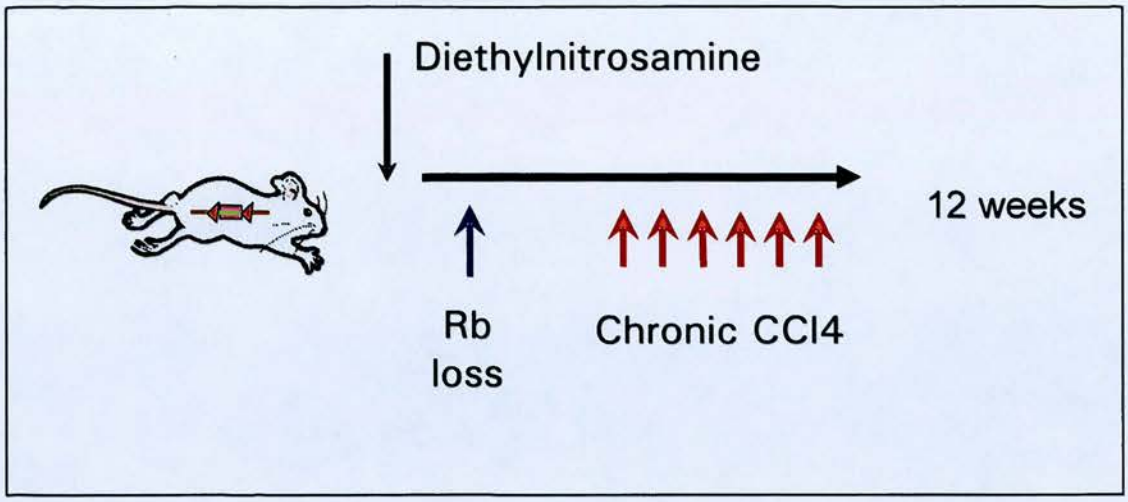


Figure 30 The carcinogenesis protocol for investigating the longer term effects of Rb deletion. DEN was given as a single intraperitoneal injection at 14 days of age and AdenoCre was administered via tail vein injection at 6 weeks of age. CCl4 was repeatedly administered weekly as an intraperitoneal injection over the following six weeks.

Rb has no effect on survival in a model of carcinogenesis

If loss of Rb is an important factor in the development of HCC, it might have been expected that Rb deletion would have an effect on mortality rates following 12 weeks of this carcinogenesis protocol. Although mortality rates tended to be higher in the AdenoCre treated group compared to the vector treated control group, this trend did not reach significance at 12 weeks, as shown in Figure 31. It is possible that a longer protocol may have increased the difference in survival between these two groups. The mortality in both of these groups is more likely to reflect the acute toxicity of the protocol, rather than a result of the histological changes occurring in the liver, because, as discussed later in this section, such lesions develop at an early stage and represent a small fraction of the total liver area.

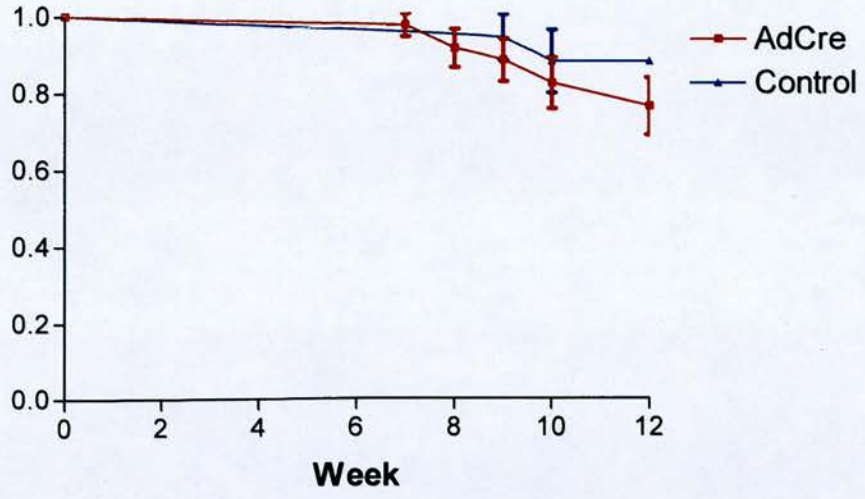


Figure 31 Survival curves comparing the effect of AdenoCre treatment vs empty vector control treatment in a carcinogenesis model of 12 weeks duration. There was no significant difference between the two curves (Log Rank test;P>0.05 p=0.3449)

Male sex is an independent risk factor for the development of preneoplastic foci in this protocol

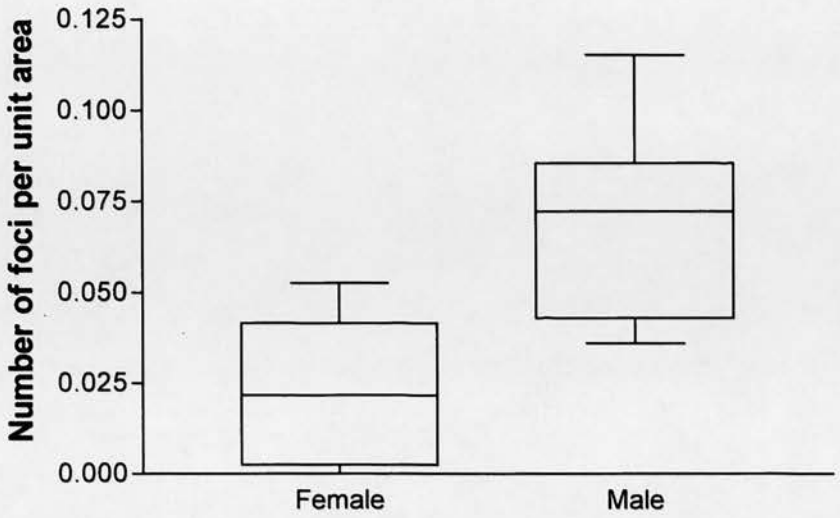


Figure 32.Comparison of the effect of sex on the development of preneoplastic foci. The number of foci per unit area tissue is expressed for male (n=12) and female (n=8) following AdenoCre treatment in the carcinogenesis protocol. ($p<0.001$ in unpaired t test).

Male sex is associated with an increased risk for the development of HCC in both mice and humans(Bosch et al. 1999a). It has been argued that such an increased risk in men is secondary to lifestyle factors, such as a higher alcohol intake (Lai et al. 1987). However, these data are more fitting with previous findings showing male sex is a strong independent risk factor for the development of HCC in mice (Ghebranious et al. 1998)

Rb loss functions to accelerate the rate of preneoplastic change in a model of carcinogenesis

There were a number of prominent histological features demonstrable in the liver following the carcinogenesis protocol. These included:

- The appearance of multiple preneoplastic foci
- High levels of hepatocyte apoptosis
- Occasional foci of dysplastic hepatocytes

The predominant histological feature was the development of multiple preneoplastic foci, typical examples of which are shown in Figure 33. These foci were made up of small, round cells and displayed either a basophilic or a clear cell phenotype.

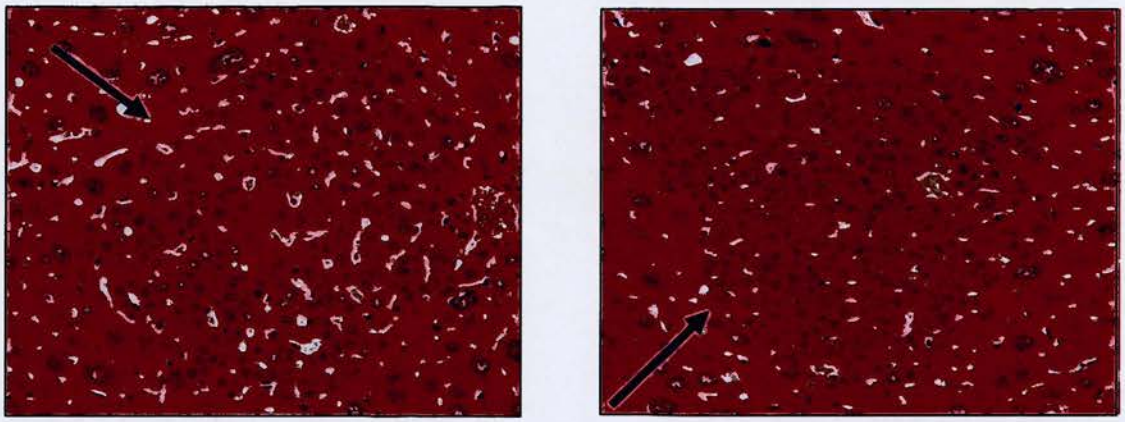


Figure 33. H&E stained liver sections showing typical preneoplastic foci comprised of non-encapsulated clusters of small basophilic staining hepatocytes.

Preneoplastic Foci stain positively for markers of cell proliferation

Preneoplastic foci are believed to represent the clonal expansion of hepatocytes that harbour mutations advantageous to tumour development. One characteristic of these foci is increased cell replication compared to hepatocytes out with the foci (Schulte-Hermann et al. 1993). To determine whether the foci seen following this carcinogenesis protocol represented hyperplastic cell populations, immunohistochemistry to detect the proliferation markers, Ki67 and PCNA, was performed (Figure 34). Positive staining for these markers was demonstrable within preneoplastic foci, but was rarely seen in the surrounding tissue. Quantification of the percentage of positive cells present in the foci of AdenoCre treated mice compared to areas of surrounding tissue demonstrated a significantly higher rate of hepatocyte division in these abnormal preneoplastic foci. (Figure 35)

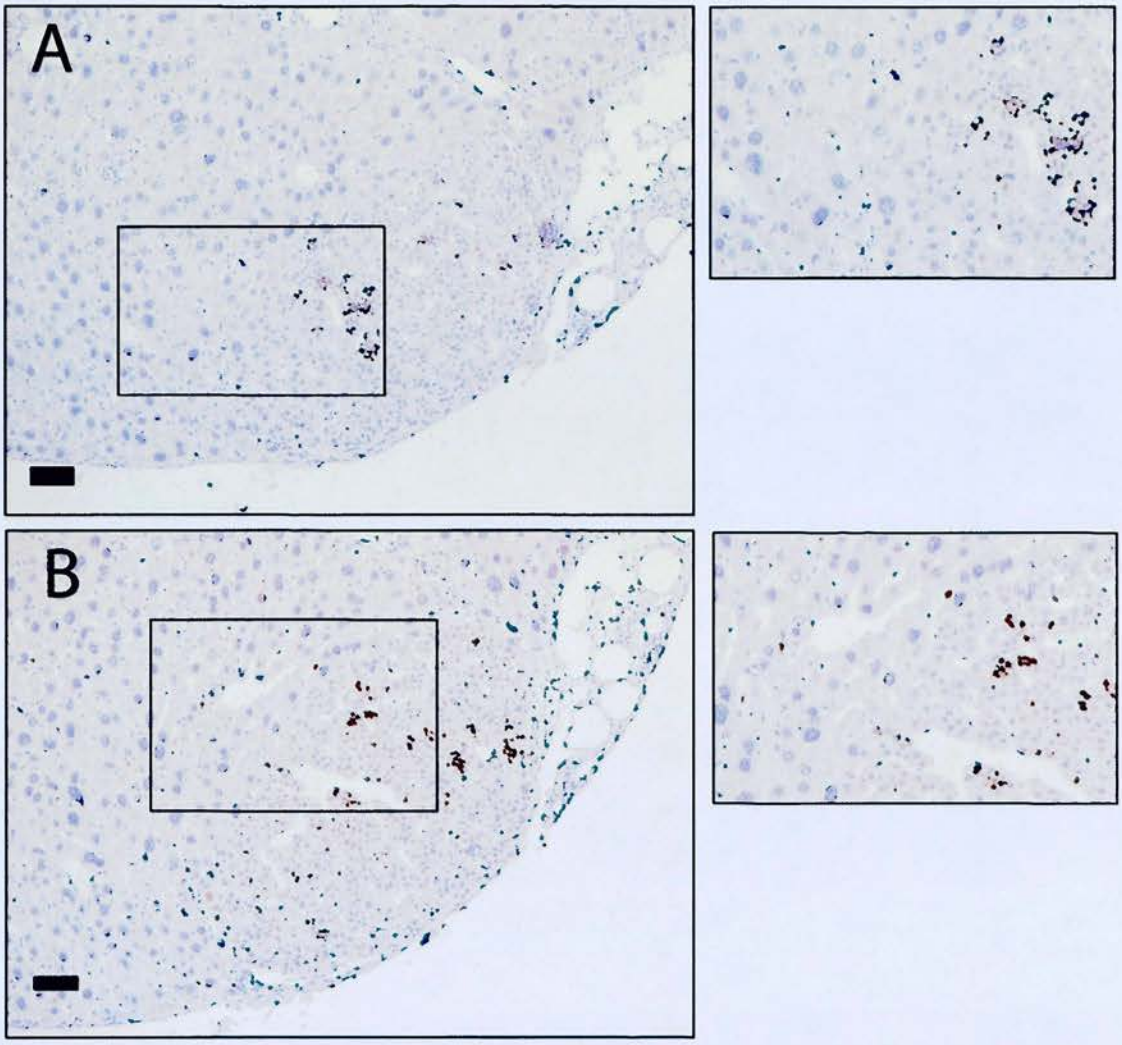


Figure 34. Immunohistochemistry of a typical preneoplastic focus showing positive staining within the focus (darkly stained cells) for the proliferative markers PCNA (A) and Ki67 (B).

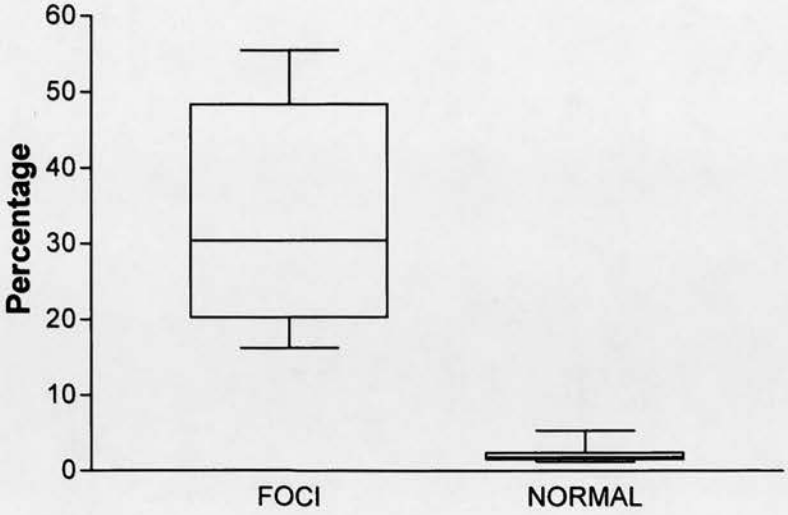


Figure 35. The total percentage of hepatocytes positively stained for PCNA and Ki67 within preneoplastic foci compared to normal tissue. The total number of foci counted was 62 across 8 different animals. Comparison was made against the percentage of positively staining hepatocytes from a minimum of 500 hepatocytes outwith the foci. (P= 0.0006; paired t-test).

Rb loss increases the frequency, size of and fractional area of preneoplastic foci in a model of carcinogenesis

In order to quantify the preneoplastic foci that characterised the liver histology following the carcinogenesis protocol, a method of image analysis was developed similar to previously described methods (Pierce et al. 2002). This allowed quantification of the size of individual foci, the number of foci per unit tissue area and the total cross sectional area made up of preneoplastic tissue.

Quantification of the size of individual preneoplastic foci in male mice demonstrated that Rb deletion (AdenoCre treatment) resulted in foci that were significantly larger compared to those in the empty vector treated control animals (83300 ± 8553 μm^2; n=12 *vs* 29980 ± 6802 μm^2; n=6; *P*= 0.0009, students t test. (Figure 36). The number of foci per unit of section were also significantly increased in the AdenoCre treated group compared to the empty vector treated control group (0.074 ± 0.011 foci/mm^2; n=12 *vs* 0.023 ± 0.006 foci/mm^2; n=6; *P*=0.0058; students t-test; Figure 37). This meant preneoplastic tissue occupied a greater proportion of the tissue in the Rb deleted (AdenoCre treated) animals compared to the empty vector treated control animals (0.656% ± 0.124%; n=12 *vs* 0.068 ± 0.022 %; n=6, *P*=0.0047; students t-test; Figure 38).

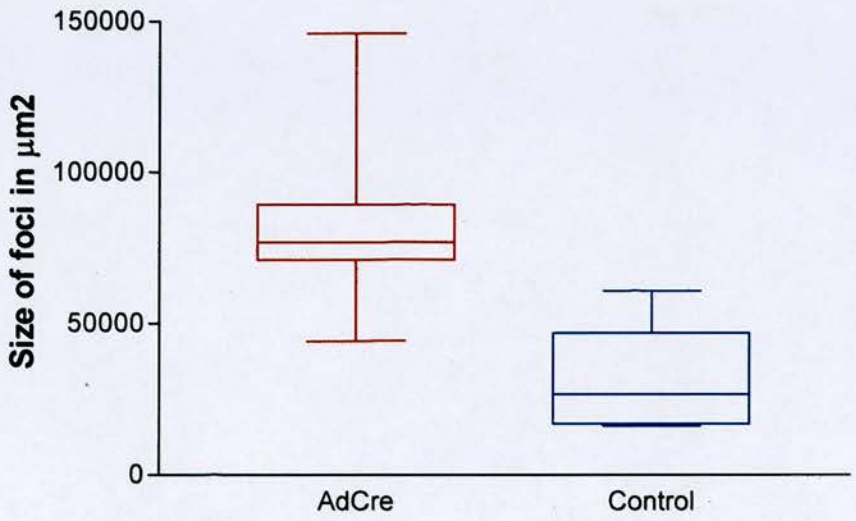


Figure 36. Effect of Rb deletion on the size of preneoplastic foci in male mice treated in the carcinogenesis protocol.

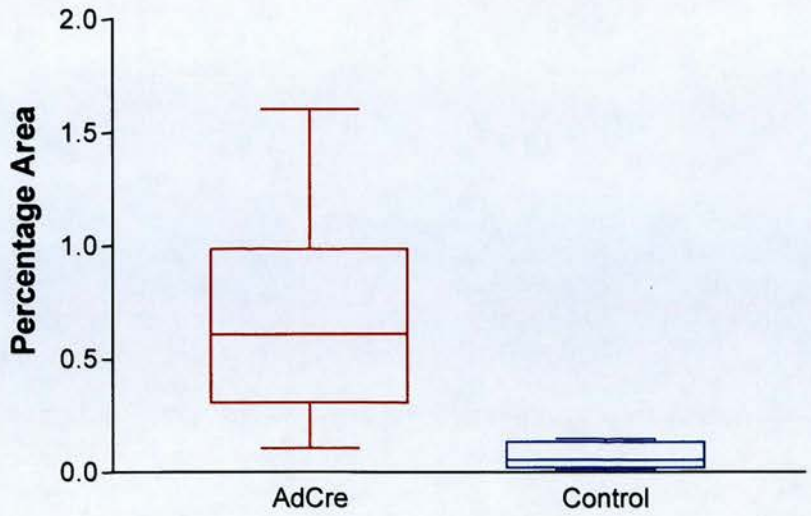


Figure 37. Effect of Rb deletion on the percentage area of preneoplastic tissue in male mice treated in the carcinogenesis protocol.

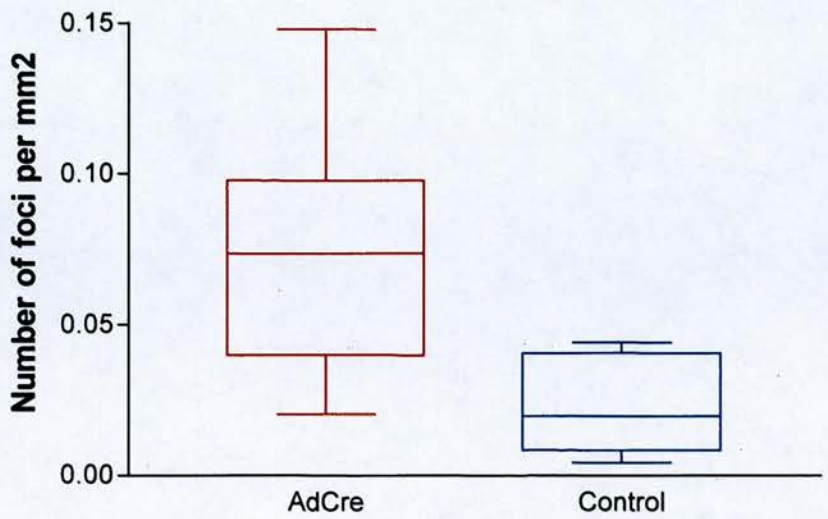


Figure 38. Effect of Rb deletion on the number of preneoplastic foci per unit area of tissue section in male mice treated in the carcinogenesis protocol.

Rb loss is associated with increased levels of apoptosis in a model of carcinogenesis

One of the notable characteristics of the livers of AdenoCre treated mice compared to the livers of empty vector treated control mice in the carcinogenesis protocol was the presence of high levels of apoptosis. Quantification of levels of apoptosis based on morphology (typical morphology is demonstrated in Figure 40) revealed that male AdenoCre treated mice (n=12) had significantly elevated levels of apoptosis compared to empty vector treated control mice (n=6; P=0.0158; students t-test; Figure 39).

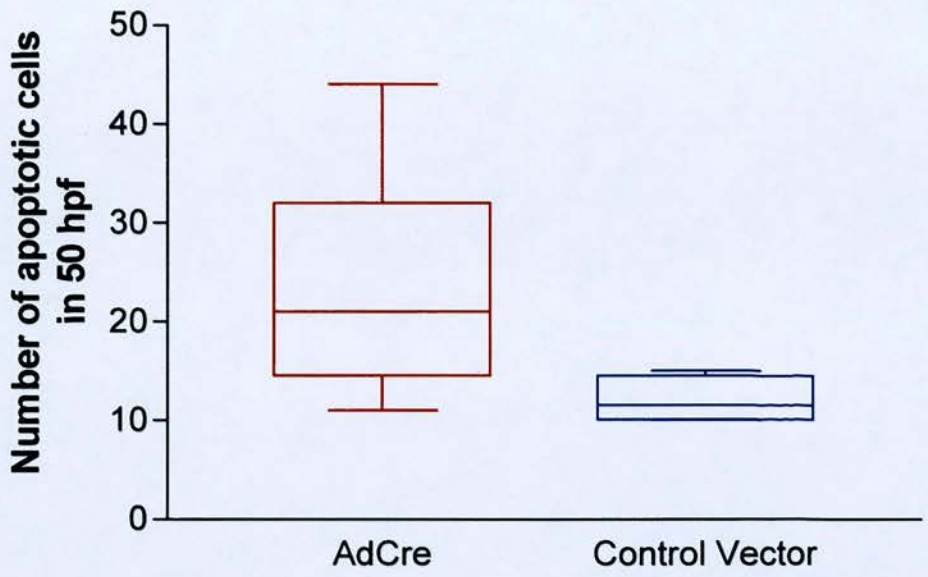


Figure 39. Quantification of apoptosis in liver sections from AdenoCre treated mice compared to empty vector treated control mice following the carcinogenesis protocol.

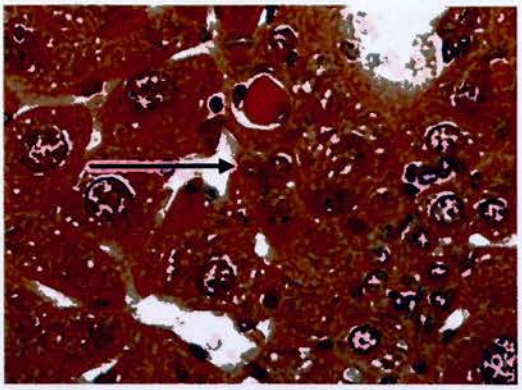


Figure 40. H&E stained liver section showing a typical apoptotic body. (x40 magnification)

Rb null cells persist following a period of chronic inflammation in a model of carcinogenesis

If Rb deletion provides a proliferative advantage to Rb null hepatocytes, then one might reasonably predict that Rb null hepatocytes should be detectable at the end of the carcinogenesis protocol, 6 weeks following AdenoCre treatment. PCR performed on genomic DNA extracted from liver at the end of the protocol demonstrated recombined DNA was present (Figure 41).

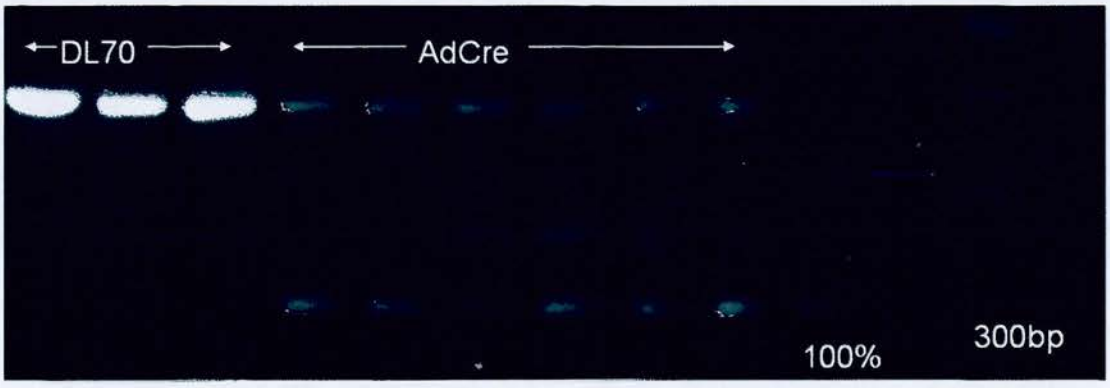


Figure 41. PCR detection of recombined Rb floxed alleles. Lanes 1-3 represent genomic DNA taken from the livers of mice following completion of the carcinogenesis protocol 6 weeks post injection of the control virus (DL-70). Lanes 4-9 represent genomic DNA taken from the livers of mice following completion of the carcinogenesis protocol 6 weeks post injection of the AdenoCre virus. Lane 10 is DNA from hepatocytes treated in vitro with AdenoCre virus (positive control). Lane 12 is molecular markers. 1.5% agarose/ethidium bromide gel

Rb loss can be demonstrated in both preneoplastic foci and in non lesional tissue in livers following the carcinogenesis protocol.

Rb deletion (AdenoCre treatment) was associated with an increase in the prevalence of preneoplastic foci compared to that in empty vector treated control mice. To determine whether these foci represented populations of Rb null cells, laser capture mico-dissection was used to isolate these foci in order to compare their cellular composition with that of surrounding tissue.(Figure 42) Rb loss could be demonstrated in both preneoplastic foci and surrounding non lesional tissue. However, it was not possible to demonstrate a non-recombined allele in neither preneoplastic foci nor the surrounding non lesional tissue. Indeed the non recombined allele could not be demonstrated in untreated laser dissected control tissue, presumably because the process of laser dissection produces DNA fragments which are too short to allow the amplification of the (longer) non recombined allele.

Figure 42. PCR detection of recombination in Rb floxed alleles in DNA extracted from laser-dissected areas of normal liver tissue (N), or areas of preneoplastic foci (F) following DEN-induced carcinogenesis and AdCre treatment. Lanes 1-6 = DNA extracted from normal tissue or foci as indicated. Bar represents dissections made from one individual slide. Lane 7 = genomic DNA from hepatocytes treated with AdCre in vitro (positive control). Lane 8 = DNA extracted from hepatocyes treated in vitro with empty adenovirus (negative control). Lane 9 = molecular markers

In mice homozygous for p53 Rb loss may accelerate dysplastic change in a model of carcinogenesis

It has been widely demonstrated that Rb loss is associated with p53 stabilisation and increased levels of apoptosis (Macleod, Hu, & Jacks 1996;Pan, Yin, Dyson, Harlow, Yamasaki, & Van Dyke 1998;Tsai, MacPherson, Rubinson, Crowley, & Jacks 2002). It has been suggested loss of p53 might, therefore, be essential for neoplastic transformation of Rb null cells (Williams et al. 1994a). To determine the effect of p53 loss in combination with Rb deletion, the carcinogenesis protocol was repeated using Rb floxed mice crossed with p53 null mice (Figure 43). Histological examination of these livers by an experienced liver pathologist demonstrated that compared with Rb loss alone, mice deficient in both p53 and Rb exhibited an additional, and striking diffuse increase in hepatocyte turnover, manifesting as increased mitosis and cell death - both apoptosis and single cell necrosis with inflammation (spotty necrosis). The cytological appearance of hepatocytes was even more extremely deranged than in the Rb-deficient animals. The changes were notably distributed throughout the liver, rather than clustered in the relatively atypical foci seen following Rb deletion alone. These changes could be attributed, to some degree, to synergistic effects between p53 deficiency and viral treatment. Because a further control of p53 null mice treated with empty adenovirus was not employed it is not possible to completely exclude this possibility. However, this seems an unlikely explanation for the gross effects seen here for two reasons; firstly, empty adenoviral treatment appeared to have only a minimal effect when given to Rb floxed p53 wild type mice and secondly, it has been demonstrated that p53 is required for adenovirus of this type to have a cytopathic effect (Hall et al. 1998).

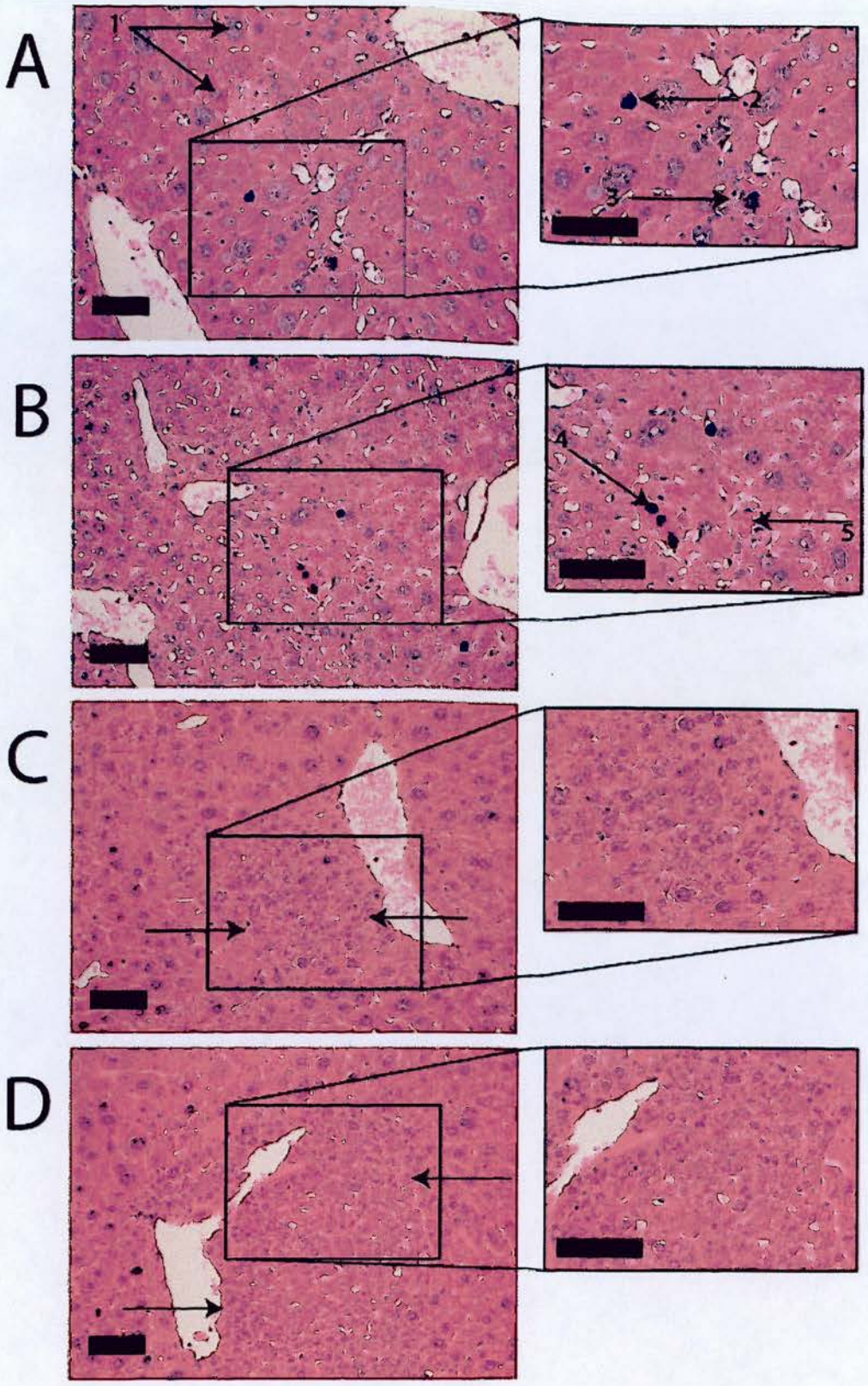


Figure 43 Altered histology of double Rb/p53-deficient livers after DEN/CCl4 injury. Loss of Rb on a p53-deleted background (A & B) was characterised by diffuse changes of increased hepatocyte size, extreme nuclear pleomorphism (arrow 1),

abnormal mitoses (arrows 2&4) and spotty hepatocyte necrosis (arrows 3&5). These changes were distinct from the phenotype produced by Rb deletion alone, where background hepatocyte nuclear enlargement and pleomorphism was present but comparatively markedly reduced (similar to p53-null hepatocytes), and features of markedly increased turnover (apoptosis, mitoses) and mitotic instability were absent (C & D; arrows indicate borders of a small hyperplastic focus (C &D inserts). Scale bar represents 50 μM.

Chapter 5 Discussion

Rb loss deregulates the processes of liver regeneration in two different models

To determine the role of Rb in the regulation of liver regeneration, two models of liver injury were developed, which, when combined with AdenoCre treatment, allowed the consequences of Rb loss on hepatocyte proliferation to be examined *in vivo*.

Both partial hepatectomy and acute necrosis following AdenoCre treatment resulted in accelerated entry into S phase of the cell cycle. Higher rates of DNA synthesis were maintained throughout the time course, with the peak of DNA synthesis occurring earlier than in controls. These observations suggest Rb negatively regulates cell cycle progression during liver regeneration. These findings are in keeping with the accepted role of pRb as a regulator of both the restriction point, and the G2/M checkpoint. Loss of Rb has a similar effect on proliferation in many different experimental systems, including, importantly, isolated primary hepatocytes *in vitro*(Sheahan et al. 2004a). However, this is the first report of such a role for Rb being demonstrated in the regulation of liver regeneration *in vivo*.

The levels of pRb were not determined in these experiments, though previous work has demonstrated levels of pRb are normally extremely low in the quiescent liver(Garriga et al. 1998) and increase dramatically during liver regeneration (Fan et al. 1995). Both hypo- and hyper- phosphorylated forms of pRb are found in the time course following PH, with levels of phosphorylation increasing rapidly during G1. The phosphorylation status of pRb controls its activity such that hyperphosphorylation induces conformational change in the pocket region of the protein, reducing its binding affinity for E2F and, therefore, permitting cell cycle progression. It is interesting that hypophosphorylated forms of pRb persist throughout liver regeneration, which suggests the rate of progression through the restriction point may be controlled by both the absolute levels of pRb, and also the

ratio of phosphorylated to hypophosphorylated forms, i.e. the balance between active and inactive forms. Such a hypothesis would fit with the observations presented in this thesis, for; if pRb was entirely hyperphosphorylated (deactivated) from the outset of, and throughout regeneration, then the somatic loss of Rb engineered here might have been expected to have had no additional effect. However, in these experiments, loss of Rb resulted in a reduction in the time between stimulus and S phase entry, indicating a shorter G1 phase of the cell cycle. In addition, the normally biphasic cell cycle entry typical of liver regeneration, was disrupted and increased rates of DNA synthesis occurred throughout the time course. Thus, it would appear Rb has an important function in regulating both rate of entry into the cell cycle and the co-ordination of hepatocyte replication.

Although no previous attempts have been made to determine the role of Rb in the regulation of liver proliferation, the role of composite members of the Rb pathway has been examined. Loss of inhibitors of Rb, including the cyclin kinase inhibitor, p21, deregulates liver regeneration in a similar manner as loss of Rb – a shortened G1 phase , associated with Rb hyperphosphorylation (Albrecht et al. 1998b;Jaime et al. 2002). Alternatively, loss of p18, belonging to the second family of cyclin kinase inhibitors, the INK4 family, has little effect on liver regeneration in isolation, but co-operates with p21 and p27 to regulate both the length of G1, and the maximum rate of DNA synthesis (Luedde et al. 2003). The role of activators of Rb has been examined using an adenoviral delivery system to increase cyclin D expression in the murine liver. This resulted in DNA synthesis, mitosis, and increased liver weight. However, the effects were only short lived, and the role of increased cyclin D expression on liver regeneration was not examined (Nelsen et al. 2001b). The phenotype of deregulated proliferation that results from Rb loss is believed to be a consquence, at least in part, of increased E2F activity. Liver specific over-expression of E2F-1 in a murine model increased the basal rate of hepatocyte proliferation, but surprisingly had little effect on hepatocyte proliferation following PH (Conner et al. 2000). This may be because Rb is known to exert a negative influence on cell cycle progression through a number of different mechanisms in addition to that of sequestering E2F family members, including directly inhibiting the transcription

machinery, such that in the regulation of liver regeneration loss of E2F1 may not equate with that of Rb loss. It is interesting that both loss of Rb inhibitors, and increased levels of Rb activators, have an effect on hepatocyte proliferation *in vivo*. Such an observation provides support for both the theory (Vogelstein et al. 2004) and *in vivo* evidence (Edamoto et al. 2003) that there operates within the development of HCC an "exclusivity principle" which suggests the loss of only one of the negative regulators, or increased activity of one of the activators, of the pathway is required to produce the same functional outcome.

The results presented in this thesis demonstrate an important role for Rb in the regulation of hepatocyte turnover, specifically during those conditions when hepatocytes are driven to proliferate. Such findings suggest loss of Rb function could be a significant event in the development of HCC. The environment of chronic inflammation which precedes the development of HCC is characterised by continual regeneration, during which hepatocytes are constantly driven to proliferate by a number of factors including ongoing hepatocyte necrosis and death, as well as increased expression of liver mitogens including TGFα and HGF (Grisham 2001a). The rate of hepatocyte proliferation correlates directly with the frequency of tumour development in the liver (Borzio et al. 1998). Loss of heterozygosity of Rb has been demonstrated in cirrhosis accompanying HCC (Ashida et al. 1997), which suggests that loss of regulation of regeneration by pRb may occur early in the development of HCC. Loss of Rb function in such an environment would provide a clear proliferative advantage allowing for clonal expansion of hepatocytes lacking functional pRb.

The most common aetiological factor in the development of HCC is infection with the hepadnavirusus. Both Hepatitis B and Hepatitis C infection drive hepatocyte proliferation as a consequence of chronic inflammation. Indeed, the inflammatory response to viral infection is required for the development of HCC. (Nakamoto et al. 1998;Nakamoto et al. 2002). In addition to this non-specific effect of viral infection, viral proteins also have specific interactions with the cell cycle machinery. (Feitelson et al. 2005) Hepatitis C core protein appears to increase pRb phosphorylation and

E2F activity *in vitro* (Ghosh et al. 1999;Hassan et al. 2004). HBx has similar effects on the function of pRb, both directly and indirectly, through p21(Sirma et al. 1999b). Therefore, hepatitis infection may be such a powerful aetiological factor in the development of HCC because it has the ability to simultaneously drive hepatocyte proliferation, while abrogating the ability of pRb to negatively regulate cell cycle progression.

In addition to conferring a proliferative advantage on hepatocytes, loss of Rb may confer a number of further advantages to incipient tumour cells. Rb has been ascribed a role in maintaining the stability of the genome through its regulation of the G2M checkpoint, interacting with the protein phosphatases 1α (Durfee et al. 1993) and Hec1(highly expressed in cancer 1), (Zheng et al. 2000) to ensure faithful chromosomal segregation. Rb may also have a role in the regulation of centrosome duplication (Borel et al. 2002a;Meraldi et al. 1999). In the absence of Rb, high rates of proliferation lead to the accumulation of markers of chromosomal damage in mouse embryonic stem cells (Zheng et al. 2002). Thus the absence of Rb may contribute directly to defects in mitosis and genetic instability. It would be of interest to determine whether loss of Rb had a similar effect on such markers of genomic instability following liver regeneration.

As well as direct effects on mitosis, the absence of Rb may also prevent replicating hepatocytes from arresting in response to DNA damage, bypassing cell cycle checkpoints and allowing the accumulation of further mutations in Rb null cells. pRb is essential for effective p53-mediated cell cycle arrest following UV and γ radiation induced DNA damage in mouse embryo fibroblasts and Rb null hepatocytes (Harrington et al. 1998;Sage et al. 2000;Sheahan et al. 2004b).In the absence of Rb, adult mouse fibroblasts continue to replicate following DNA damage induced by chemotherapeutic agents (Bosco et al. 2004). The process of liver regeneration appears to involve considerable genotoxic stress and oxidative injury. PH is associated with increased levels of p21 and the accumulation of markers of DNA damage in regenerating liver (Sigal et al. 1999), suggesting the activation of the ATM/p53/p21damage response pathway. It would be interesting to determine in the

regeneration model presented in this thesis, whether loss of Rb was associated not only with an increase in deregulated proliferative activity, but also an impaired response to the cell cycle arrest signals which appear to operate during liver regeneration.

The mechanism through which Rb loss deregulated liver regeneration in the experiments presented here was not examined. A substantial body of evidence suggests deregulated cell proliferation following Rb loss is mediated through deregulated E2F activity. Downstream targets of E2F include those genes which control the machinery of DNA synthesis (DeGregori et al. 1995a), therefore it is reasonable to assume the link between Rb loss and increased DNA synthesis demonstrated here is secondary to increased E2F activity, though it would be of interest to examine how the time course of E2F was altered during the regenerative period. A further consequence of Rb loss is increased apoptosis (reviewed (Chau et al. 2003), which is also believed to be mediated through increased E2F activity. There are several potential pathways through which E2F may mediate apoptosis, both p53 independent and p53 dependent. That apoptosis may result from loss of Rb remains a puzzle because such an observation conflicts with the role of Rb as a tumour suppressor. Whether Rb loss resulted in increased levels of hepatocyte apoptosis in the liver was not determined in these experiments. In order to be confident of the implications for development of HCC of acute Rb loss in the liver, it would be interesting to determine whether the increased proliferative activity seen in the results of this thesis was associated with increased levels of apoptosis in the liver.

The premise of this thesis was that as a negative regulator of cell cycle progression, the role of Rb in the liver would only be apparent when hepatocytes were dividing. Therefore, models of liver regeneration and carcinogenesis were used to test this hypothesis. There is evidence to suggest Rb has a role not only in governing proliferation, but also in the control of quiescence (Sage et al. 2003). A recent paper describes the use of very similar techniques to those used here, in order to examine the effect of Rb loss on the maintenance of quiescence in the liver (Mayhew et al. 2005). Mayhew *et al* demonstrated that acute Rb loss in quiescent liver was

sufficient to produce hepatocyte DNA synthesis, but not mitosis, and implied acute loss of Rb could be an initiating event in the development of HCC. However when considered in combination with the observation that the levels of pRb are extremely low in quiescent liver (Garriga et al. 1998), and that, in the absence of a regenerative stimulus, acute Rb loss simply leads to cell cycle arrest (Mayhew et al. 2005), it seems unlikely that isolated loss of Rb in the quiescent liver is likely to be a transforming event..

Regulation of polyploidisation by the Retinoblastoma Gene

The experiments described here demonstrate that when regeneration occurred following Rb loss, the process of polyploidisation which normally accompanies rapid liver growth was altered. Although polyploid nuclei could certainly be demonstrated during the regeneration which followed both AdCre and empty vector treatment, the fraction of polyploid hepatocytes was decreased following Rb loss, and the fraction of diploid nuclei was increased. This was an unexpected result for several reasons. Firstly, in keeping with initial experiments and those of others (Fukasawa 2005;Nigg 2002a) the rapid replication of hepatocytes following PH is normally associated with an increase in the fraction of polyploid nuclei. Therefore, the increased levels of proliferation seen following Rb loss might have been expected to produce an increase, rather than a decrease, in levels of ploidy. Secondly, if Rb contributes to the maintenance of genomic stability (Bosco et al. 2004), and if polyploidisation can be assumed to be a marker of chromosomal instability, then loss of Rb might have been predicted to increase polyploidisation. In addition, the results of this thesis directly contradict previous results of *in vivo* and *in vitro* work that examined the effect of Rb loss on polyploidisation in the liver. *In vitro,* the loss of Rb is associated with an increase in both the rate of hepatocyte proliferation and in the levels of hepatocyte polyploidisation (Sheahan et al. 2004c). Similarly *in vivo* experiments have demonstrated liver-specific Rb loss resulted in increased levels of hepatocyte polyploidisation (Mayhew et al. 2005).

There are a number of possible explanations for these results. The first is that regeneration following loss of Rb results in the loss of the normal polyploidising growth pattern characteristic of hepatocytes. A second explanation is that the increased fraction of diploid nuclei represents not a change in the growth pattern of mature adult hepatocytes, but instead the emergence of a diploid progenitor population whose normal growth pattern is non-polyploidising, and whose replication is exaggerated by Rb loss, such that they expand to make up a larger fraction of the analysed nuclei.

The interpretation of these results is somewhat restricted by limitations of the methods employed here to determine levels of ploidy. The Vindelov preparation involves the labelling of individual nuclei following their isolation from whole cells by a trypsin digest of multiple liver aspirates (Vindelov et al. 1983b). The DNA content of individual nuclei is then examined by flow cytometric analysis. Therefore, it is only possible to comment on the change of ploidy of individual nuclei, rather than the DNA content of individual whole cells. In the adult mouse, a large proportion of hepatocytes are binuclear (Brodsky et al. 1977), and polyploidisation occurs through generation of a binuclear intermediate (Guidotti et al. 2003). As the Vindelov method does not allow analysis of the DNA content of whole cells, it is not possible to know whether the apparent increase in the fraction of diploid nuclei represents a genuine diploid cell population, or the expansion of binuclear hepatocytes of 2n2n ploidy. However, it is possible to say the inverse; that there is a fall in the prevalence of nuclei whose DNA content is 4n or greater.

The significance of polyploidy is not yet fully known, nor are the mechanisms that govern this process. A popular, and long standing, theory in cancer biology is that polyploidisation, particularly the formation of tetraploid cells, may represent an intermediate step in the development of aneuploidy and cancer. This theory was initially proposed almost 100 years ago (Boveri T 1914), and is the foundation for the proposed "tetraploidy checkpoint", a putative, possibly p53-controlled checkpoint, that directs cells which undergo cleavage failure into cell cycle arrest. The theory is that if this checkpoint fails, tetraploid intermediates that possess an

abnormal number of centrosomes are more likely to undergo an abnormal mitosis resulting in aneuploidy (Doxsey 2001;Fukasawa 2005;Nigg 2002b;Pihan et al. 1998). Abnormal centrosome number has been observed in a number of different human tumours (Lingle et al. 1998;Pihan et al. 1998), and *in vitro* experiments have demonstrated abnormal centrosome segregation and aneuploidy following artificially induced cell cycle arrest (Borel et al. 2002c;Meraldi et al. 2002). Rb has been attributed a role in the control of centrosome duplication and in maintaining cell cycle arrest as part of the tetraploidy checkpoint (Margolis et al. 2003). It is an attractive hypothesis that loss of Rb could lead directly to both aneuploidy and deregulated cell cycle regulation. However, actual *in vivo* evidence for an obligatory tetraploid intermediate in the development of cancer is limited to one tumour type (Galipeau et al. 1996).

The ubiquity of polyploidisation in the liver seems to provide direct evidence contrary to the theory that tetraploid cells are obligatory cancer intermediates. Polyploidisation in the liver appears to represent a normal physiological growth pattern. In healthy liver, ploidy increases gradually throughout development to the point where hepatocytes of 4n or greater represent the majority of hepatocytes in the liver (Brodsky et al. 1977), and unless the normal regulatory processes of liver growth are perturbed through chronic inflammation, liver cancer is unusual (Grando-Lemaire et al. 1999). This suggests that in the liver, polyploidisation represents a specialised growth strategy which presumably serves some advantage. Polyploidy occurs unusually out with the liver (Vliegen et al. 1995) suggesting hepatocytes show a specialised upregulation of polyploidising pathways.

An alternative theory is that polyploidisation in the liver may serve as a biological defence mechanism, directing hepatocytes that have acquired DNA damage towards senescence rather than apoptosis, as an economical alternative to maintain the homeostatic function of the liver. The presence of polyploidy in the liver would then be indicative of the on going replenishment of hepatocytes in a toxic environment, rather than representing pathological intermediates in the development of cancer.

There are a number of lines of evidence which support this theory and can explain the presence of polyploid hepatocytes in both healthy and diseased liver. If polyploidisation occurs in the liver as a response to DNA damage, and DNA damage is an inevitability of cell replication, then rates of polyploidy might be expected to increase in parallel with growth. Basal rates of hepatocyte renewal are slow, but are associated with steady increases in levels of polyploidisation throughout the life of the adult (Kudryavtsev et al. 1993). During periods of rapid growth, as occurs during post natal development and regeneration, the rate of polyploidisation is increased in parallel with rates of replication and presumed acquired DNA damage. A rapid increase in polyploidisation with a concomitant increase in markers of DNA damage occurs following PH (Gorla et al. 2001a). Manipulation of the basal rates of hepatocyte replication would be expected to increase polyploidisation, and this is evident in transgenic mice that over-express mitogens (Shiota et al. 1994;Webber et al. 1994), and in mice which are treated with exogenous thyroid hormone (Torres et al. 1999).

Polyploidisation would also be expected to increase in conditions where the rate of acquired DNA damage is increased, or where defects in DNA repair pathways occur. Polyploidisation increases on weaning(Brodsky et al. 1977), presumably due to an increase in the xenobiotic load, and following drug and toxin exposure (Bohm et al. 1981;Kato et al. 1996;Madra et al. 1995;Vindelov et al. 1983b). Increased levels of polyploidisation have also been recapitulated *in vitro* in response to oxidative injury (Gorla et al. 2001a). Conversely, polyploidisation is reduced following PH in transgenic mice expressing antioxidants (Nakatani 1997). Loss of the DNA repair gene, ERCC (excision repair cross-complementing rodent repair deficiency, complementation group 1), results in gross pleomorphism and polyploidisation in the murine liver (Chipchase et al. 2003;Nunez et al. 2000).

Hepatocytes of higher ploidy express markers of cell senescence and are less sensitive to mitogenic stimulation (Brodsky et al. 1977;Mossin et al. 1994). It has been suggested that transplanted hepatocytes of higher ploidy are less able to regenerate diseased liver than those of lower ploidy (Sigal et al. 1999), though this

has been disputed (Weglarz et al. 2000). Together these findings make polyploid hepatocytes unlikely progenitors in the development of carcinogenesis. Those hepatocytes of high ploidy which have been demonstrated in CAH (Toyoda et al. 2005), cirrhosis, and in the liver adjacent to HCCs, may simply be indicative of a preventative strategy designed to limit the consequences of increased hepatocyte turnover in an environment of continuous DNA damage. It may be then, that loss of polyploidisation is an important step in the development of HCC, in the same way that loss of apoptosis or deregulated proliferation are considered obligatory steps in the development of cancer (Hanahan et al. 2000). Loss of polyploidisation in the liver would lead to the emergence of diploid hepatocytes that are more sensitive to mitogenic signals and carry markers of DNA damage, which could represent advantageous mutations.

It is not obvious why, when regeneration occurs in the absence of Rb, as demonstrated in these experiments, there is a loss of polyploidisation, whereas previous work has demonstrated an increase in polyploidisation following Rb loss, both *in vitro* and *in vivo*. As already discussed, the increased proliferation seen following AdCre treatment might have been expected to increase rates of polyploidisation. The purported increase in genomic instability associated with Rb loss, may have been expected to have a similar effect, if indeed the mechanism of polyploidisation is protective. Rb is not required for polyploidisation, as polploidisation still occurs, albeit to a lesser degree in these experiments, so the effect seen here is likely to be an indirect effect of Rb loss, acting in concert with other factors through, as yet undetermined, pathways. The unique factor which may explain the difference between these results and previous work may be the presence of a powerful regenerative drive for hepatocyte proliferation that was not present in previous experiments. The protective process of polyploidisation may, therefore, be over ridden when a combination of factors, such as PH, and loss of tumour suppression function, are combined.

Supportive of the idea of the importance of the regenerative drive in combination with Rb loss in deregulating polyploidisation, is the observation that the fall in the

fraction of polyploid nuclei that occurred following PH in this study was greater than that following CCl4 treatment. This may be because regeneration is less synchronous following CCL4 treatment, and so the difference between groups at a particular time point is less obvious. It may also be because CCL4 treatment is considered to provide less of a regenerative drive than PH.

The effect of Rb loss in a pro-proliferative environment on the amelioration of the process of polyploidisation and the emergence of a diploid population is in keeping with a role for Rb in the development HCC as discussed in the first section. Loss of Rb in the environment of chronic inflammation may select for the development of diploid rather than polyploid hepatocytes. The pattern of HCC is characterized by the emergence of such diploid populations (Oriyama et al. 1998;Saeter et al. 1988a;Schwarze et al. 1984c), which have also been demonstrated in preneoplastic lesions (Saeter et al. 1988b). Similarly, transplantation of DEN-treated hepatocytes results in the development of diploid tumours in recipient mice (Saeter et al. 1989). It would be of interest to characterise the diploid cells that appear to dominate following Rb loss in the experiments presented here, and, in particular, to examine their neoplastic potential.

The pathways through which Rb loss may affect polyploidisation were not explored in these experiments. The effects of acute Rb loss are usually attributed to increased E2F activity (reviewed Mulligan & Jacks 1998). Although liver specific over-expression of E2F-1 does not have a significant effect on hepatocyte proliferation following hepatectomy (Conner et al. 2000), conditional expression of E2F-1 at supraphysiological levels is associated with a loss of polyploidisation *in vivo*(Conner et al. 2003). It may be that, in addition to their role in maintaining normal liver physiology, the absolute levels of E2F expression are important in determining the fate of hepatocytes in terms of polyploidisation. It is possible that there is a threshold of activity below which the process of polyploidisation is triggered, and above which polyploidisation fails. The level of such a threshold may then be modulated by the environment. Certainly the theory of a "threshold" limit for the proliferative effects of Rb loss in the mediation of apoptosis is well described (Trimarchi et al. 2002).

A second explanation for the increased fraction of diploid nuclei seen following Rb loss in a model of regeneration, is the that these nuclei may actually represent the expansion of a diploid cell population on which Rb loss has a greater effect than native hepatocytes. The preferential expansion of a trans-differentiated progenitor population is one possibility.

Although the existence of a progenitor population, derived from intra and extra hepatic "stem cells" has been described previously in the liver (Evarts et al. 1987;Sell 1990a), the extent to which such a population contributes to regeneration is a contentious issue, and an uneasy consensus exists at present that acknowledges the presence of such a population, but apportions it a limited contribution to regeneration in the normal liver (Fausto 2004a). The prevalence of this population is greatly increased in certain experimental systems where the replication of adult hepatocytes is inhibited (Sell 2001;Thorgeirsson 1996). It has also been suggested that the contribution of this cell population to the maintenance of liver function is increased in disease states (Crosby et al. 1998;Libbrecht et al. 2000;Roskams et al. 2003), and that this "stem cell" population may be the progenitor for the development of HCC, rather than the mature hepatocyte (Dumble et al. 2002;Sell et al. 1989;Sell 1993). It has not been determined in the experiments herein whether a significant progenitor population exists, or if Rb loss has a preferential effect on the expansion of this population compared to the mature hepatocyte. These would be very interesting questions to pursue, particularly in light of the stem cell theory of HCC (reviewed (Roskams 2006).

Role of Rb in a chemical model of carcinogenesis

Having determined a role of Rb in the regulation of liver regeneration and polyploidisation, a further aim of this thesis was to determine directly what role loss

of Rb may play on the development of HCC. Returning to the founding premise of this thesis, that the role of Rb as a regulator of cell cycle progression would be most obvious when hepatocytes were replicating, we designed a model to recapitulate the environment of chronic liver disease from which HCC develops. We achieved this using DEN as a DNA damaging agent, followed by repeated administration of CCl4 as a necrotic agent to produce a period of sustained hepatocyte proliferation. Using AdCre we then imposed on this environment acute Rb loss. Similar methods involving chemical carcinogenesis have been used to determine the effect of loss of other tumour suppressor genes on HCC development (Finnberg et al. 2004;Jensen et al. 2003;Lukas et al. 1999). In these experiments, Rb loss was associated with a distinct phenotype characterised by a number of histological changes in the liver, including the increased prevalence of mitotic figures, apoptotic bodies, and, most strikingly, the accelerated development of foci of preneoplastic hepatocytes. These foci were larger and more frequent in Rb deleted mice than in controls, and represented a significantly higher proportion of the liver cross sectional area. These preneoplastic foci were shown to have higher proliferative indices than surrounding tissue, and thus represent hyperplastic cell populations. Hyperplastic foci represent pre-malignant clones, and the frequency of such foci in human liver has been positively correlated with the multiplicity of HCA (Sugitani et al. 1998). Thus, loss of the regulatory function of pRb appears to accelerate the preneoplastic phase of hepatocarcinogenesis. These changes were demonstrable after the relatively short period of promotion of only 6 weeks.

The observations that Rb loss could be demonstrated in laser captured liver preneoplastic foci and in surrounding normal tissue, and that preneoplastic foci also occurred in control mice, demonstrates that Rb loss is not required for foci development in this model. DEN treatment causes indiscriminate DNA damage, and it is probable that the larger, more frequent foci demonstrated following AdCre treatment harbour additional mutations as well as loss of Rb. Together these observations make it unlikely that Rb loss alone is sufficient to initiate the development of HCC. The larger fraction of preneoplastic foci in AdCre treated animals clearly demonstrates Rb loss has a significant effect on the expansion of this

preneoplastic cell type, presumably through deregulated proliferation, and possibly through increased genomic instability, as discussed earlier. It would be of interest to further characterise these foci, looking in particular for further mutations, such as that of p53.

The experimental protocol for these carcinogenesis experiments was necessarily short so that the additive effect of p53 loss in addition to that of Rb could be assessed. It would obviously be of interest to repeat these experiments using a much longer protocol to determine whether the effect of Rb loss was associated with an actual increased tumour burden compared to controls.

The pattern of preneoplastic development in these livers was typical of previous descriptions of DEN-induced carcinogenesis, with foci made up of small diploid cells (Sell 1993;Vesselinovitch et al. 1978;Vesselinovitch et al. 1983). The shift between a polyploidizing growth pattern to a mitotic one is characteristic of hepatocarcinogenesis (Schwarze et al. 1984a), and it is attractive to hypothesise that the loss of polyploidisation seen in these regeneration experiments following Rb loss may play a role in switching between growth patterns consequently seen in the carcinogenesis model.

Intriguingly, there is also the possibility that the lineage of these foci of diploid cells is not that of the mature hepatocyte but that of a liver progenitor. The lineage of hepatocellular carcinoma remains undetermined and, as mentioned above, different animal models have suggested different candidates for the origin of HCC. As discussed in the second section of this discussion, the preferential expansion of a progenitor lineage could explain the emergence of a diploid population seen following regeneration. Whether clonal expansion could occur from this population to produce preneoplastic foci has not been determined, but it would be of interest to examine these foci more closely in an attempt to determine their lineage.

In terms of the relevance of these results to the development of HCC in the human, it has been demonstrated recently that the pattern of gene expression which occurs in murine liver tumours following DEN is similar to that of clinically aggressive human HCCs (Lee et al. 2004;Lee et al. 2005). This suggests the effect we demonstrate in these experiments of Rb loss in a model of DEN initiation, may be directly indicative of the potential effect of Rb loss in human tumours. Nevertheless, it would be of interest to determine the role of Rb in alternative non toxic models of HCC, such as the HBx transgenic mouse.

Increased apoptosis following Rb loss in a chemical model of carcinogenesis

Rb deletion in combination with a period of sustained liver damage in the carcinogenesis model induced higher levels of apoptosis in these experiments. Increased rates of apoptosis in parallel with accelerated development of preneoplastic foci have been demonstrated in human liver during the development of HCC (Park et al. 2001). Increased apoptosis has been shown to have a negative correlation with prognosis of HCC (Ito et al. 1999b). Elevated levels of apoptosis may be a consequence of the changes in the liver architecture that occur during the development of preneoplastic foci (Grasl-Kraupp et al. 1997). However, in our model, increased rates of apoptosis may be a result of Rb loss. Apoptosis is a characteristic feature of Rb loss in a number of different systems (Almasan et al. 1995;Clarke et al. 1992) and is believed to be mediated via p53 dependent and independent mechanisms leading to increased E2F activity. (Tsai et al. 1998). Increased apoptosis following loss of pRb appears to contradict the role of pRb as a tumour suppressor, and it has been suggested that loss of multiple apoptotic pathways must occur concomitantly for loss of pRb to be a significant event in oncogenesis (Sherr et al. 2002).

Loss of p53 in addition to that of Rb produces a characteristic phenotype

To determine whether there was a synergism between p53 loss and Rb loss in the development of HCC, the carcinogenesis model was repeated using p53 null mice that were treated with AdCre. The additional loss of Rb produced a phenotype which could be clearly distinguished from that due to Rb loss alone, with features of additional increased cell turnover, multiple abnormal mitoses, gross pleomorphism and a shift from foci of hyperplastic cells to a more diffuse pattern of abnormalities. Concurrent alterations in both p53 and Rb pathways have been demonstrated in a significant proportion of human HCCs, and tend to be more common in poorly differentiated tumours. (Edamoto et al. 2003).

Final Conclusion

In summary, the development of a liver specific conditional knockout has allowed a demonstration that loss of Rb deregulates the normally tightly controlled process of hepatocyte proliferation which occurs during liver regeneration. In addition Rb loss brings about a switch in the growth pattern of hepatocytes following regeneration with a loss of polyploidisation and the emergence of a diploid cell population. Deregulation of these two processes would be expected to contribute to hepatocarcinogenesis which has been demonstrated directly through a model of promotion of hepatocyte regeneration following Rb loss which accelerated the development of premalignant changes in the liver. These findings suggest Rb loss may be a critical event in the development of liver cancer.

Appendix

Solutions for flow Cytometry

Citrate Buffer pH7.6
85.5g Sucrose
11.76g Trisodium Citrate
50ml DMSO
Dissolved in 800ml dH20
pH adjusted to 7.6 and made up to 1 litre.

Stock Solution pH7.6

2g Trisodium Citrate (Sigma)
121mg Tris (Sigma)
1.044 Spermine tetrahydrochloride (Sigma)
2ml NonidetP40 (Sigma)
Dissolved in 1.8L dH20
pH adjusted to 7.6 and made up to 2 Litres with dH20

Vindelov A, pH 7.6
500ml Stock Solution
15mg Trypsin (Sigma)
(stored at -20°C and brought to room temperature)

Vindelov B, pH 7.6
500ml Stock Solution
250mg Trypsin Inhibitor (Sigma)
50mg RNaseA (Sigma)
(stored at -20°C and brought to room temperature)

Vindelov C, pH 7.6
500ml Stock Solution
208mg PI (Sigma)
500mg Spermine Tetrahydrochloride
(stored at -20°C and brought to room temperature, protected at all times from direct light

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