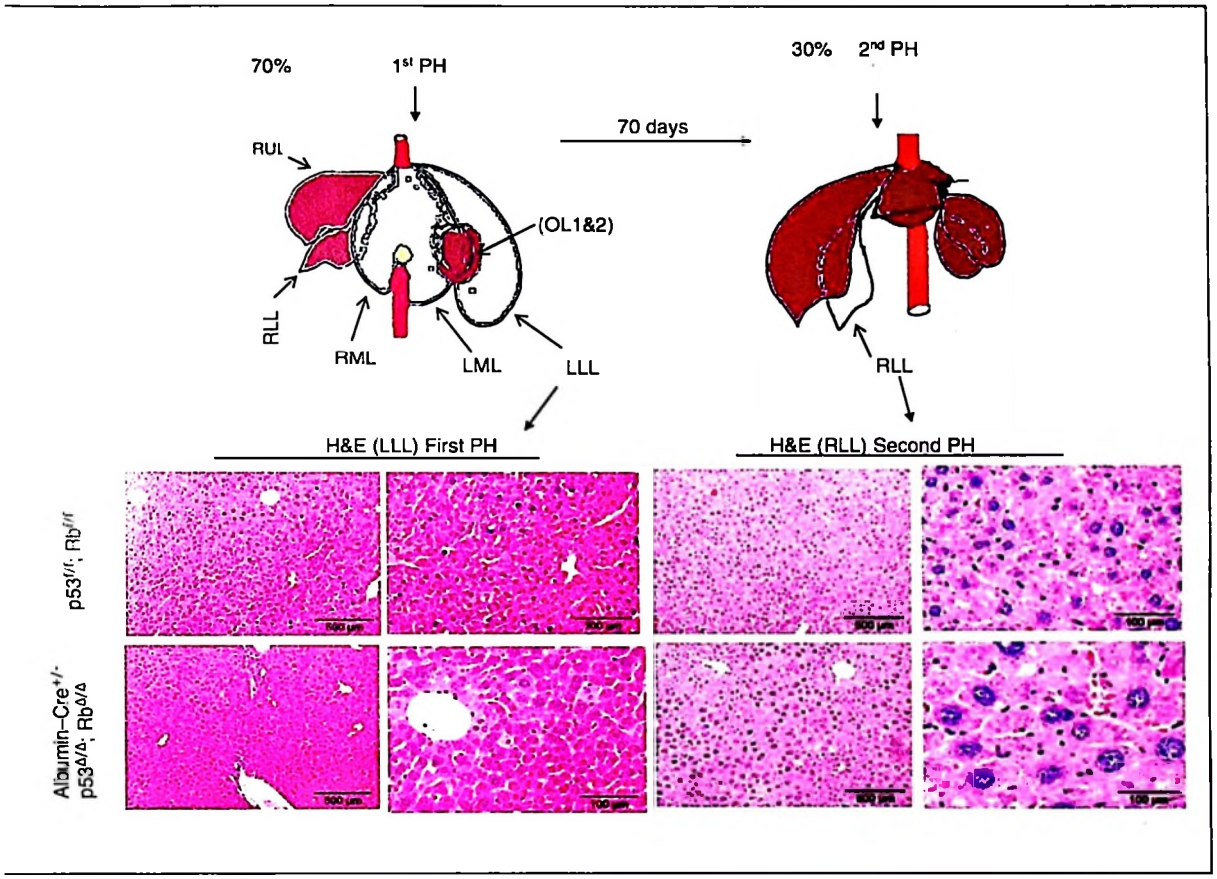
Role of p53 and Rb in liver cancer



Master of Science Thesis

in
Animal Pathology

Ramadhan B. Matondo

August 2008

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Dedication

To my family, who sacrificed their love, offered me unconditional support and stood by me throughout the course of this thesis.

Contents

CHAPTER 1

Introduction

Liver organization and function

Liver, the largest single organ in the body plays a central role in metabolism with a number of functions including, synthesis, storage and redistribution of carbohydrates, fats, and vitamins. Also, the liver produces large numbers of serum proteins including albumin, acute-phase proteins, enzymes and cofactors (1). Anatomically and functionally, the liver can be divided into hepatic lobules which are functional units and hexagonal in shape (Figure 1), (2).

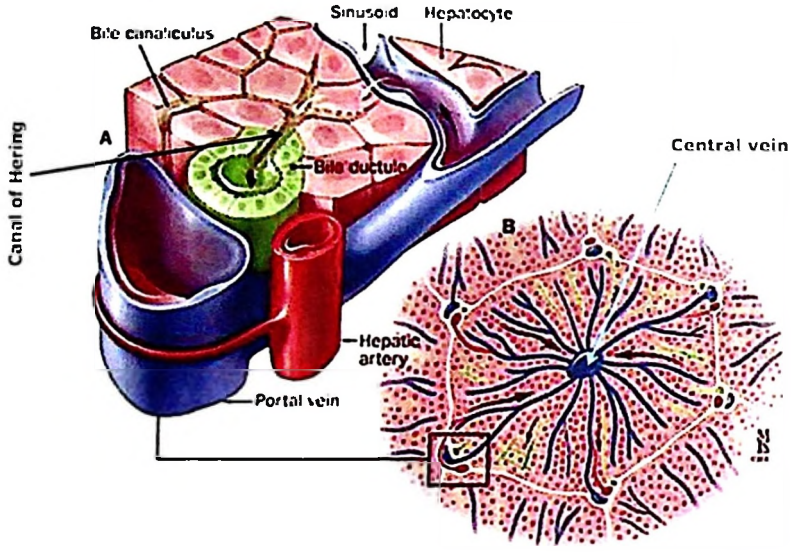

Figure 1.Liver organization structure. Magnified portal area (A), and Liver lobule (B).

Each lobule has a central vein (terminal hepatic venule), which is a tributary of hepatic vein at the centre, and portal area at the angles of the hexagon. Portal area contain bile ducts, branches of portal vein, the hepatic artery, nerves and lymph vessels, all supported by a collagenous stroma. The limiting plate forms the outer boundary of the

portal area and is made up of mainly fibrous connective tissues as well as fibroblasts. From this area, portal vein and hepatic artery project into the liver parenchyma.

The bile canaliculi, a modified portion of hepatocyte membranes in two adjacent hepatocytes forms lumen for bile secretion and these canaliculi collect bile from hepatocytes and drain into cholangioles (canal of Hering). Just outside the limiting plate, cholangioles converge into bile duct. The canal of Herring is lined with low cuboidal epithelial cells, where as bile duct are lined with high cuboidal epithelial cells. Within the canal of Herring, other cells considered to be hepatic progenitor also called oval cells in rodents, are found

Hepatocytes are the major cell type in the liver and are arranged in branching plates radiating from the central vein. Hepatic plates are separated by vascular sinusoids where blood from the terminal afferent branches of hepatic artery and portal vein mixes and flows to the central vein. The liver also has non-parenchyma cells which include endothelial cells, Kupffer cells, lymphocytes and stellate cells. The gap between endothelial cells and hepatocytes is called space of Disse. Within these spaces and between hepatocytes at the edge of space of Disse other cells called hepatic stellate cells, also known as Ito cells can be found. These cells at a resting state store vitamin A, and during liver regeneration these cells produces supporting tissues stroma to the newly formed hepatocytes and biliary cells. However, in case of massive hepatocyte necrosis and or chronic liver injury, these cells become a principle source of collagen type 1 contributing to liver fibrosis (3).

Liver regeneration

Adult hepatocytes normally do not undergo cell division but they maintain the ability to proliferate in response to toxic injury, infection, or surgical reduction of liver mass. This process is referred to as liver regeneration. This process is also referred to as compensatory liver growth as it involves replacement of the missing liver tissue to regain the original mass. A wide variety of genes are differentially expressed during liver regeneration. For convenience, these genes are divided into three categories: cytokines, growth factors, and genes with metabolic functions (4). Early responses to decrease in liver mass include induction of cytokines and growth factors, an event called 'priming'. During this phase liver mRNA and serum levels of tumour necrosis factor alpha (TNF-α) as well as interleukin-6 (IL-6) increases (5). Other genes activated during this phase includes nuclear factor-kappa B (NF-kB) and signal transducer and activator of transcription 3 (STAT3) (6,7).This was further confirmed by an observation that DNA replication is inhibited by anti-TNF antibodies (1), IL-6 and TNF receptor type I (Tnfr1) Knockout in mice (7, 8).

Primed hepatocytes and biliary cells, mobilizes energy and nutrients required for progression from resting state (G0) into the cell cycle (G1). Growth factors involved during the next phase called 'proliferative phase', includes epithelial growth factor (EGF), transforming growth factor α (TGFα), heparin binding EGF like growth factor (HB-EGF), growth hormone (GH), insulin growth factor binding protein 1 (IGFBP1) (9), thyroid hormone (T3) and Serotonin (5-hydroxytryptamine, 5-HT). While 5HT can play multiple roles as a neural transmitter, growth factor and as a hormone (10), T3 doesn't

have direct role in neural transmission but plays more supportive roles in cell proliferation by supporting cellular metabolism (11).

T3 activity, depends on the binding to a nuclear thyroid hormone receptors (TRs) (TRα and TRβ), and both receptors preferentially bind to 3, 5, 3'-triiodothyronine (T3). However, the main secretory product of the thyroid gland is 3, 5, 3', 5'-tetraiodothyronine or thyroxin (T4) which has relatively low binding affinity to TRs. The availability of receptor-active T3 and thyroid hormone activity, depends on (mono) de-iodination, where one iodine is removed from the outer ring, the process known as outer ring de-iodination (ORD) or from the inner ring, inner ring de-iodination (IRD) of a iodothyronine molecule. The de-iodination process can lead to activation as well as inactivation of thyroid hormones. Outer ring de-iodination of T4 is the only way to produce active T3 and therefore ORD is important as an activating pathway. However, Inner ring de-iodination of T4 and T3 can only lead to inactive iodothyronines, namely reverse T3 (rT3), and 3, 3'-diiodothyronine (T2) respectively. Therefore, IRD is exclusively an inactivating pathway. The enzymes catalysing de-iodination of thyroid hormones have first been described in rats. So far, three types have been identified: iodothyronine de-iodinase type I (D1), type II (D2) and type III (D3). Type I is a multifunctional enzyme that catalyses both ORD and IRD. Its preferred substrate is rT3 (above T4 and T3). Type II exclusively catalyses ORD, with a substrate preference for T4 over rT3. Type III is a pure IRD enzyme with a substrate preference for T3 over T4. Expression of these enzymes is tightly regulated depending on the metabolic status and requirements of the cell (12).

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5-HT actions on cellular activities are mediated through receptors which belong to the group of G-protein coupled receptors including 5-HT2A and B which are important for liver regeneration through activation of the Akt pathway (13, 14-15).The action of serotonin is potentiated with other growth factors (16). The main site of serotonin synthesis in the body is the enterochromaffin cells located in the gastro intestinal epithelial cells and synthesized serotonin is stored mainly in platelets by serotonin transporter (SERT) re-uptake mechanism. Lack of SERT, abrogates the ability of platelets to store serotonin (17), serotonin induced exocytosis, and consequently impairs cell cycle progression (Figure 2) (18).

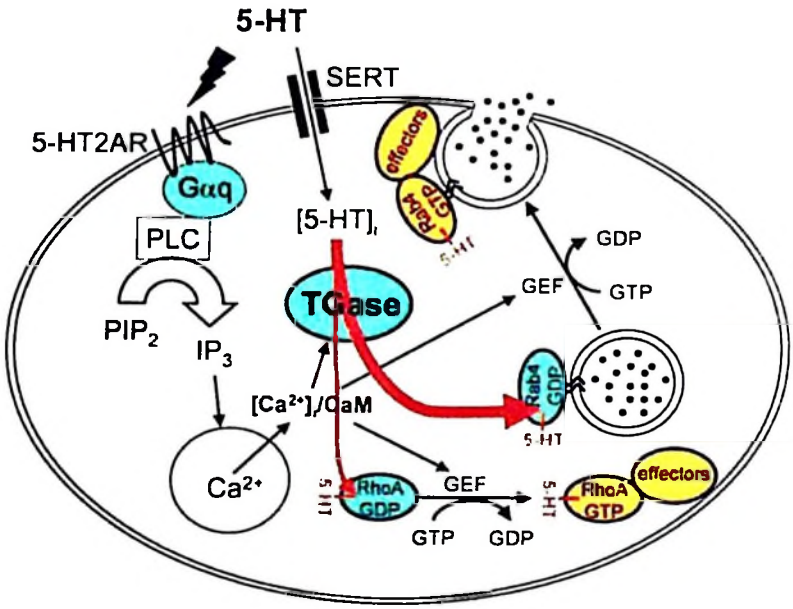

Fig. 2. 5-HT Induced exocytosis of platelet α-granules.

Both 5HT (15) and T3 lead to induction of cyclin D1. For example, studies have shown that treatment of rats with T3 caused an increase in cyclin D1 mRNA and protein levels (19). D-type cyclins forms complex with cdk4 or cdk6 to drive the cells into G1 phase by

phosphorylating the Retinoblastoma (Rb)–HDAC repressor complex which is normally bound to the E2F–DP1 transcription factors. Phosphorylation of Rb by cdk4/6 and cdk2 dissociates the Rb from the repressor complex, leading to the release of bound E2F from Rb. Freed E2F drive the transcription of S-phase genes encoding for proteins that switch the cell status from G1 to S phase for DNA replication (20).

Breakdown of extracellular matrices accomplished by metalloproteinases from pericytes in response to hepatocytes growth factor (HGF) stimulation, allows proliferating hepatocyes to form avascular clusters. Stellate and endothelial cells proliferate later than hepatocytes and also form clusters adjacent to hepatocyte. Later hepatic stellate cells produce extracellular matrix and with stimulation of TGFβ and hypoxia, cell proliferation is terminated. Up regulation of Hypoxia induced factor (HIF-1), vascular endothelial factor (VEGF), and basic fibroblast growth factor (b-FGF), stimulate establishment of microcirculation and angiogenesis including proliferation and migration of endothelial cells from neighbouring vessels and recruitment of endothelial precursor cells (EPC) from bone marrow (9).

Rodent models of liver regeneration.

Rodent model of liver regeneration dates back to 1931 when Higgins and Anderson demonstrated that, the rat can survive 2/3 partial hepatectomy (21).Since then this techniques involving removal 2/3 of the total liver have been improved and changed in different ways reflecting change in knowledge, technology and to suit various research needs (22-23). It is known that, after removal of 60-70% of the liver, the liver can grow back to its original mass within two weeks in mice (24). This ability of the liver to

regenerate, not only have turned out to be a useful tool of studying molecular mechanisms underlying hepatocyte proliferation, aging and hepatic diseases but also offer a promising tool to study the possibility of gene therapy to treat liver diseases such fulminant liver failure and liver cancer. Different approaches have been used to study genes involved in liver regeneration and or diseases. Among the methods used to determine whether a particular gene of interest is essential for liver regeneration is either to modify its expression by over-expressing the gene in transgenic mice, functional inactivation or deletion (23). With advance in knowledge and technology, it is nowadays possible to perform a targeted gene deletion or inactivation in specific organ of interest including the liver. In the liver, one of the methods used to achieve liver specific gene deletion is by using a cre recombinase enzyme mediated excision at lox P sites of the target gene(s) and a stop codon up stream of the lacZ reporter gene, driven by albumin promoter (25). LacZ reporter gene present in Rosa 26 locus (R26) and encoding β-galactosidase is exclusively expressed after the excision of a lox P-flanked (floxed) stop codon. β-galactosidase, is an enzyme which specifically cleave 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal) to produce galactose and 5-bromo-4-chloro-3-hydroxyindole. With immunohistochemical staining techniques, x-gal can be oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, giving the tissue expressing Cre and Lac Z gene a blue colour. The tissue expressing LacZ reporter indicates successful excision and recombination of lox P sites as well as deletion of the target gens. This type of gene deletion can be organ specific, and is known as conditional gene deletion. In this thesis, two approaches have been used, whereby rats with germ line deletion of SERT and mice with liver specific conditional deletion of p53 and Rb were used to study liver

regeneration, and the role of tissue repair in liver carcinogenesis respectively (Chapter 2 and 3).

Role of p53, pRb and liver regeneration in cancer

The cell cycle can be divided in four growth (G) phases, namely resting (G0), preparation for chromosome duplication (G1), chromosome duplication (S), pre-mitotic phase (G2), and mitotic (M) phases. G1/S restriction point is a checkpoint which DNA is checked for errors and repaired before duplication, whereas G2/M check point checks for any damage or duplication errors before progression to mitotic phase. These check points are regulated by Retinoblastoma (Rb) and protein 53 (p53) (26). Rb play role in inducing cell cycle arrest in an event of DNA damage to facilitate repair, as well as arresting proliferation to maintain tissue homeostasis. Exposure of mouse fibroblasts which are Rb-proficient to DNA damaging agents, induce cell cycle arrest at 4N DNA content, where as Rb-deficient cells by-pass this mitotic block and accumulate higher ploidy (26-27). On the other hand, upon DNA damage, p53 can directly induce cell cycle arrest through induction of cdk inhibitor $p21^{CIPI}$, and dephosphorylation of Rb or eliminate the damaged cells by p53 mediated apoptosis (26, 28). This indicates that, p53 and Rb work synergesticaly, and cells lacking one or both of these proteins are likely to by-pass cell cycle control mechanism, and are more prone to carcinogenesis. However, the integrity and function of these proteins depends on other protein networks (Figure 3) (26). For example, the Cdk2 inhibitor, $p27^{kip1}$(CDKN1B) is expressed at high levels in quiescent cells and suppress expression of Cyclin E-Cdk 2 activity important for progression of the cell cycle after entry into G1 phase.

Mitogenic stimulation which induces Cyclin D1 expression regulates its assembly with Cdk4 thereby sequestering $p27^{kip1}$ and activates Cyclin E-Cdk2 complex. $P27^{kip1}$ is then phosphorylated by Cyclin E-Cdk2 in late G1 leading to its degradation to allow S-phase entry. Other players in regulation of p53, Rb-E2F pathways are the INK4 family of proteins which includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$. Mice lacking $p16^{INK4a}$ are tumour prone and develop wide spectrum of cancers by continuous activation of Cyclin D-Cdk4/Cyclin E-Cdk2 complexes. The INK4a locus encodes a gene product, in part from an alternative reading frame (ARF) that overlaps sequence encoding $p16^{INK4a}$.The ARF protein in mouse ($p19^{ARF}$) is a potent activator of p53 by cancelling the effect of murine double minute (mdm2), a negative regulator of p53. Inhibition of mdm2 can lead to either stimulation of apoptotic signals or p53 mediated induction of Cdk inhibitors, and Rb dependent cell cycle arrest to allow DNA repair before progression to next phase of cell cycle, Figure (5)(26), (26-30).

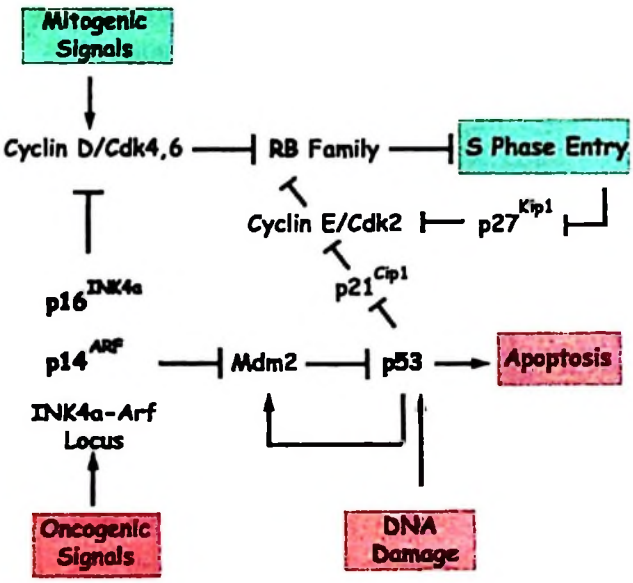

Figure 3. Rb and p53 Regulate Cell Cycle Checkpoint Controls

Development and progression of liver cancer is a mult-step process, and involves complex mechanisms. Hepatocellular carcinoma (HCC) is among the important primary liver cancers, which has been linked to various aetiologies, and most of them involves either inactivation of tumour suppressor genes, and or inducing chronic cycles of necrosis and regeneration. (Figure 4) (31), (32-35). Involvement of hepatocyte proliferation in hepato-carcinogenesis has been linked to telomere attrition in absence of cell cycle check points, the situation which contributes to ploidy and accumulation of mutations (36).

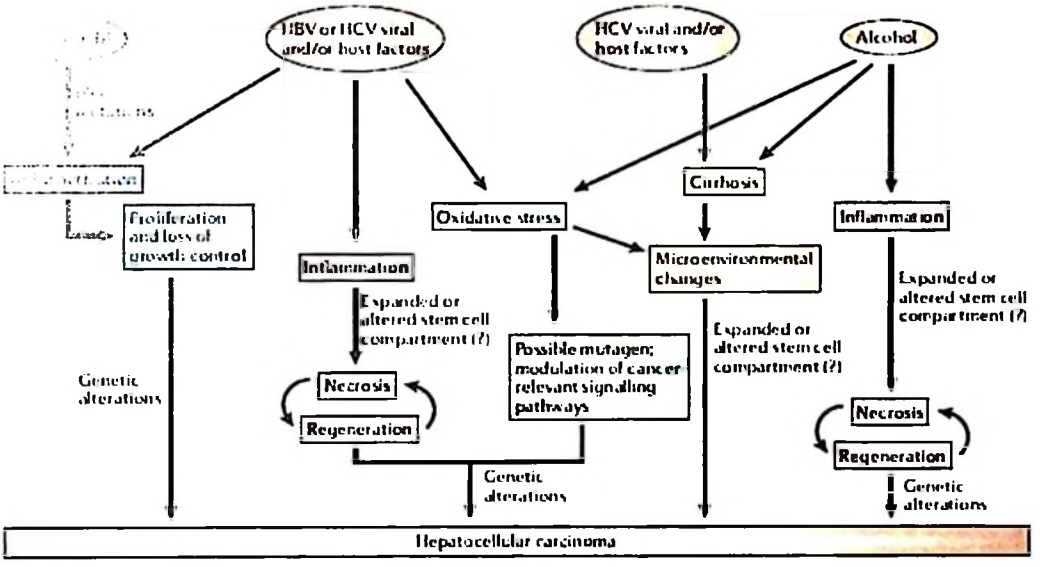

Figure. 4. Mechanisms of hepatocarcinogenesis. The suspected mechanisms of hepatocarcinogenesis for the various risk factors are shown. Commonalities are indicated using the same colour. In addition to these mechanisms, hepatitis B virus (HBV) and aflatoxin B1 share the characteristic of affecting the genome.HBV and HBC can integrate into the host genome and Aflatoxin B1 is a mutagen.

Although hepatocyte proliferation plays part in carcinogenesis, the mechanism and condition under which liver regeneration contributes to HCC remains a subject to be investigated .In this thesis the, the ability of liver regeneration to induce liver cancer in absence of chronic liver diseases or chemical induced DNA damage have been investigated (Chapter 2)

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CHAPTER 2

Tissue repair induces cancer in p53 and Rb deficient livers

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*Equal contribution

Manuscript in preperation

ABSTRACT

Hepatocellular carcinomas are often associated with mutations of the tumor suppressor genes *p53* and *Retinoblastoma* (*Rb*). To determine whether mutations of *p53* and *Rb* can cause liver cancer, we deleted both genes specifically in the liver of mice. Interestingly, simultaneously ablation of p53 and Rb in the liver was insufficient to induce liver cancer, indicating that besides inactivating these tumor suppressors other factors are required for cancer initiation. We therefore performed two subsequent partial hepatectomies to induce liver regeneration in these mice. Undifferentiated hepatocellular carcinomas in p53/Rb deficient livers were observed with high incidence (>60%) 4-5 weeks after the second hepatectomy. Strikingly, all tumours occurred at the same anatomic location, namely within the remaining liver stump of the first hepatectomy. Some tumours metastasize to regional lymph nodes and surrounding tissues and histologically the neoplastic cells resembled oval cells, the progenitor cells of hepatocytes and bile duct cells. Surrounding non neoplastic liver regions show marked oval cell proliferation particularly adjacent to the portal triads. Expression analysis of tumour tissue revealed increase expression of alpha-fetoprotein, an oval cell marker, as well as the transcription factor E2F1, a downstream partner of Rb, which is often upregulated in human hepatocellular carcinomas. Our findings demonstrate that livers deficient of p53 and Rb induces oval cell proliferation and undifferentiated hepatcellular carcinomas upon partial hepatectomy.

Introduction

Hepatocellular carcinoma (HCC) is a primary cancer of the liver reported worldwide in human population, with differences in distribution, and incidences depending on prevalence of associated risk factors (1, 2).

Various aetiologies have been linked to HCC development, the most prominent of which include chronic hepatitis B (HBV) and C (HCV) viral infections (3-5), chronic alcohol consumption, and aflatoxin-B1-contaminated food. Most of these aetiologies are capable of inducing chronic hepatitis, with repeated rounds of necrosis and regeneration, as well as liver cirrhosis.

Aflatoxin-B1 induced hepatocarcinogenesis is mostly associated with carcinogenic mutations (2, 6). Apart from causing chronic inflammatory reaction, HCV are capable of evading host immune response by interacting with various factors involved in the process such as tumour necrosis factor alpha (TNFα), and Interferon alpha (INF α). Furthermore, HCV viral proteins, NS3 and NS4A can use their proteases to cleave components of immune response (2, 7).

In addition to the risk factors described above, various genetic events have been linked with the development of HCC (8, 9). Notably, protein 53 (p53), and Retinoblastoma protein (Rb) tumour suppressor genes are either functionally inactivated, or deleted in higher percentages of HCC. For example, oncoprotein gankyrin can target and inactivate both p53 and Rb (10). In addition to that, there are reports indicating that, HBV (4) and HCV (11) viral proteins, HBx and NS5 respectively, can bind and inactivate p53 function whereas fungal toxin aflatoxin B1 is associated with increased mutation in

p53 codon 249 (2,9,12). Other frequent observations in majority of liver developing HCC include increased ploidy (9-15), chromosome segregation defects (16), and telomere attrition (17, 18).

The reason as to why p53 and Rb are targets of functional inactivation or deletion is most likely linked to their role in cell cycle control check points. Rb plays a critical role in cellular proliferation control and inhibition of oncogenic transformation (15), by encoding a nuclear phosphorylation protein (pRb) that negatively regulates G1-S phase of the cell cycle through formation of a repressor complex with other proteins to inhibit E2F transcription factors, and therefore inhibit E2F responsive genes, including those important for cell cycle progression (14,18-20).

This role in relation to cancer development is particularly important where cell cycle arrest is required in an event of DNA damage to allow repair to take place. This argument is supported by report that disruption of Rb accelerates G2–M progression in the presence of DNA damage by elevating the expression of a set of mitotic regulatory genes (21-22). On the other hand, p53 act as a break, it is activated in response to DNA damage to induce cell cycle arrest by induction of Cyclin dependent kinase inhibitor $p21^{cip1}$ to allow DNA repair. Irreparable cells are eliminated by induction of p53 dependent apoptotic pathway. Loss of p53 control allows cells to escape p53 dependent cell cycle arrest and apoptosis (22-24). Rb and p53 cell cycle control can also be deregulated by lack of functional $p16^{INK4a}$ and or $p19^{ARF}$. Lack of functional $p16^{INK4a}$ deregulates cell cycle control by causing persistent activation of cyclins, leading to unregulated Rb phosphorylation, whereas loss of $p19^{ARF}$ lead to induction of murine double minute protein (mdm), a negative regulator of p53 (13, 25-26). Increased

expression of mdm causes increased p53 export from nucleus and becomes a target for ubiquitin proteasome degradation pathway, and promotes carcinogenesis (26-27). These examples can serve to indicate the central role played by p53 and Rb tumour suppressors in the cell cycle control, and why many oncogenic processes target p53/Rb pathways.

The link of liver regeneration in HCC development is based on among others, the observations that many HCC are associated with repeated rounds of hepatocyte necrosis and regeneration caused by chronic liver damage (2-6), and recurrence of colorectal liver carcinoma in patients after surgical resection (28). It has been shown that recurrence of colorectal liver carcinoma occurs during the late phase of liver regeneration after partial hepatectomy (29), coinciding with cytokines mediated regulation of re-establishment of liver structures in the newly regenerated liver tissues. This cytokine network includes transforming growth factor-β (TGF-β), vascular endocelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), hypoxia inducible factor-1 alpha (HIF1α), and the enzyme matrix metalloproteinases (MMPs). They together regulate modelling of hepatic parenchyma, extracellular matrix formation, angiogenesis and vasculogenesis (29-30). Despite of this advance in knowledge, the mechanism involving liver regeneration in the liver carcinogenesis is poorly understood. Moreover, mice with conditional knock out of p53 or Rb do not result in liver cancer (2, 15). To determine whether liver regeneration can promote development of HCC, we have performed two rounds of partial hepatectomy (PH) in mice with conditional deletion of p53 and or Rb in the liver. The choice of conditional knockout system was stemmed on previous reports, that mice with germ line deletion of p53 have short life span due to

development of thymic lymphoma and sarcoma. Similarly, mice with germ line deletion of Rb die at early embryonic stage (31). In order to avoid complications associated with germ line deletion of these proteins, we decided to use mice where p53, and or Rb have been deleted conditionally in the liver.

Materials and methods

Genotyping

Was done to confirm the presence of the desired transgenes using genomic DNA extracted from ear tissues obtained during ear notching (Figure 1B). Primer sequences and conditions are as follows: *Cre*$^{+/-}$ alleles (260 bp), forward primer: 5'-ATG CTT CTG TCC GTT TGC CG-3', reverse primer: 5'-CCT GTT TTG CAC GTT CAC CG-3', PCR parameters were 94 ^{0}C for 3minutes, 33 cycles of 94 ^{0}C for 30 seconds, 55 ^{0}C for 30 seconds, and 72 ^{0}C for 45 seconds followed by 72 ^{0}C for 3minutes and hold at 7 ^{0} C. For p53$^{f/f}$ band (584 bp); forward primer, 5'–AAG GGG TAT GAG GGA CAA GG-3', reverse 5'–GAA GAC AGA AAA GGG GAG GG-3', with parameters; 95 ^{0}C for 3minutes, 45 cycles of 94 ^{0}C for 30 seconds, 58 ^{0}C for 30 seconds, and 72 ^{0}C for 1 minute followed by 72 ^{0}C for 3minutes and hold at 7 ^{0} C. For Rb$^{f/f}$ alleles (283 bp); Rb exon 18; 5'–GGC GTG TGC CAT CAA TG -3', Rb exon 19; 5'–CTC AAG AGC TCA GAC TCA TGG-3' with parameters; 95 ^{0}C for 3minutes, 45 cycles of 94 ^{0}C for 30 seconds, 57 ^{0}C for 30 seconds, and 72 ^{0}C for 50 seconds followed by 72 ^{0}C for 3minutes and hold at 7 ^{0} C. For LacZ reporter (R26$^{f/f}$), 260 bp, a combination of three primers was used; IMR315: 5'-GCG AAG AGT TTG TCC TCA ACC-3' , IMR316: 5'-GGA GCG GGA GAA ATG GAT AT-3', and IMR883: 5'-AAA GTC GCT CTG AGT TGT TAT-3' with protocol of 94 ^{0}C for

3minutes, 35 cycles of 94 ^{0}C for 30 seconds, 60 ^{0}C for 30 seconds, and 72 ^{0}C for 1 minute followed by 72 ^{0}C for 3minutes and hold at 7 ^{0}C

Partial hepatectomy (PH)

Mice with age ranging between 9.5 to 14.5 weeks underwent two times PH at an interval of 10 weeks. For first PH 60-70% of the liver was removed, LLL, RUL, and LUL liver lobes (Figure 2A). Preparations, induction and maintenance for anaesthesia were done as previously described (32), with oxygen flow rate of 0.4L/min, and air 0.3L/min. All mice were injected with 0.01mg Buprenophine hydrochloride per 100grams of body weight before starting surgery and at most 24 hours after surgery. Surgical techniques for first PH involved midline incision of skin approximately 2-3 centimetres from xiphoid region (2_SI.Fig 1A) followed by muscles and peritoneum along the linear alba. The ligament which connects the RUL, LUL, gall bladder and diaphragm was dissected as previously described (32). To externalize the RUL, LUL and LLL, gentle pressure was applied above the xiphoid region with the middle fingers facing each other from either directions pressing downwards at the junction of sternum and xiphoid cartilage, with the thumbs gently pressing inwards and backwards from lateral sides to allow the LLL to be externalized at the posterior end of the incision (2_S1. Fig 1B&C) with correct incision size, allows the three liver lobes (RUL, LUL and LLL) to be exteriolized while keeping other lobes and viscera inside the abdominal cavity (2_SI.Fig.1C). The exteriolized liver lobes were laid on wet sterile gauze soaked in sterile physiological saline (2_SI.Fig.1D). The ligament located on the visceral surface of the left lower lobe was severed to free the lobe from other connections (2_SI.Fig.1E). This lobe was then ligated and resected (2_SI.Fig.1F & G). The median lobes were ligated with one ligature at the common

pedicle and resected (2_SI.Fig.1H). Redundant tissues and blood clots were removed as previously described (32). Peritoneum, abdominal muscles and subcutaneous were all closed in one layer using Polyglactin 910 (Vicryl, ® suture size 4-0, Johnson Johnson, Belgium) and finally the skin was closed using same type of suture. During the second PH, approximately 30% was removed involving the RLL. After midline incision, the intestines were exteriolized to create enough space and access to the RLL. Exteriolized intestines were protected from drying up with sterile gauze soaked in sterile physiological saline. To prevent fat tissues, prostate glands or urinary bladder from blocking access to the lobe, a small piece of sterile gauze, soaked in saline was gently inserted in the space between the liver lobe and these organs. This also served to absorb blood coming from resected liver lobe. Then a small ligament connecting the base of RLL and hepatic artery underneath was cut to allow better positioning of the ligature and prevent vascular damage during ligation and resection. After ligation and lobectomy of RLL, the abdomen was cleaned, and the intestines were returned and finally the incisions were closed in a similar manner as described in first PH. We did not use the microscope during these surgical procedures.

Determination of of LacZ reporter gene expression by X-gal staining

Liver samples for x-gal staining were embeded in Tissue-Tek OCT compound, briefly immersed in liquid nitrogen cooled isopentane and then temporariry stored in liquid nitrogen, before freezing in -80 °C. Staining to detect Lac Z expression was done on freshly cut frozen sections 5µm thick, at -20 °C, and staining for x-gal was performed as described in (33).

Routine and Immunohistochemical staining

Liver samples harvested during first and second PH, as well as from sacrificed mice, were fixed in 10% neutral buffered formalin for 24 hours, paraffin embedded, and cut into 5-µm sections for immunohistochemistry, routine H&E and Masson's trichrome staining. Staining for Ki67 was done using Envision method for Rabbit anti-Ki67; clone SP6 for paraffin sections (Lab vision, RM-9106-S0) as previously described (34). Antibody dilution was 1:50 for anti-ki67 in phosphate buffered saline (PBS).

Quantitative real-time PCR

Total RNA was isolated using Qiagen RNAeasy kit for fibrous tissues, and the protocol for muscle tissues according to the manufacture's recommendation. Reverse transcription of total RNA was performed using Superscript III reverse transcriptase (Invitrogen) and RNAse Inhibitor (Roche) according to the manufacturer's protocol. Quantitative real-time PCR was performed to determine expression level of E2F1-8 and α-fetoprotein using a BioRad iCycler, and reactions were performed in triplicate as previously described (35). Relative expression was normalized to GAPDH. A complete list of primers is provided as supplementary information (SI.Table 2).

Histograms and statistical analysis

Data in histograms are presented as mean ± standard deviation, and all histograms were prepared by using Microsoft exel®. Subsequent statistical analysis was performed by using a free software (R 2.7.0) package downloaded from http://cran.r-project.org/. Survival curve comparing occurrence of cancer between groups was determined by using Cox's proportional hazard analysis method, and statistical difference was

determined by using log rank test, the differences were considered significant when $p < 0.05$, at 95% confidence interval.

Results

Generation of liver specific Rb and p53 deficient knockout mice.

To generate conditional p53 and Rb knockouts in the liver, mice harbouring floxed alleles of p53 at exons 2-10, floxed alleles of Rb at exon 19 and a floxed stop codon upstream of the Lac Z reporter (Rosa 26), were crossed with mice expressing Cre recombinase under the control of Albumin promoter, which is specifically expressed in the liver (Figure 1 A). The Cre recombinase enzyme specifically recognizes and cleaves floxed alleles at lox P sites (36) (Figure 1B).

Nuclear size of hepatocytes increase in .Albumin-Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$ mice

To study the effect of p53 and Rb ablation in cell cycle regulation in the liver of adult mice after PH, we analysed hepatocytes morphology on H&E stained liver sections in all mice in each stage of partial hepatectomy (PH). Right median (RML), left median (LML), and left lower (LLL) liver lobes removed during first PH showed nuclear hypertrophy in p53 and Rb deficient (*Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$*) compared to p53 and Rb competent (*p53$^{f/f}$; Rb$^{f/f}$*) *mice*, (Figure 2A). 10 weeks later, right lower lobe (RLL) removed during second PH, showed nuclear hypertrophy also in *p53$^{f/f}$; Rb$^{f/f}$* , though not as large as in *Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$* (Figure 2A).

Liver regeneration and conditional deletion of p53 and Rb, leads to liver cancer

Between 70 and 158 days after first PH, neoplastic lesions developed in compound mutants: Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{f/\Delta}$, and Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$ but not in p53$^{f/f}$; Rb$^{f/f}$ mice. Interestingly, all neoplasia developed on the same location, at the site of

ligation, on the residual median lobe (Figure 3A). Most neoplasia were observed between 4-5 weeks after second PH, the earliest was observed 28 days after second PH, or (98 days) from first PH in Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$, whereas in Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{f/\Delta}$ the earliest lesion developed 42 days after second PH, or (112days) from first PH (Figure 3B). (3/12) of the observed neoplastic lesions in Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$ had metastases to the diaphragm, omentum, and gastro-intestinal lymphoid tissues. In addition to icterus observed in all mice with neoplasia, mice with metastasis also developed ascites with amount of fluid ranging from 3 to 3.5mL in the abdominal cavity. Total incidence of liver cancer during the duration 158 days counted from the day these mice underwent first PH in mice were (0/19), (3/8), and (12/19) for p53$^{f/f}$; Rb$^{f/f}$, Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{f/\Delta}$, and Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$ respectively (Table 1),.

Neoplastic lesions shows wide spectra of phenotypes

Neoplastic lesions observed in both Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{\Delta/\Delta}$ and Albumin–Cre$^{+/-}$; p53$^{\Delta/\Delta}$; Rb$^{f/\Delta}$ mice, they all show features ranging from well differentiated hepatocellular carcinoma (Figure 4A, right panel) to poorly differentiated hepatocellular carcinoma (Figure 4A middle panel). The lesions also display various morphological features including spindle shaped cells. These spindle shaped and other neoplastic cells does not react to Masson's tri-chrome staining for collagen, only the surrounding stroma, was labelled by this staining (Figure 4B,). In addition, some parts of the neoplastic lesion and surrounding liver lobes show features of cholangiolocellular carcinoma, which includes small cords resembling cholangioles and ductular reaction-like anastomosing glands in abundant fibrous stroma (figure 4B left panel, and D).

Furthermore cholangiocytes and hepatocytes in neoplastic lesion and surrounding liver lobes, express Lac Z reporter gene (Figure 4C).

Ablation of p53 and Rb deregulates E2Fs and elevate cell cycle progression in the liver of adult mice, neoplastic tissue and surrounding liver lobes

The effect of somatic Rb ablation in the liver has been described. Rb ablation results in E2F target gene deregulation and elevated cell cycle progression during postnatal growth, but compensation eventually occurs such that the expression of E2F targets is repressed and hepatocytes becomes quiescent in adult livers independent of Rb genes (15). We show that, liver removed during first PH and subsequent PH from Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$ mice aged between 9.5 -14 weeks , have elevated E2Fs mRNA level compared to their age mates with $p53^{f/f}$;$Rb^{f/f}$ genotype (Figure 5A-H). In addition these mice also show elevated α-fetoprotein (Figure 5I), and cell proliferation as indicated by ki67 staining (Figure 6A). Liver samples collected from neoplastic tissue as well as surrounding liver lobes, also shows elevated E2Fs, α-fetoprotein and proliferation, but proliferation in the surrounding liver lobes is mainly confined to the portal area (Figure 5A-I, and 6B).

Discussions

Liver regeneration models of hepato-carcinogenesis

Cycles of hepatocyte necrosis, and regeneration leading to fibrosis, and cirrhotic micro environment incited by chronic liver injury, and or toxicosis is known to promote hepato-carcinogenesis (1-6, and 37-38). Rodent models supporting this pathogenesis are available. These models are consistent with large percentage of HCC which also share the same pathogenesis, involving cirrhotic stage and or fibrosis. However, the occurrence of cancer in normal liver, or recurrence of HCC in patients after surgical

resection, does not fit well in these models as this type of cancer does not necessarily involve liver fibrosis and or liver cirrhosis during pre-cancerous stage. In addition, use of chemical agents may produce additional un- specified mutations not known to the researcher, further complicating the interpretation of results in liver toxicosis-PH models. To circumvent this complexity, we did not include additional treatment to the mice after PH during this liver regeneration-carcinogenesis study. To our knowledge liver specific p53/Rbnull-PH approach in studying liver carcinogenesis has never been reported.

Liver specific loss of p53 and Rb deregulates E2F transcription and cell proliferation

Because of known evidences indicating existence of synergism between p53 and Rb in suppressing carcinogenesis (39-40), in our study, we focussed on mice with conditional ablation of p53 and Rb in the liver. The role of p53 and Rb in carcinogenesis has been reviewed (21-27), and conditional ablation Rb in the liver does not upset E2F regulation and cell cycle control in adult mice, due to compensatory mechanisms by other Rb family of pocket proteins p107, and p130 (15). Mouse embryonic fibroblast cells (MEFs) deficient of activator E2F1-3 and $p21^{CIPI}$ could pass through G1/S transition, but not G2/M transition and deletion of p53 in these cells restored their ability to pass through G2/M, suggesting E2Fs mediated regulation of cdk activity through a $p21^{CIPI}$ mediated negative feedback loop. Eventually, hypo-phosphorylation of Rb family of pocket proteins occurs, leading to E2F target repressions (35, 43-44). Consistent with these reports, and as reported elsewhere in hepatocytes primary culture (45), we show that conditional deletion of both p53 and Rb in the liver, lead to increased nuclear size, ploidy, E2Fs transcripts as well as cell proliferation in adult mice (Figure 2B), (Figure 5A-H, black bars), and (Figure 6A).

Liver regeneration, conditional ablation of p53, and Rb leads to liver cancer

To investigate whether the observed cell cycle derangement at first PH will lead to hepato-carcinogenesis by inducing another round of cell proliferation, we performed second PH 70 days later. As in first PH, we observed the same pattern of high level of E2F transcripts in Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$ and Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{f/\Delta}$ mice compared to level observed in $p53^{f/f}$; $Rb^{f/f}$ at first PH (Figure 5A-I, grey bars).

It is known that, over-expression of E2F1 in murine liver results into centrilobular nuclear hyperchromasia, hepatocytes with multiple prominent nucleoli and hepato-carcinogenesis. In addition, they show small round cells which always appeared in close proximity to portal region (46). Consistent with this report, we show persistence high levels of activating E2F1-3a and portal cellular proliferation (5A, 6A and 6B, left lower panel).

It is also considered that, liver regeneration after PH involves replication of mature hepatocyets and biliary cells (37, 47). However, in an event of massive liver loss or limited ability for hepatocytes and biliary cells to take part in regeneration process, and bi-potential oval cells are activated which have the ability to differentiate into hepatocytes and biliary cells. Alternatively, in case of insufficient or failure of oval cells to respond, multi-potent bone marrow stem cells are activated, which can differentiate into hepatocytes, biliary cells and other liver cells (48).

Therefore, it is most likely that, the regional proliferation observed may be a consequence of the limited capacity of hepatocytes and biliary cells to replicate as indicated in (Figure 6A, arrow head) and hence, the activation of oval cells located in the canal of Herring. This is supported by the observation that, neoplastic lesion and

surrounding liver lobes, show increased proliferation of small round shaped cells in portal region, migrating into hepatic parenchyma (Figure 4A, middle panel, B left panel, Figure 4D and 6B, left lower panel). Furthermore, the requirement for oval cells to undergo significant rounds of proliferation and subsequent complex differentiation process (37), may be the reason for the high level of activator E2Fs (Figure 5A-C), and E2F4, 5 and 6 transcripts (Figure 5D-F) which are known to be involved more in cell differentiation (47,49-50). In addition to that, the activation of oval cells is further supported by an elevated level of a-fetoprotein transcript (Figure 5I), which is a known oval cell marker in rodents (51-52)

Liver injury as important factor in liver carcinogenesis, and the diversity of neoplastic lesions

Liver regeneration pass through three phases namely; priming, proliferation and maturation phases (53). Primed cells have the ability to respond to growth factors and proliferate. Priming factors are produced after liver injury, and are inflammatory mediators, mainly TNF-α, IL-6 and their downstream targets. Finally, in maturation phase, inhibition of proliferation occurs to allow formation of supportive structures such as extracellular matrix and blood vessels. This process is also cytokine mediated involving TGF-β, HIF1-α, VEGF, and hypoxia created by avascular newly formed liver and biliary cells (30).

In our results we have shown that, primary neoplastic lesion occurs on the ligation site, in the residual median lobe in the mechanism which is not understood (Figure 4A). We can only speculate that, local injury, and hypoxia on the residual median lobe created during PH, repetitive exposure to priming factors, growth factors and maturation factors

during the two PH may contribute to carcinogenesis in this location. In addition, the presence suture materials on the ligation site in this specific area could spark local chronic inflammatory process, including recruiting Kupffer cells and lymphocytes, which are known to produce priming factors, TNF-α and IL-6. Continuous local production of these factors would then create high concentration gradient, and confers proliferative advantage to the oval cells around the ligation site leading to expedition of the carcinogenic process. In particular, TNF-α and IL-6 are known for their role as activators of oval cells proliferation. Inhibition of TNF-α with gadolinium chloride or dexamethasone treatment in rats with 2AAF/PH can inhibit oval cell response in bile duct ligated rats (37). While IL-6 can directly activates oval cell proliferation, TNF-α activates oval cell proliferation by induction of STAT3 and NFkB. Oval cells isolated from 2AAF/PH rats shows activation of STAT3 and NFkB, downstream targets of TNF-α (54). The involvement of oval cells may be the reason for the diversity of neoplastic lesions observed ranging from well differentiated to poorly differentiated hepatocellular carcinoma (Figure 4A, right and middle panel). These lesions also show spindle shaped cells, which has been described in human Hepatocellular carcinoma with various names including spindle cell carcinoma, sarcomatoid carcinoma, pseudo carcinoma and carcinocarcinoma (55). (Figure 4A, middle panel). Masson's trichrome staining for collagen show that, only tissue stroma contains collagen, and not the neoplastic cells (Figure 4B). Other phenotypes resemble features of cholangiolocellular carcinoma (56), including small cords resembling cholangioles and anastomosing glands in abundant fibrous stroma. The stromal tissues, stain blue with Masson's tri-chrome staining for collagen (Figure 4B, left panel) and H&E staining (Figure 4D).

This observed heterogeneity of cancerous cells indicate the possibility of this carcinogenic process to start early before differentiation of the oval cells. This theory is further supported by the expression of LacZ in neoplastic cells. Expression of LacZ occurs after Albumin-Cre$^{+/-}$ mediated excision of lox P sites and recombination (36). Because LacZ gene is expressed in hepatocytes, biliary and neoplastic cells (Figure 4C). The excision of lox P sites in the liver can only occur in albumin expressing cells, which is not the case with bile duct epithelial cells. This means that, excision and recombination took place early in oval cells before differentiation. This implies that, p53 and Rb was deleted in oval cells. This is argument is supported by evidences that oval cells can express albumin (51-52), a promoter which drive the expression of Cre recombinance enzyme. Deletion of p53 and Rb gene in oval cells may interfere with normal proliferation and differentiation programme of these cells; and this may be the cause of diversity of cellular morphology we see in neoplastic lesions.

Conclusion

Diversity of the lesions observed in this study and involvement of oval cells may be useful in future studies for understanding the molecular mechanisms involved in activation of liver stem cells, and neoplastic behaviour oval cells derived liver cancer. Further molecular characterization of all the cells in these lesions would be helpful and may give better understanding of their origin, behaviour, and may provide more knowledge in the possible pathogenesis of these lesions. Site specific location of primary lesion observed in this study, is interesting and may have implications on the treatment of HCC patient by surgical resection, especially if the surrounding liver tissues contain mitotic incompetent cells and or transformed cells. Liver specific p53/Rbnull-PH model may be a valuable tool in future studies of liver carcinogenesis at various stages of development, progression and the underlying molecular pathogenesis.

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Legends

Fig. 1: **(A)** Schematic drawing showing Liver specific conditional gene targeting approach. The targeted exons (floxed exons with lox P sites) for each gene are indicated in brackets. Active *Cre* recombinase enzyme (green shedding), recognises and excise the lox P sites shown as solid arrow head, and pointed with green arrow. Excision and recombination site are shown as red dotted line. Blue shedding indicates expression of Lac Z reporter gene after Cre mediated excision of stop codon up-stream of the Rosa 26 locus. **(B)** Genotyping to confirm the presence of transgenes and the targeted alleles before setting breeding pairs.

Fig. 2: Partial hepatectomy schedule and histology. **(A)** 70 days after first partial hepatectomy (PH), the second PH was introduced. Grey shedding indicates liver lobes removed at each hepatectomy. Right panel show the remained liver lobes after two hepatectomies and the regenerated segment of the median indicated by an arrow as it was observed during second PH. **(B)** H&E staining of the liver, Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$ and Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{f/\Delta}$ show nuclear hypertrophy in hepatocytes in the liver collected at first PH from mice, 9.5-14.5 weeks (Left panel). 70 days later, $p53^{f/f}$; $Rb^{f/f}$ also show nuclear hypertrophy but at lesser a degree compared to p53 and Rb knockouts (Right panel). The liver lobe used for histology in each case is shown in brackets.

Fig. 3 **(A)**. Schematic drawing of normal liver and location of neoplastic lesion (Top panel). Insitu, and external appearance normal liver and neoplastic lesion. Note that the lesion has merged with the surrounding liver lobe. **(B)** Survival curve prepared using Cox-proportional hazard analysis method, showing occurrence neoplasia. No neoplasia was observed in $p53^{f/f}$; $Rb^{f/f}$ (black line). Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$ mice (shown in red) developed lesions earlier compared to Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{f/\Delta}$ (shown in blue).

Fig. 4 A. H&E staining: Liver at first PH (left), neoplastic segment of the median lobe (middle panel) and sorrounding liver lobe (right panel). Note the gland like ductular reaction (arrow head) and spindle shaped neoplastic cells (arrow) in the neoplastic segment of the median lobe as well as well differentiated Hepatocellular carcinoma in the sorrounding liver lobes (arrow)

B. Masson's trichrome staining: Neoplastic segment of the median lobe (middle panel), surrounding liver lobes (left and right panels). Hepatocytes are stained with violet to indigo colour, collagen are stained blue and weakly stained neoplastic cells with indigo/violet colour.Stromal tissues around neoplastic cells are strongly stained blue for collagen (arrow).

C. X-gal staining to demonstrate expression of LacZ. Normal liver (top panel), and magnified portal area (top left). Neoplastic lesion (middle and lower panels) at low and high magnifications showing expression of LacZ in neoplastic spindle shaped cells (arrow) and bile duct cells (arrow head),

D. H&E staining of portal area: low and higher magnification. Show proliferation of cells in the bile duct (arrow), and surrounding areas. Proliferating cells in the area surrounding the bile duct show different sizes and shape (arrow head)

Fig. 5. Real time PCR results at fisrt PH (black bars), Second PH (grey bars), neoplastic lesion (red bars), and liver lobes surrounding the neoplastic lesion (blue bars). E2F1-8, and a-fetoprotein expression is higher in mice deficient of p53 and Rb compared to expression of these genes in $p53^{f/f}$; $Rb^{f/f}$ at first PH. E2F1 (**A**), E2F4 (**D**), E2F6 (**F**), and a-fetoprotein (**I**) mRNA expression is higher in the neoplastic segment and surrounding liver lobes. (**C**) E2F3a show similar pattern but with variations among individual mice. (**B**) E2F2 is constituently higher in majority of the mice from time of second PH and maintained in neoplastic tissues and the sorronding liver lobes. (**E**) E2F5 show relatively constant expression at first, and second

PH as well as in the neoplastic and surrounding liver lobes. E2F7 (**G**) show high expression from first PH, second PH and surrounding liver lobes compared to neoplastic lesion whereas, E2F8 (**H**) show variable but more expression in the neoplastic and surrounding liver tissues

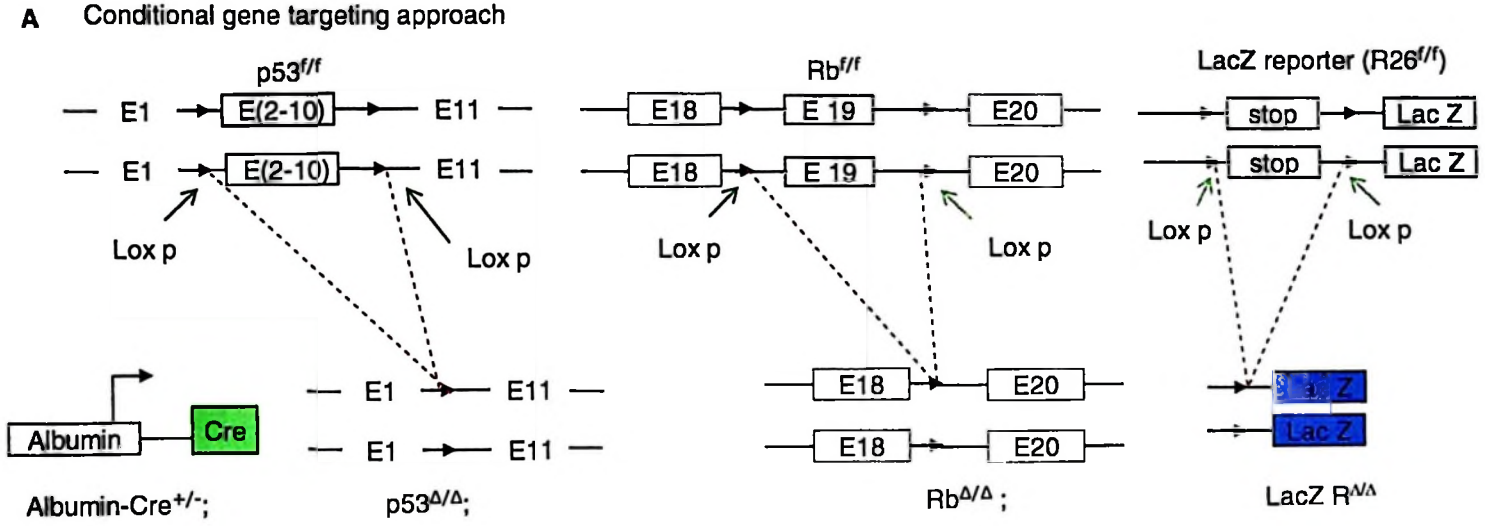
Fig. 6. (**A**) Liver collected at first PH show more proliferation in the liver of Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$ compared to $p53^{f/f}$; $Rb^{f/f}$ .This proliferation involves small round to oval cells (arrow) and few hepatocyes which display lobulated nuclei (arrow head). (**B**) Schematic drawing of neoplastic segment of the median lobe and surrounding lobes, long arrows indicates origin of the liver sample for histology (top). Ki67 staining show high proliferation in the surrounding liver lobes involving mainly the portal area, and around bile ducts (arrow), lower left panel. Ki67 staining of the neoplastic lesion, show resting and proliferating cells, mainly spindle shaped cells and few oval to round cells (arrow), lower right panel

Table 1. No lesion was observed in $p53^{f/f}$; $Rb^{f/f}$ mice. 3 out of 8 mice developed neoplastic lesion equal to 37.5% where as 12 out of 19 Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$ mice, equal to 63.2% developed neoplastic lesion and 25% of the lesions were found to be spreading to other organs in the abdominal cavity.

Supplimentary informations (SI)

2_SI.Fig.1. PH procedure. (**A**) Midline incision of skin. (**B**) Applying pressure to exteriorize the liver lobes to be removed. (**C**) Liver lobes are out ready to be removed. (**D**) Median lobes (ML) are secured with wet gauze; enable the left lower lobe (LLL) to be easily dealt with. (**E**) Cutting the ligament on the visceral surface of the LLL. (**F**) Ligation of LLL. (**G**) Lobectomy. (**H**) Placing the suture, the two ML right (R) and left (L) are ligated with one ligature.

2_SI. Table 2. Oligonucleotide primers used for real-time PCR.


A Conditional gene targeting approach
p53f/f
E1
E(2-10)
E11
Lox p
Albumin
Cre
Albumin-Cre+/-;
p53Δ/Δ;
Rbf/f
E18
E 19
E20
Rb Δ/Δ ;
LacZ reporter (R26f/f)
stop
Lac Z
LacZ RΔ/Δ

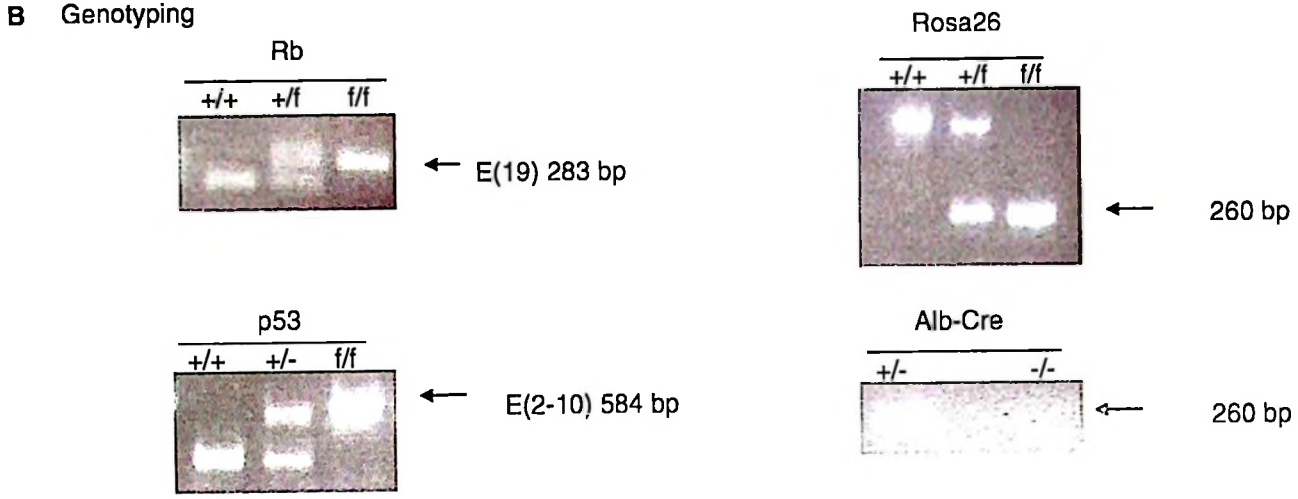

B Genotyping
Rb
+/+ +/f f/f
E(19) 283 bp
Rosa26
260 bp
p53
+/+ +/- f/f
E(2-10) 584 bp
Alb-Cre
+/- -/-
260 bp

Fig. 1

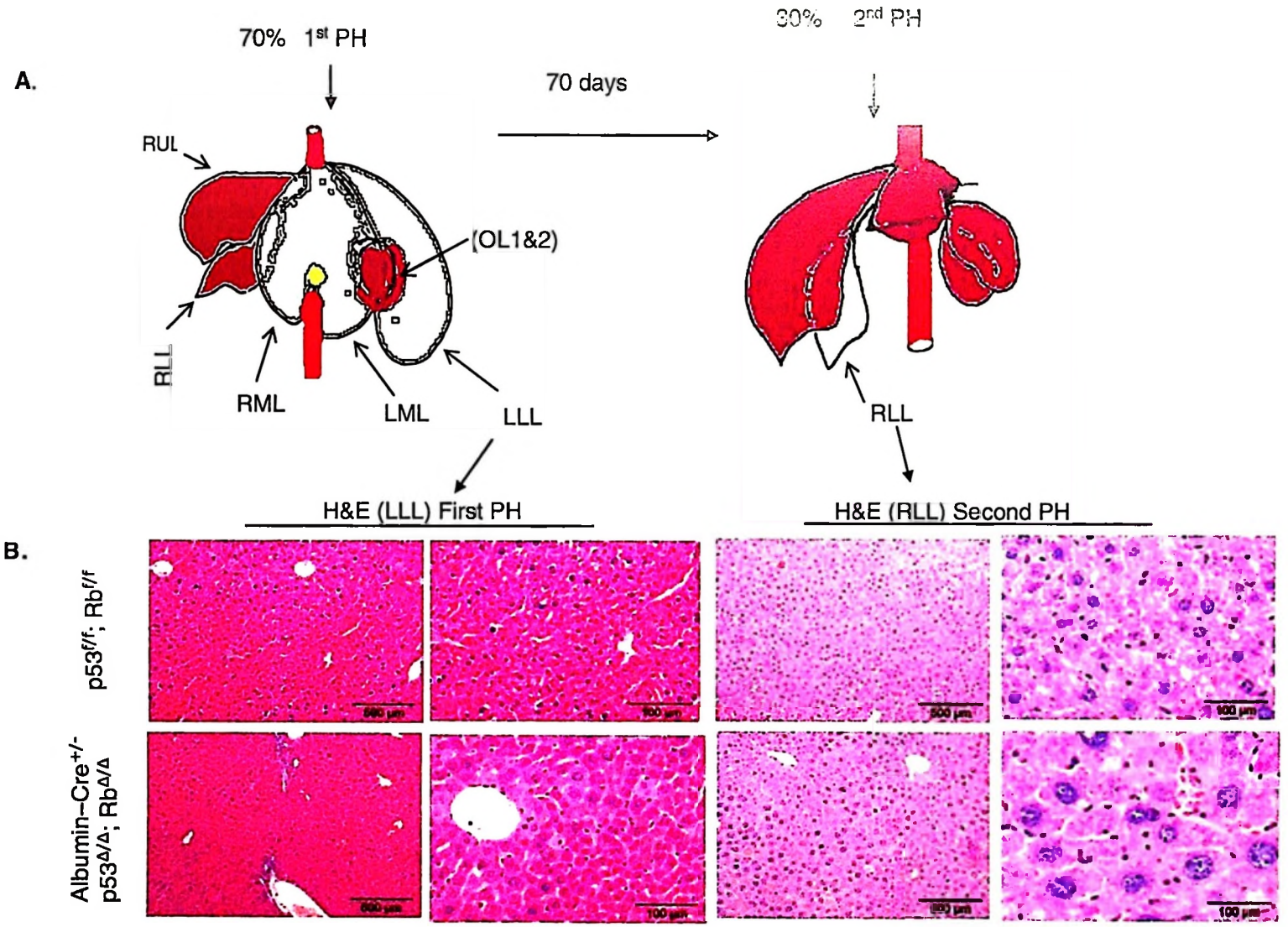

A.
70% 1st PH
70 days
30% 2nd PH
RUL
(OL1&2)
RLL
RML
LML
LLL
RLL
H&E (LLL) First PH
H&E (RLL) Second PH
B.
p53f/f; Rbf/f
Albumin-Cre+/-
p53Δ/Δ; RbΔ/Δ

Fig. 2

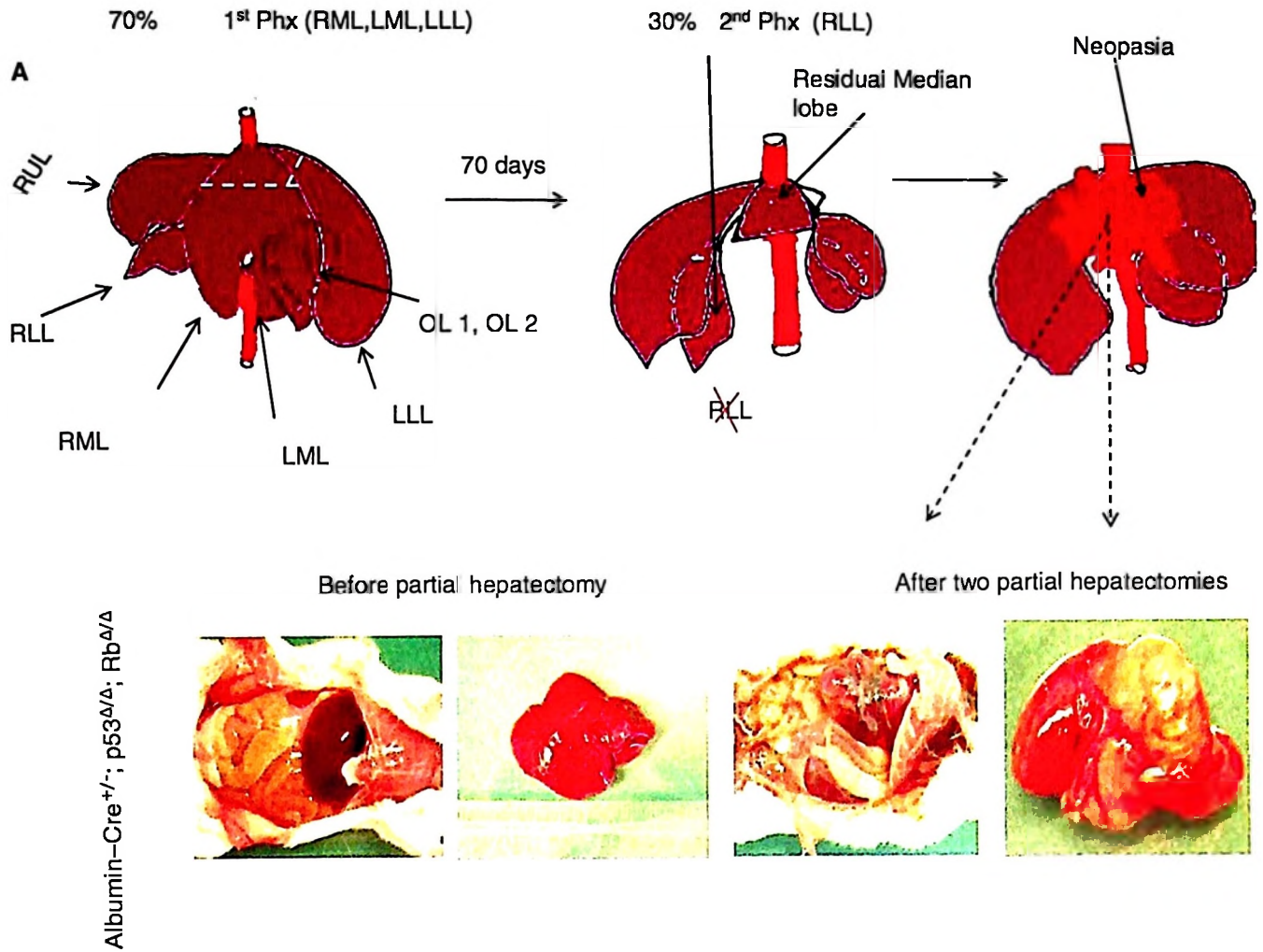

70%
1st Phx (RML,LML,LLL)
30%
2nd Phx (RLL)
A
RUL
RLL
RML
LML
LLL
OL 1, OL 2
70 days
Residual Median lobe
RLL
Neopasia
Before partial hepatectomy
After two partial hepatectomies
Albumin–Cre+/-; p53Δ/Δ; RbΔ/Δ

Fig. 3

B. Occurence of liver cancer

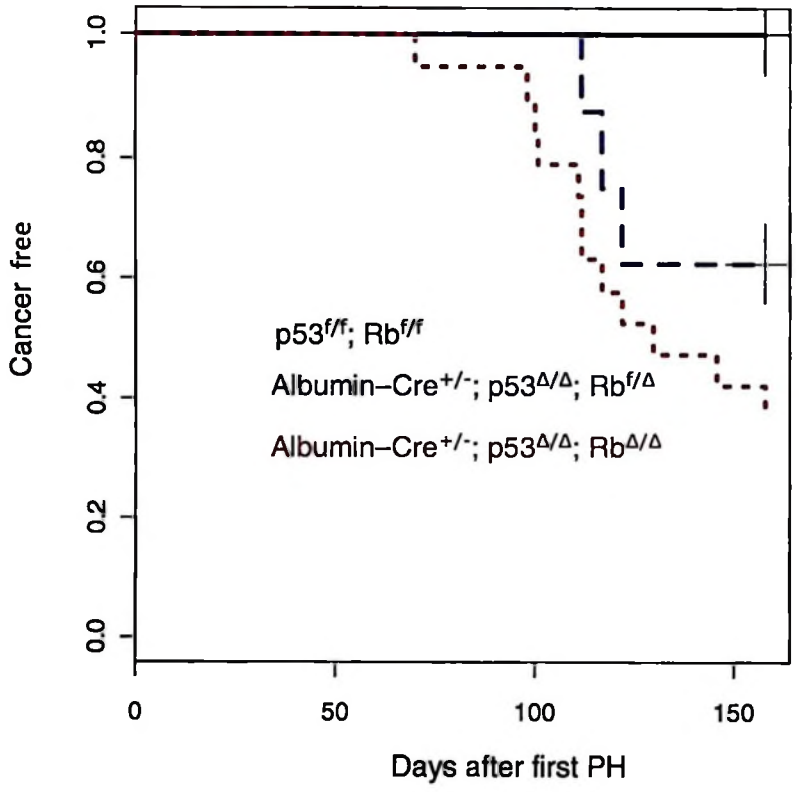


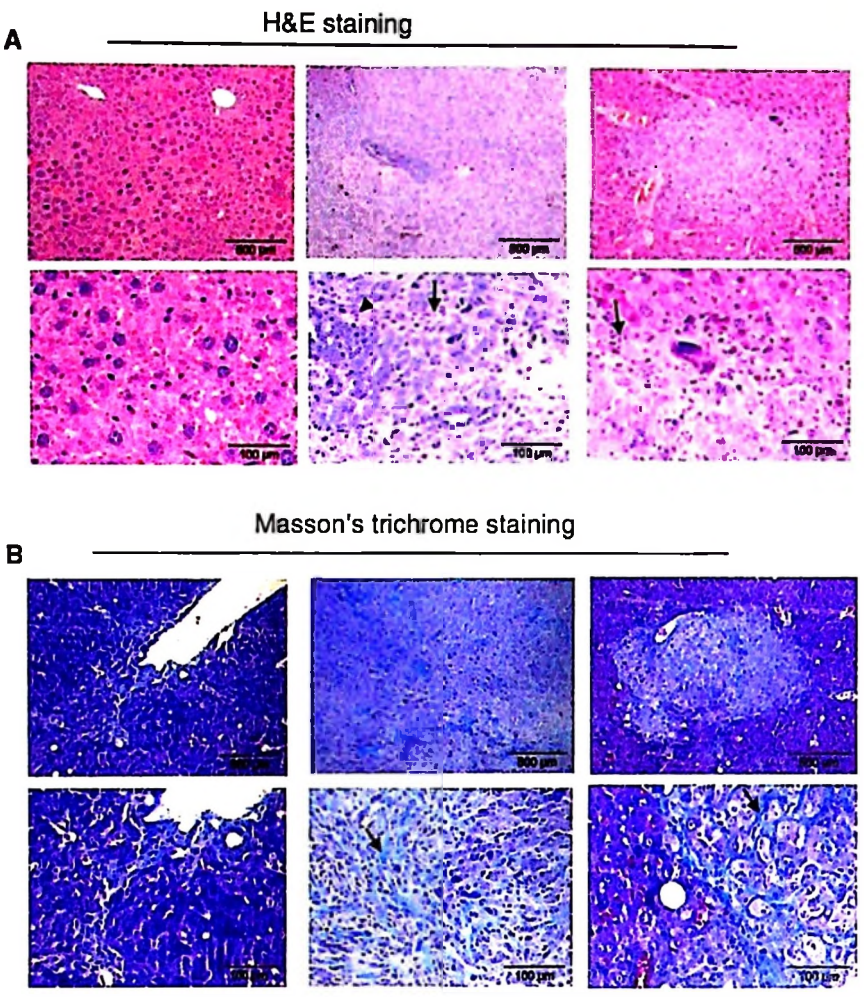
Table 1. Occurrence of liver cancer: All mice compared with $p53^{f/f}$; $Rb^{f/f}$

Genotype	Total	Neoplasia	Metastasis
$p53^{f/f}$; $Rb^{f/f}$	19	0/19	0/0
Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{f/\Delta}$	8	3/8 **	0/3
Albumin–$Cre^{+/-}$; $p53^{\Delta/\Delta}$; $Rb^{\Delta/\Delta}$	19	12/19 ***	3/12

Log rank test, significance level: $p < 0.05$

**$p < 0.005$, and ***$p < 0.0005$

Fig. 3


A
H&E staining
B
Masson's trichrome staining

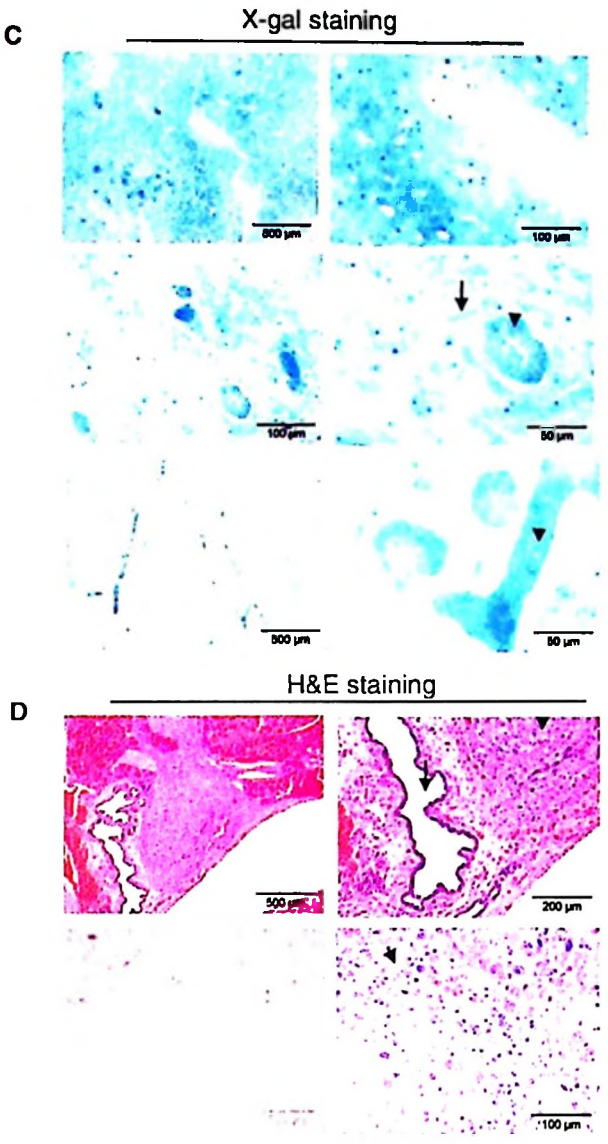

C
X-gal staining
D
H&E staining

Fig. 4

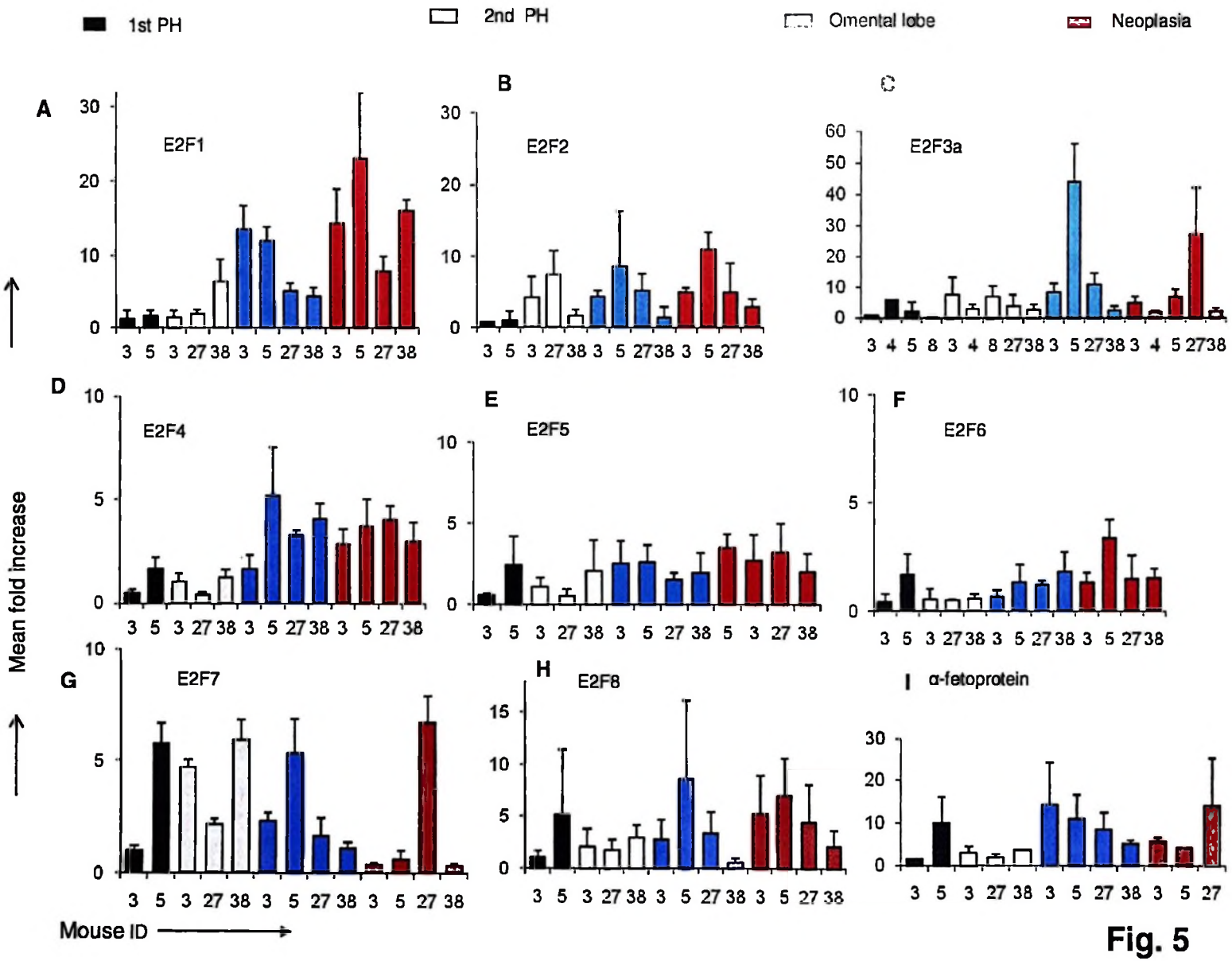
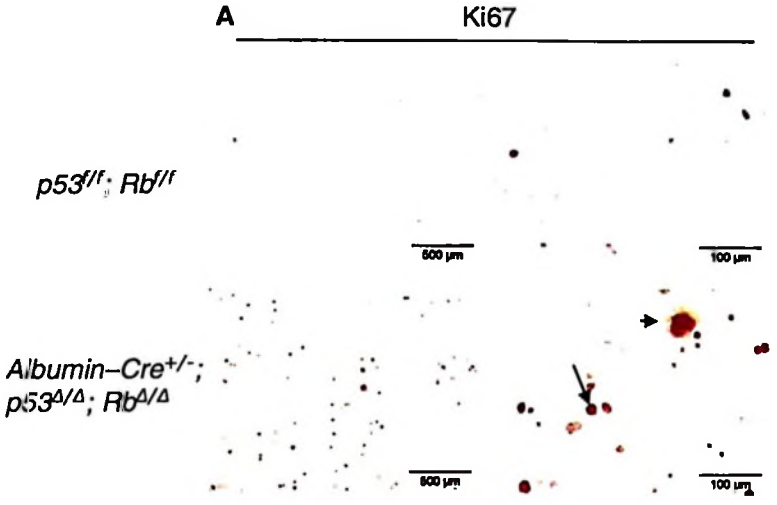

1st PH
2nd PH
Omental lobe
Neoplasia
A
E2F1
B
E2F2
C
E2F3a
D
E2F4
E
E2F5
F
E2F6
G
E2F7
H
E2F8
I
α-fetoprotein
Mean fold increase
Mouse ID

Fig. 5



First partial hepatectomy

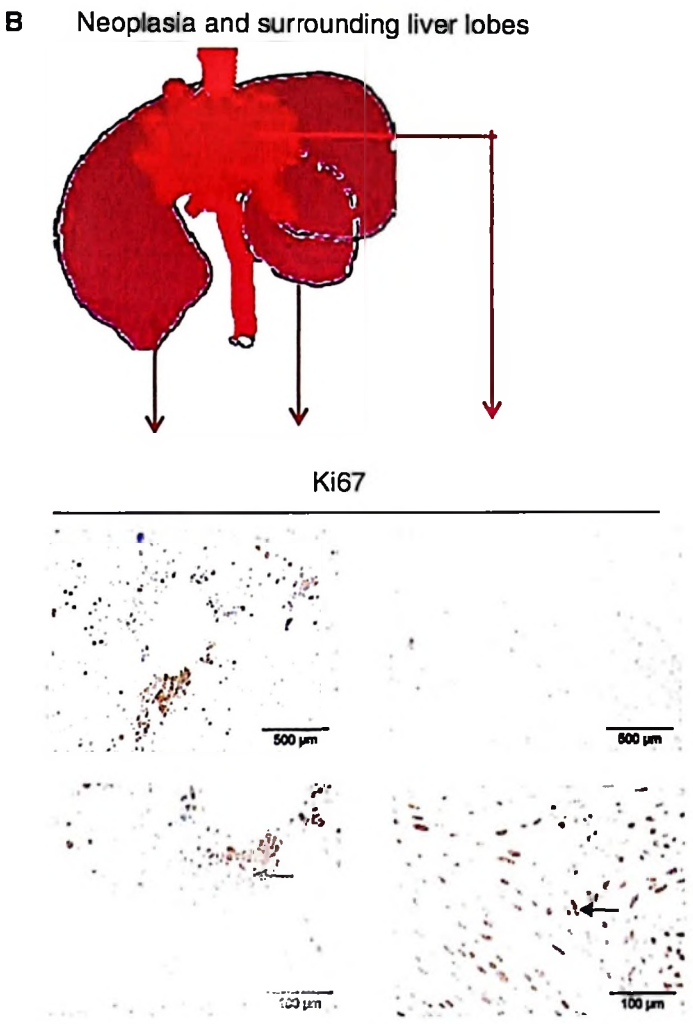
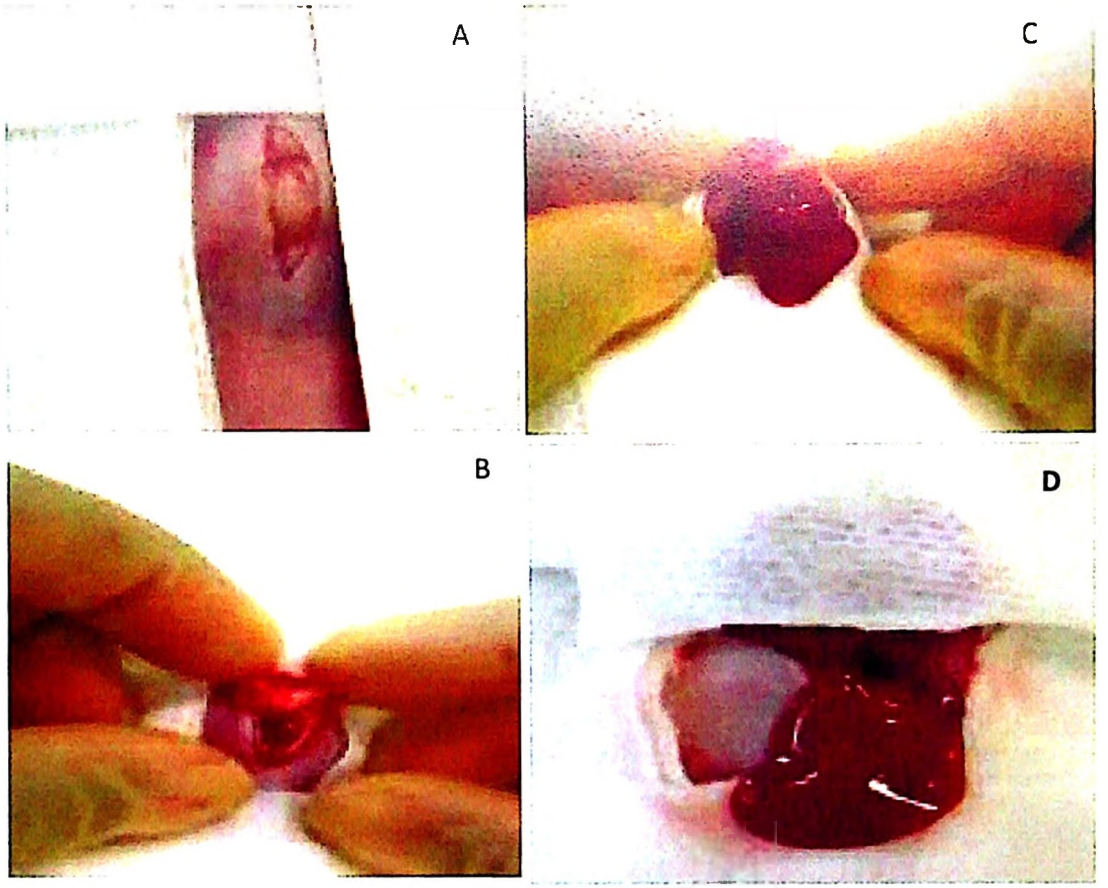
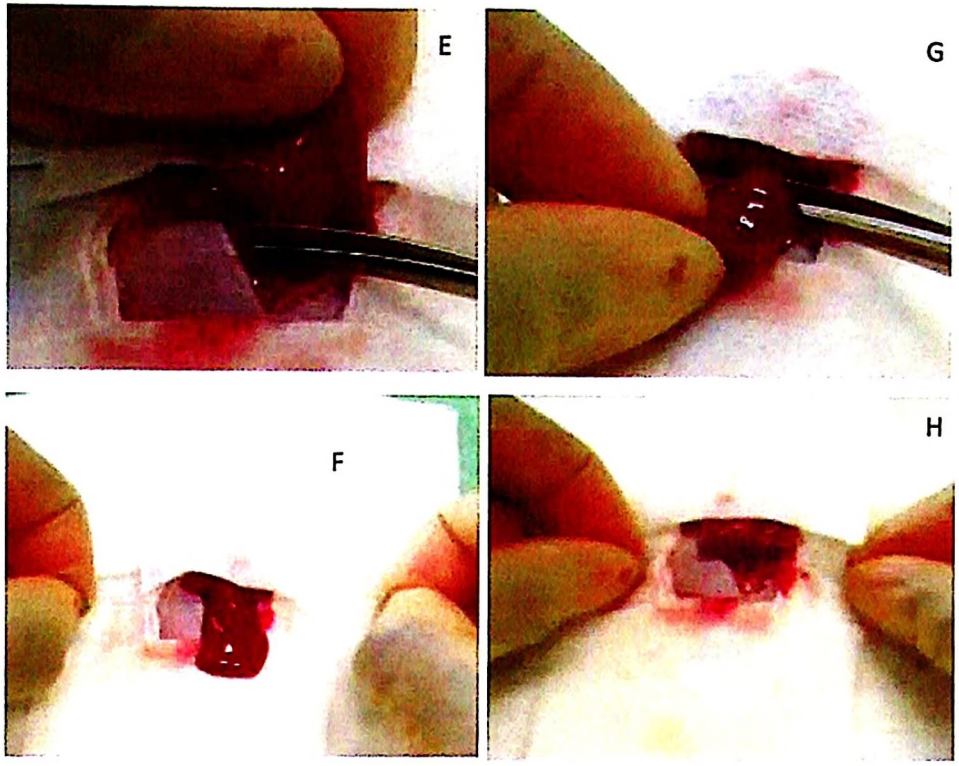


Fig. 6

Sl.Fig.1 Partial hepatectomy procedures



2_Sl.
Fig.1



2_Sl. Fig.1

SI.Table 2

Gene	Code	Melting (Tm)	Forward		Reverse
E2F1	ST-151	63.5 °C	5'- GCCCTTGACTATCACTTTGGTCTC -3'	ST-152	5'- CCTTCCCATTTTGGTCTGCTC -3'
E2F2	ST-197		5'- TAGGGAGATGTGGAGGATTCGG -3'	ST-198	5'- AACTCAGGGTGGACAAACAAACAC -3'
E2F3a	ST-191		5'- GCCTCTACACCACGCCACAAG -3'	ST-192	5'- TCGCCCAGTTCCAGCCTTC -3'
E2F4	ST-159		5'- CTTCTACCTCCTTTGAGCCCATC -3'	ST-160	5'- TCACAGACACCTTCACTCTCGTCC -3'
E2F5	ST-161		5'- ACCTGATGACCTCACACAGCCTTC -3'	ST-162	5'- GGGGTAGGAGAAAGCCGTAAAAG -3'
E2F6	ST-163		5'- CATCCACAATGCCCAGAGAAAG -3'	ST-164	5'- CAATAGAGCACAAGAGCACTCCG -3'
E2F7	L2		5'- CCCCCGAGATCCACACCTAC -3'	R2	5'- CAGAGCCAGGCTGGTCAGAA -3'
E2F8	L2		5'- GCCCAGAAATCAGCCCAAAC -3'	R2	5'- GCTGGGAGCGGAACTGATCT -3'
α-fetoprotein	222	57 °C	5'- CGGAACTCTCTAACAAGTATGG -3'	223	5'- AGGTTCTGGAAACTGGAAGG -3'
GAPDH		60.3 °C	5'- CGGTGTGAACGGATTTGGC -3'		5'- TTTGATGTTAGTGGGGTCTCGC -3'

SI.2. Masson's Trichrome Staining Protocol for Collagen Fibers

Description: This method is used for the detection of collagen fibers in formalin-fixed tissues, paraffin-embedded sections, and may be used for frozen sections.

Liver tissue fixation: 10% buffered formalin.

Section: Paraffin sections at 5 μm.

Solutions and Reagents:

Weigert's Iron Hematoxylin Solution:

Stock Solution A:

Hematoxylin ---------- 1 g

95% Alcohol ---------- 100 ml

Stock Solution B:

29% Ferric chloride in water --------- 4 ml

Distilled water ---------- 95 ml

Hydrochloric acid, concentrated ---- 1ml

Weigert's Iron Hematoxylin Working Solution:

Mix equal parts of stock solution A and B. This working solution is stable for 3months

Biebrich Scarlet-Acid Fuchsin Solution:

Biebrich scarlet, 1% aqueous --------- 90 ml

Acid fuchsin, 1% aqueous ----------10 ml

Acetic acid, glacial ---------- 1 ml

Phosphomolybdic-Phosphotungstic Acid Solution:

5% Phosphomolybdic acid ---------- 25 ml

5% Phosphotungstic acid ---------- 25 ml

Aniline Blue Solution:

Aniline blue ---------- 2.5 g

Acetic acide, glacial ---------- 2 ml

Distilled water ---------- 100 ml

1% Acetic Acid Solution:

Acetic acid, glacial ---------- 1 ml

Distilled water ---------- 99 ml

Procedure:

1. Deparaffinize and rehydrate through 100% alcohol, 95% alcohol 70% alcohol.
2. Wash in distilled water.
3. Stain in Weigert's iron hematoxylin working solution for 10 minutes.
4. Rinse in running warm tap water for 10 minutes.
5. Wash in distilled water.
6. Stain in Biebrich scarlet-acid fuchsin solution for 15 minutes. Solution can be saved for future use.
7. Wash in distilled water.
8. Differentiate in phosphomolybdic-phosphotungstic acid solution for 15 minutes or until collagen is not red.
9. Transfer sections directly (without rinse) to aniline blue solution and stain for 5-10 minutes. Rinse briefly in distilled water and differentiate in 1% acetic acid solution for 2-5 minutes.
10. Wash in distilled water.
11. Dehydrate very quickly through 95% ethyl alcohol, absolute ethyl alcohol (these step will wipe off Biebrich scarlet-acid fuchsin staining) and clear in xylene.
12. Mount with resinous mounting medium.

Results:

Collagen ----------blue

Nuclei ---------- black

Muscle, cytoplasm, keratin ---------- red

CHAPTER 3

Liver regeneration and platelet aggregation in rats lacking the serotonin transporter

Ramadhan B. Matondo, Carine Punt, Judith Homberg, Mathilda J.M. Toussaint, Ronald Kisjes, Edwin Cuppen, and Alain de Bruin

Manuscript in preperation

ABSTRACT

The liver demonstrates a remarkable capacity for regeneration and following 70% partial hepatectomy in rodents is able to almost completely restore its lost mass within 14 days. Recent studies indicate that the vasoconstrictor serotonin plays a role in liver regeneration and platelet aggregation. In the blood 95% of serotonin is actively taken up by the platelets expressing the serotonin transporter, where it is stored in intracellular vesicles and released in response to variuos stimuli. We have recently generated a serotonin transporter knockout rats and demontrated that the platelets of these rats lack serotonin. Here we show that the serotonin transporter defcient rats had marked reduced levels of serotonin levels in the blood and liver associated with prolonged bleeding times. Surpringsly, these rats showed normal platelet aggregation and liver regeneration upon partial hepaectomy. These results indicate that a release of high levels of serotonin from platelets is not required for liver regeneration, but rather low levels of serotonin in blood and/or liver are sufficient for liver regeneration

Introduction

5-Hydroxytryptaminie (5HT) also known as Serotonin, is a neurotransmitter as well as a mitogen (1), synthesized mainly in enterochromaffin cells (ECC) of the gastrointestinal tract (2, 3). Outside the gastrointestinal tract, 5HT is also synthesized in neurones of the central nervous system (4). 5HT is involved in inducing platelet alpha granule release of von Willebrand factor (vWF) from platelets, platelet adhesion, and aggregation (2, 5) through activation of serotonergic receptor 5HT2 (6, 7).

Mice lacking serotonin transporter (SERT) exhibited reduced platelet aggregability (8), and inhibition of 5HT receptor (5HT2A) in rats, results in inhibition of 5HT-induced vascular contraction (9). This led to a suggestion that, 5HT function as a hormone, involved in vasoconstriction and platelet function (2, 10). Therefore, it has been suggested that synergism exist between the primary platelet stimuli and the products of platelet release for an efficient platelet aggregation and haemostasis (3, 6,11).

In addition to its role described above, 5HT has been reported to play an important role in liver regeneration through 5HT2A receptors. Ketanserin, a 5HT2 blocker can arrest liver regeneration in rats when administered at 16th hour after 60–70% partial hepatectomy (PH) (12). Other reports describing mitogenic potential of 5HT has been described elsewhere (13-14). Likewise, platelet derived 5HT has been reported to be crucial in mediating liver regeneration (15). However, other conflicting reports are emerging on the role of 5HT in hepatocyte proliferation, including resent report suggesting that, 5HT alone is not enough to drive hepatocyte proliferation, but can strongly induce DNA synthesis in the presence of other growth factors (16).

Platelets possess a high-affinity SERT (17-18), and are loaded with 5HT when they pass through the intestinal circulation by SERT mediated reuptake mechanism (3,11). About 95% of 5HT found in blood is stored in platelets. (19-20). Although it is known that ablation of SERT in rodents results into loss of 5HT reuptake into platelets (21), the consequences this ablation in rat liver regeneration is not known. To establish the role of platelet and platelet derived 5HT in rat liver regeneration, PH was performed both in wild type and SERT knockout rats. The consequence of 5HT deficiency in platelet on megakaryocytopoiesis, blood platelet counts, aggregation and bleeding time was also investigated.

Materials and methods

Animals

Generation of SERT knockout rats and genotyping was done as previously described (17, 22). 37, 12 weeks old, littermate Wistar rats were used for hepatectomy experiments. 33 animals were used for 70% PH, and to study percentage of BrdU uptake in hepatocytes at 20, 24, 48 and 96hours after PH.

The number of animals used at each time point per group is as indicated in brackets. 20 hours ($SERT^{+/+}$, n=5; $SERT^{-/-}$, n=4), 24 hours ($SERT^{+/+}$, n=5; $SERT^{-/-}$, n=5), 48 hours ($SERT^{+/+}$, n=3; $SERT^{-/-}$, n=5), 96 hours ($SERT^{+/+}$, n=3; $SERT^{-/-}$, n=3). In addition, 4 rats (2 $SERT^{+/+}$ and 2 $SERT^{-/-}$) were used to study platelet activation in the liver at 0 and 5 minutes after PH.

This experiment was approved and performed in accordance with the University of Utrecht Animal Experiment ethics committee; animals were housed at University of Utrecht Animal facility at a 12 hours cycle of light and dark with free access to food and water

Blood sampling for Enzyme Linked Immunosorbent Assay (ELISA), and platelet counts

Blood sample for platelet counts was collected from the tail vein by using a 25 gauges hypodermic needle and then directly mixed with 10% of Tri- sodium Citrate Dehydrate (TSCD) in a ratio of 1 part TSCD and 9 parts of blood sample. For ELISA, blood was drawn from portal vein and vena cava just before the animals were sacrificed. All blood collection was performed under 2% Isoflurane (Abbott, England) anaesthesia, 0.4 and 0.3Litres/minute of oxygen and air respectively.

Bleeding time

Assesment of bleeding time was done as previously described with minor modifications (9). Animals were anaesthetized with 2% isoflurane in oxygen and air, followed by cleaning of the tail with water and Chlorohexidene gluconate (SLL Health care, Netherlands). After this step, the tail was dried with a piece of gauze. Bleeding was then assessed by recording time from the moment the tail was incised until bleeding stopped completely.

Platelet counts, platelet volume, density and aggregation

Platelet counting, platelet volume and density were determined by using an automated Bayer ADVIA^{TM} 120 analyser at University of Utrecht, Small animal Haematology

Laboratory, as previously described (23), with minor modifications. Whole blood sample collected in 10% TSCD was used in a ratio described under blood sampling section. Preparation of platelet rich plasma, and collagen induced platelet aggregation assay was performed as described previously with minor modifications (24). Platelet concentration was adjusted to make 2.5 x 10^8 cells/L in presence of 1mM of $Cacl_2.2H_2O$ end concentration. Aliquots of 291 µL were warmed to 37 °C for 5minutes followed by addition of 3µL of collagen (10µg/mL) and 6µL of fibrinogen (0.5µg/mL), each sample in duplicate. Aggregation was monitored continuously for 11 minutes at 900 revolutions per minute, in an optical aggregometer (Model 570 VS, Chrono-Log Corporation, Havertown, PA, USA).

ELISA

Preparation of serum, and plasma samples as well as determination 5HT in blood by ELISA, was performed according to protocol as described in ELISA kit (KAPL 10-0900, Biosource, Belgium). For 5HT and its metabolite, 5 hydroxy indole acetic acid (5HIAA) in the liver, 100mg of liver was homogenized in 1mL ice cold 0.05N HCL. Amount of protein in the samples were normalized after quantifying the total protein by Pierce BCA protein assay reagent kit. ELISA assay for 5HT in the liver was performed as described in the protocol for measuring 5HT in plasma in the ELISA kit (Cat. KAPL 10-0900, Biosource, Belgium). 5HIAA in the liver was determined by using the protocol for determination of 5HIAA in urine as described in the ELISA kit (Cat. KAPL10-1900, Biosource, Belgium).

Partial hepatectomy and 5-Bromo-2-deoxyuridine (BrdU) injection

Rats were induced by isoflurane 3%, oxygen 0.4L/min, and air 0.3L/min. Preparation of of incision area was followed by injection of Buprenophine hydrochloride (Temgesic®)

analgesic at the dosage of 0.01mg/100grams of body weight before starting surgery. 70% partial hepatectomy was performed at 2 % isoflurane. Surgical procedures involved midline incision of skin approximately 3-4 centimetres from xiphoid region, followed by muscles and peritoneum. The ligament connecting diaphragm and median lobes was removed as previously described (25), (3_SI.Fig.1A). Gentle pressure was applied to exteriolize the liver as described in chapter 2 of this thesis. To remove median lobes; two ligatures were applied on the vessel approximately 5 mm apart on its visceral surface close to the hilus (3_SI.Fig.1 B-F). The blood vessel was freed from other tissue carefully to permit the passage suture (Mersilene®) size 4-0 (Johnson Johnson, Belgium) with the help of tissue forceps. The vessel was then separated by cutting between the two ligatures. A third ligature was laid by hand by passing the suture around the common pedicle of the median lobes. Finally a knot was tied between the first two ligatures and the lobes resected proximal to the third ligature. After cutting the ligament on the visceral surface of the left lower lobe, a ligature was laid with hands around its pedicle, and the lobe was ligated and resected as described in chapter 2 of this theis. Redundant tissues and blood clots were removed as previously described (25) and the incisions were closed. 2 hours before the rats were sacrificed, BrdU (SIGMA Aldrich, Germany) dissolved in PBS was injected at a dose of 50mg/kg body weight, intraperitoneally, and a carbon dioxide chamber was used for sacrifice at 20, 24, 48 and 96hours respectively, after 70% PH

Histology and Immunohistochemistry.

Processing for routine histological evaluation and immunohistochemistry was performed with minor modifications of the methods described previously. (9, 26-29) Briefly, liver samples and bones were fixed in 10% buffered formalin. Liver samples for cryopreservation, were attached on filter paper containing OCT tissue-Tek, and flooded with the same fluid to submerge the sample before brief immersion into liquid nitrogen cooled isopentane. After this step, the sample was in stored in liquid nitrogen, or frozen in -80°C for long term storage. Demineralization of the bone was performed by keeping bone samples in 10% EDTA solution for 2 weeks as previously described (29). Routine Haematoxylin–Eosin (H&E), immuno-histochemical staining for Von Willebrand Factor (vWF) and BrdU staining on paraffin and frozen sections respectively, were done on 5 µm thick sections. For BrdU staining, paraffin sections were deparaffinised and hydrated for 5minutes for each step, washed with phosphate buffered saline (PBS). Cryosections of the liver were allowed to reach room temperature and then fixated in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Slides from paraffin sections and cryosections were incubated with 0.3% Hydrogen peroxide in methanol followed with subsequent rinsing in PBS/Tween-20. Paraffin sections were then pre-treated with 10mM Citrate buffer in R-4S56 microwave (Sharp, Germany), followed with cooling and rinsing in PBS/Tween-20. Goat serum diluted in PBS at ratio of 1:10 was used for blocking in all sections. Paraffin sections were then incubated with anti-BrdU, dilution 1:50, while Cryosections were incubated with Rabbit anti Factor VIII (dilution 1:400) in PBS overnight at 4°C. Thereafter, paraffin sections were rinsed in PBS/Tween-20 where as cryosections were rinsed in PBS before incubation with Envision goat anti-

mouse (Dakocytomation K4001 for 45minutes, and K4003 for 10 minutes) for paraffin and cryosections respectively. After washing, slides were developed in chromogen 3, 3'diaminobenzidine (DAB) substrate and rinsed in tap water before counterstaining with Haematoxylin. Finally the sections were dehydrated in graded series of alcohol and xylene before mounting and drying at room temperature for microscopic evaluation.

BrdU and vWF positive nuclei were randomly counted in ten fields with the help of eye piece with square grids (Olympus). Photo slides were acquired using microscope attached with camera and image acquisition software (Cell B, Olympus).

Statistics

Histograms were prepared using Microsoft excel® and unless stated otherwise, data are presented as mean ± standard deviation. Further statistical analysis to compare SERT deficient and SERT proficient rats, was done by using Mann Whitney U test method with R software version 2.7.0 for Windows. Results were considered significant when $p < 0.05$, at 95% confidence interval.

Results and discussion

Lack of Serotonin transporter does not inhibit platelet aggregation

Platelet aggregation and vasoconstriction are among the important events in the process preceding haemostasis. To understand effect of SERT ablation on the status of platelet function and haemostasis in our model, bleeding time and *in vitro* platelet aggregation were investigated. SERT deficient platelets exhibited normal aggregation *invitro*, and animals lacking SERT had prolonged bleeding time (Figure 1A and 1B). Vasoconstriction

occurs during haemostasis to reduce the flow of blood towards the damaged vessel and this process is mediated by a number of factors including 5HT. In support of this theory, it has been shown that, rodents deficient of 5HT in peripheral circulation, exhibit impaired haemostasis (2, 30). To determine whether the prolonged bleeding time in rats lacking SERT is a result of low 5HT in peripheral circulation, we measured 5HT levels in portal vein and vena cava. Indeed, these rats exhibited very low blood 5HT level compared to their wild type counter parts (Figure 1C). To rule out the possibility of thrombocytopenia as the cause of prolonged bleeding in SERT deficient rats, platelets counts were compared between SERT deficient and wild type littermate rats. These rats exhibited same level of platelets counts (Figure 1D). Activated platelets increase their mean platelet volume (MPV), and decrease contents, measured as mean platelet contents (MPC) by releasing their cytosolic contents, and consequently activated platelets form micro clots which may lead to pseudo thrombocytopenia as a result of failure for the automated machine to recognize clumping platelets (31-32). To cross check the accuracy of our blood sample handling and possible effects on platelet count results, MPV and MPC results obtained simultaneously with results of platelet counts from the same sample were analysed, and as shown in (Figure 1E & 1F), the results are comparable. These findings supports previous reports that, the prolonged bleeding was a result of low 5HT in peripheral circulation (9), not due to SERT deficiency or reduced platelets aggregation (6, 8). Also, because of the observed differences in 5HT level in this study and the previous report that 5HT can induce megakaryocytopoiesis (13), we examined histological H&E sections of bone marrow which showed no difference in number of megakaryocytopoiesis in both genotypes (data not shown).

SERT deficiency does not impair liver regeneration

Lack of SERT in rats impair 5HT uptake in platelets (21). To test whether lack of 5HT in platelets will inhibit liver regeneration, we performed PH and compared the ability of hepatocytes to enter S-phase in both wild type and SERT knockout rats. Interestingly, rats deficient of SERT exhibited accelerated liver regeneration as indicated by the percentage of BrdU incorporation in hepatocyes 24 hours after PH (Figure 2A and 2B, p=0.008). The difference in other time points were not statistically significant (20hrs, p=0.73; 48hours, p=0.07; and 96hours, p=0.2). It is known that, lack of SERT does not impair 5HT synthesis in ECC of the gastro intestinal tract (33). Since 5HT is not stored in SERT deficient platelets, and 5HT is known for its mitogenic role, we investigated whether the accelerated liver regeneration may be caused by increased flow of blood 5HT from the ECC to the liver through portal vein. As in other peripheral vessels, 5HT in portal vein of SERT deficient rats was lower compared to wild type rats (Figure 1C). It has been reported that, 5HT is important at the G1/S interphase, and that inhibition of 5HT receptor 16 hours after PH, inhibited liver regeneration. In addition, 5HT has been reported to increase and attain maximum level in the liver 24hours after PH (12). We considered the possibility of temporal increase and direct 5HT transport by other organic transporters into the liver (34), we measured 5HT levels and its metabolites in the liver, and results show low 5HT in the liver of SERT deficient rats compared to the control group, though no notable difference shown at metabolite level (Figure 3A, and 3B). Previous reports show that, platelets accumulate in the residual liver in the early period after PH (26, 35). We performed PH and investigate the ability of platelets to form aggregates *in vivo* with the help of vWF staining of the liver samples harvested

immediately after ligation (0min) and 5 minutes later (Figures 3B and 3C). These results show that platelets in SERT$^{-/-}$ can still be activated and aggregates in *in vivo* as in wild type rats. More studies need to be done to distinguish, and ascertain the signal observed in (Figure 3B), whether is due to small vWF protein molecules present in sinusoidal plasma, large multimeric forms stored in the Weibel-Palade bodies of endothelial cells or is actually present on the surface of platelets (2, 36). Staining for vWF along with platelet specific markers may help to distinguish activated platelets from other vWF positive cells or background. However, morphology and location of cells in the aggregates suggest that these cells are indeed platelets, and formation of aggregates suggest 5HT deficiency does not affect platelets activation and adhesion *in vivo* contrary to previous reports (2, 8), (Figure 3B&C).

Conclusion

The results show that the release of platelet derived serotonin is not required for liver regeneration. This is due to the fact that the liver could still regenerate in absence of 5HT in platelets, as well as low circulating and liver 5HT. Similarly high circulating and liver 5HT did not confer any advantage in wild type rats in terms of liver regeneration. In addition, rats lacking 5HT in platelets as well as low liver and circulating 5HT did not show impaired platelet aggregation *in vitro* and *in vivo* (Figure 1B and 3B). All together, the data presented in this thesis suggests that, platelet derived serotonin is dispensable for liver regeneration in rats and that; small amount circulating 5HT may be required to support liver regeneration along with other growth factors. Although we did not show vWF specifically on the platelet surface to exclude other non-cytosolic platelet derived

vWF, at the same time, our data does not directly support previous report that high cytosolic 5HT is required for efficient activation and release of platelet α-granules (2) and aggregation (8). However, because SERT deficient platelets can still aggregate, it is possible that only basal level of circulating 5HT is enough to initiate α-granule exocytosis through direct activation of serotonin receptor (5HT2A) as suggested previously (2). In general, further study is required to establish the exact mechanism underlying the acceleration of liver regeneration in SERT knockout rats as well as the exact role played by 5HT in liver regeneration.

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Legends

Fig. 1. (A) Bleeding time measured in seconds. SERT-/- (grey bar) show prolonged bleeding time compared to $SERT^{+/+}$ (black bar). Data presented as mean ± standard deviation. (B) Collagen induced invitro aggregation. $SERT^{-/-}$ show slight delay initially in forming aggregates but later aggregates to the same level as $SERT^{+/+}$. Data presented as percentage of maximum light transmittance in the sample compared with platelet poor plasma. (C) 5HT level in blood as measured in serum obtained from portal vein and vena cava as well as platelet free plasma from vena cava, show very low level of 5HT in $SERT^{-/-}$ (grey bars) compared to $SERT^{+/+}$ (black bars). The amount of platelet free plasma sample used for assay is five times higher than serum. Data presented as mean ± standard deviation. $SERT^{-/-}$ (grey bar), and $SERT^{+/+}$ (black bar) show same level of Platelet counts (D), mean platelet volume (MPV) (E), and mean platelet content (MPC) (F). Data presented as mean ± standard deviation.

Fig. 2. BrdU incorporation afer PH. (A) 20hrs after PH both $SERT^{+/+}$ and $SERT^{-/-}$ show increasing BrdU incorporation but the difference between the two groups was not significant (p= 0.7302). 24hrs after PH, $SERT^{-/-}$ exhibited accelerated Brdu incorporation (p=0.008). At 48h BrdU incorporation was found to be decreasing and the difference between $SERT^{-/-}$ and $SERT^{+/+}$ was not significant at 48h (p=0.0719) and 9hrs (p=0.2) respectively. (B) $SERT^{-/-}$ (grey bars) show elevated BrdU incorporation at 24h after PH compared to $SERT^{+/+}$ (black bars). Data presented in histograms represents mean BrdU positive hepatocyte (%) ± standard deviation. Statistical analysis was done

by using Mann Whitney U test, and differences are significant if $p<0.05$ at 95% confidence interval.

Fig. 3. (A) 5HT in the liver was found to be high in $SERT^{+/+}$ (black bar) compared to $SERT^{-/-}$ (grey bar) at 24h after PH, (B) no difference was noted in 5HIAA, the product of 5HT metabolism. (C) The ability of platelets to aggregate *in vivo* was also not affected in both $SERT^{-/-}$ and $SERT^{+/+}$ (upper panel, arrow). (D) Quantification of small round cells, not in aggregates but positive for von Willebrand factor also did not show differences between the two groups (lower panel).

Supplimentary information (SI)

3_SI. Fig.1 PH procedure. (**A**) Cutting the ligament to free the median lobes. (**B-D**) Placing two ligatures approximately 5 millimeters apart. (**E**) Applying the third ligature between the first two. (**F**) Cutting the median lobes above the third ligature and between the first two ligatures.

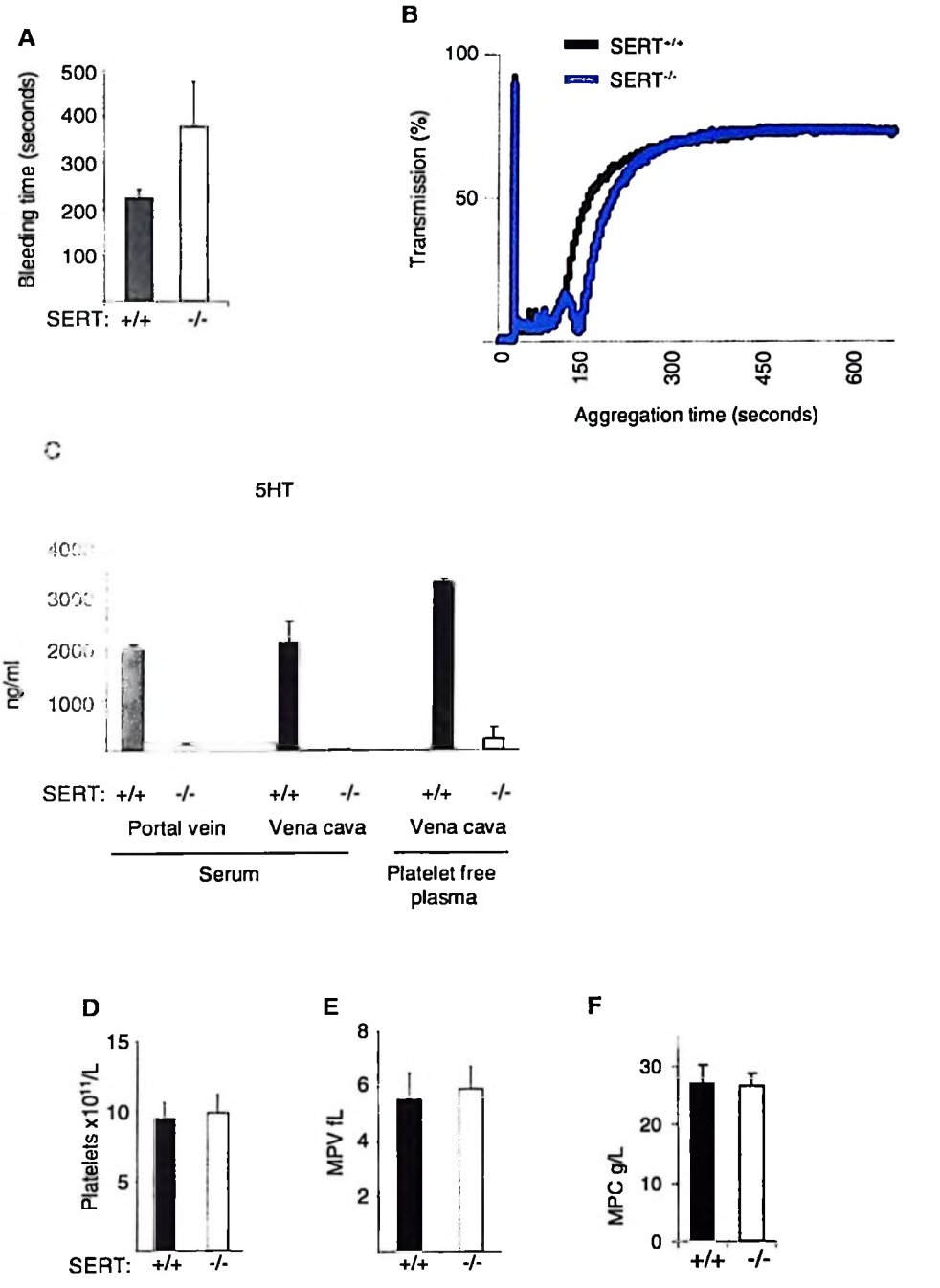
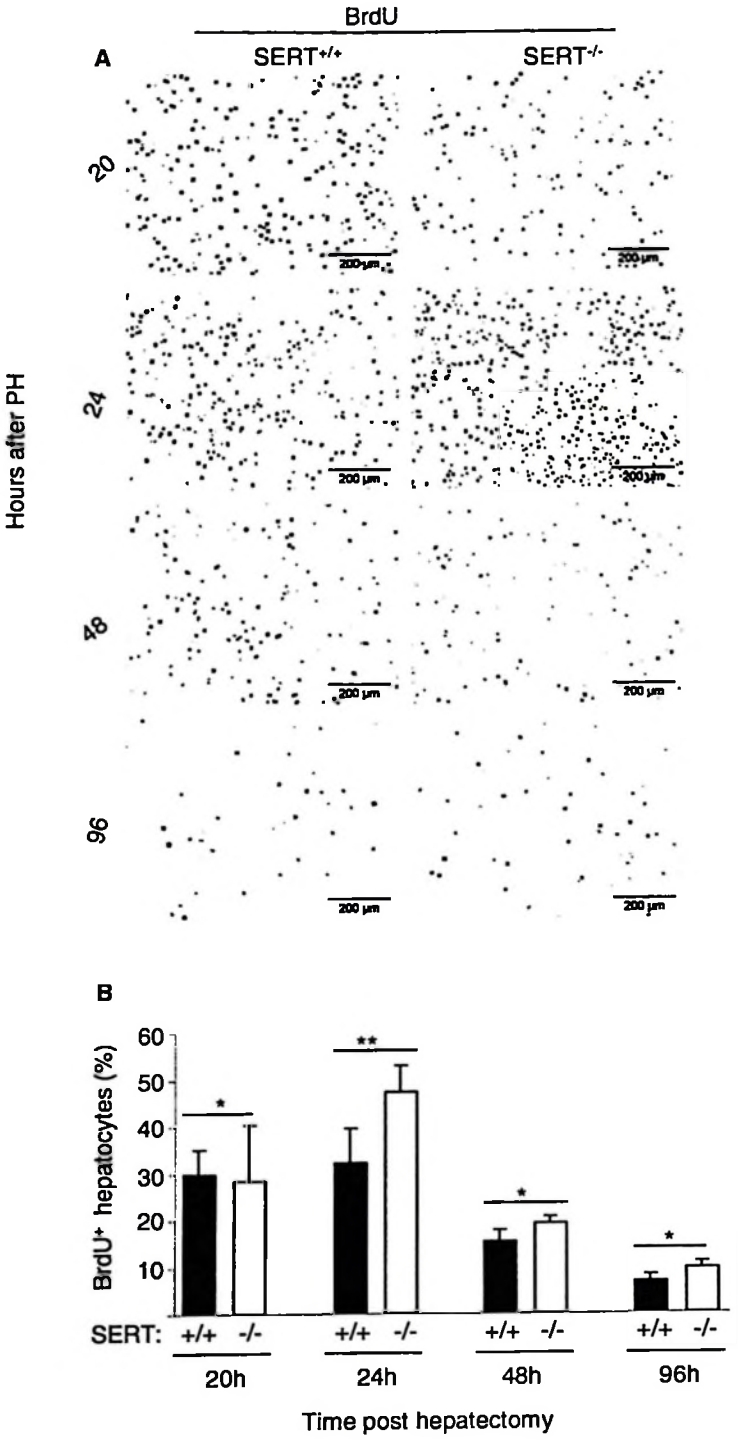

A
Bleeding time (seconds)
500
400
300
200
100
SERT: +/+ -/-
B
SERT+/+
SERT-/-
100
50
Transmission (%)
0
150
300
450
600
Aggregation time (seconds)
C
5HT
ng/ml
1000
SERT: +/+ -/- +/+ -/- +/+ -/-
Portal vein
Vena cava
Vena cava
Serum
Platelet free plasma
D
Platelets x10¹¹/L
15
10
5
SERT: +/+ -/-
E
MPV fL
8
6
4
2
+/+ -/-
F
MPC g/L
30
20
10
0
+/+ -/-

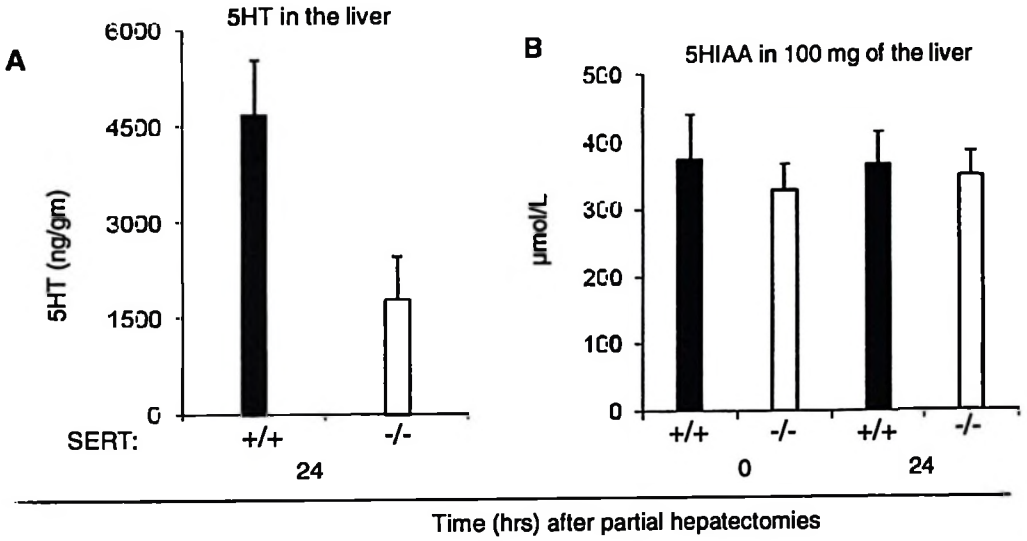
Fig. 1

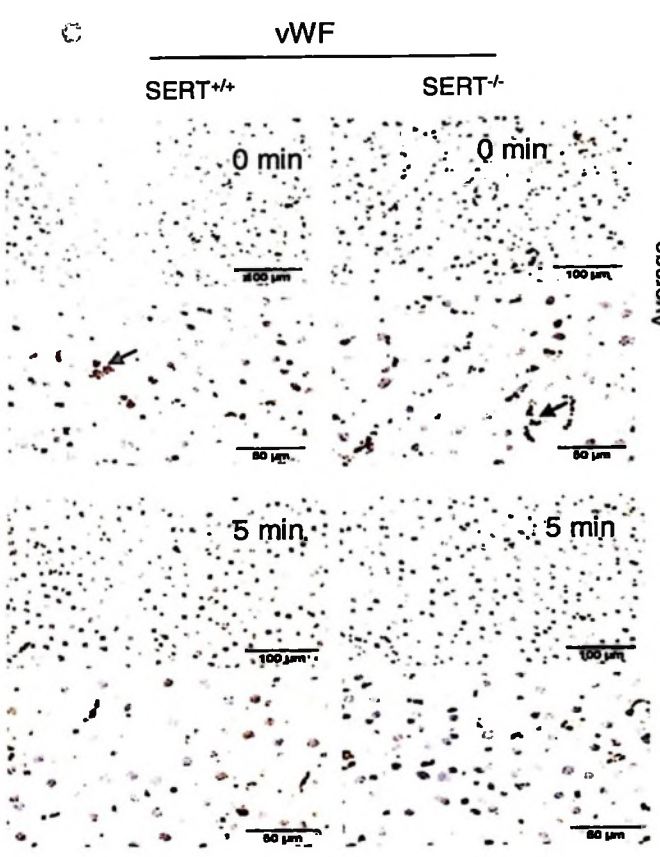


Mann Whiteney U test, signicance level:p<0.05, 95% Confidence interval

* p>0.05 ** p=0.008

Fig. 2


A
5HT in the liver
6000
4500
3000
1500
0
5HT (ng/gm)
SERT:
+/+
-/-
24
B
5HIAA in 100 mg of the liver
500
400
300
200
100
0
µmol/L
+/+
-/-
+/+
-/-
0
24
Time (hrs) after partial hepatectomies


C
vWF
SERT+/+
SERT-/-
0 min
0 min
5 min
5 min

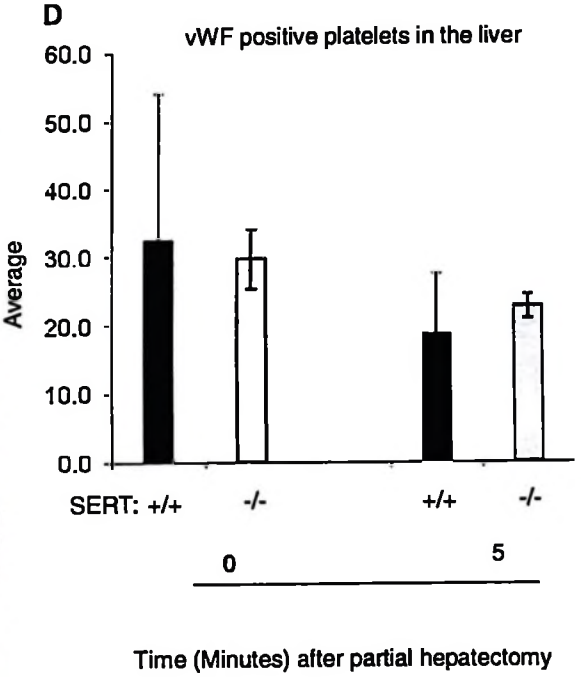
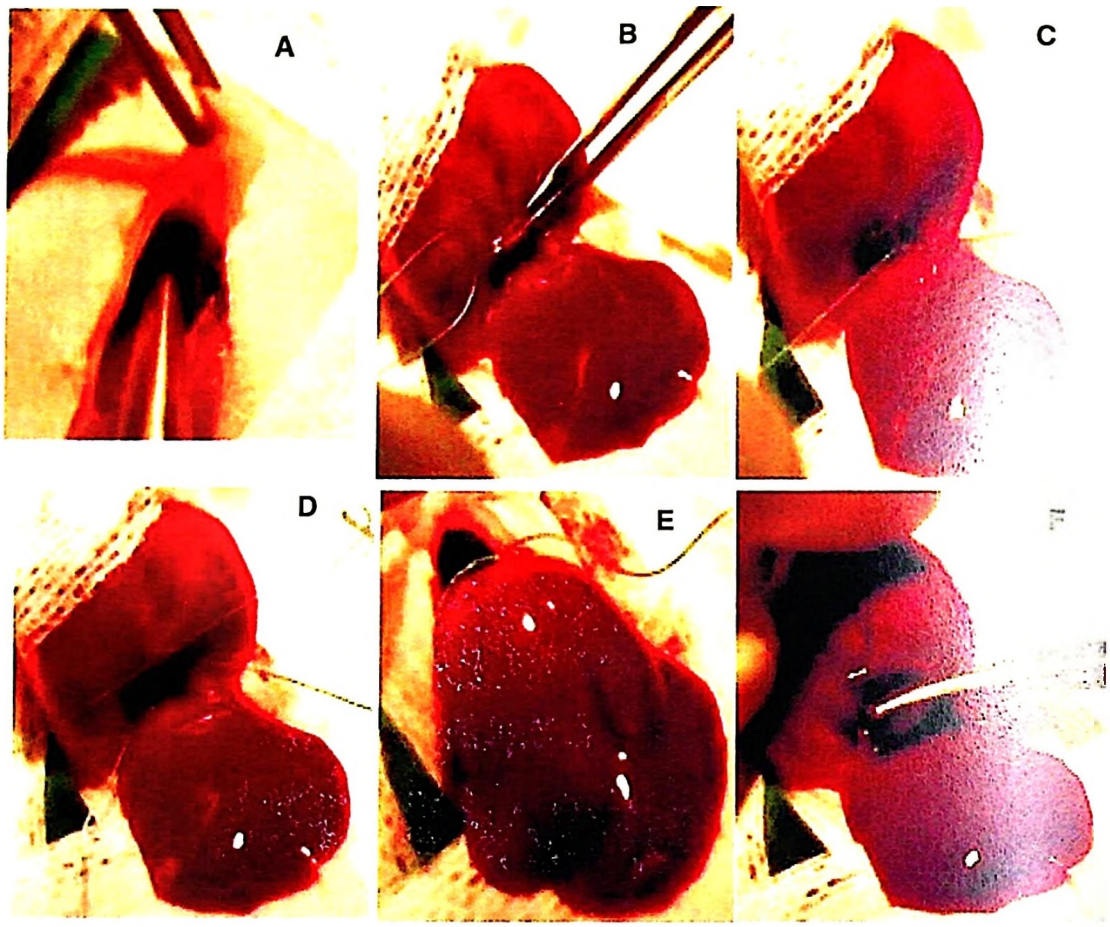

D
vWF positive platelets in the liver
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Average
SERT: +/+
-/-
+/+
-/-
0
5
Time (Minutes) after partial hepatectomy

Fig. 3

Rat PH : Cutting ligament and ligation procedure



Sl.Fig.1

CHAPTER 4

Large Induction of Type III Deiodinase (D3) Expression after Partial Hepatectomy in the Regenerating Mouse and Rat Liver.

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Short title: Type III Deiodinase Expression Induced during Liver Regeneration.

Key words: Deiodination; Hepatectomy; Liver; Tissue Regeneration

DISCLOSURE STATEMENT: The authors have nothing to disclose.

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In revision at Endocrinology

ABSTRACT

The deiodinases type I (D1) and type II (D2) catalyzes the activation of T4 to T3, whereas type III deiodinase (D3) catalyzes the inactivation of T3 and T4. D3 plays a key role in controlling thyroid hormone bioavailability. It is highly expressed during fetal development, but also in other processes with increased cell proliferation, e.g. in vascular tumors. Since tissue regeneration is dependent on cellular proliferation and associated with activation of fetal genes, we evaluated deiodinase activities and mRNA expression in rat and mouse liver after partial hepatectomy (PH), and determined the local and systemic thyroid hormone status after hepatectomy.

We observed that in rats, D3 activity was increased 10-fold at 20 h and 3-fold at 48 h after PH; D3 mRNA expression was increased only 3-fold at 20 h. The increase in D3 expression was associated with maximum 2 to 3-fold decreases of serum and liver T3 and T4 at 20 h after PH. In mice, D3 activity was increased 5-fold at 12 h, 8-fold at 24 h, 40-fold at 36 h, 15-fold at 48 h; and 7-fold at 72 h after PH. In correlation with this, D3 mRNA was highest (6-fold increase) and serum T3 and T4 were lowest at 36 h. Furthermore, as a measure for cell proliferation, BrdU incorporation peaked at 24 h in rats and at 36 h in mice. No significant effect on D1 activity or mRNA expression was found after PH. D2 activity was always undetectable.

In conclusion, we found a large induction of hepatic D3 expression after PH which was correlated with an increased cellular proliferation. The mechanism of D3 induction appears to be mainly post-transcriptional. Our data suggest that D3 is important in the modulation of thyroid hormone levels in the regenerating liver, in which a decrease in cellular T3 permits an increase in proliferation.

Introduction

A strict regulation of thyroid hormone levels is essential for normal development of different tissues, as well as for the metabolic function of these tissues. The bioavailability of thyroid hormone is regulated by three iodothyronine deiodinases (D1, D2 and D3). D1 and D2 are thyroid hormone activating enzymes that catalyze the conversion of the prohormone thyroxin (T4) to the active hormone 3,3',5-triiodothyronine (T3). D1 is expressed in liver, kidney, thyroid and pituitary, and plays an important role in the production of serum T3 (1). D2 is mainly expressed in brain, pituitary and brown adipose tissue. In tissues such as brain, this enzyme is important for the local activation of T3 (1). D3 is the major (voorstel:)enzyme in the inactivating pathway, catalyzing the degradation of T3 to 3,3'-diiodothyronine (T2) and the conversion of T4 to the inactive metabolite reverse T3 (rT3). It is abundantly present in fetal tissues such as liver and brain, and in placenta, pregnant uterus and umbilical arteries and vein (1, 2). The high expression in the fetal compartment suggests that D3 plays an essential role in the regulation of fetal T3 levels, as a mechanism to protect the fetus from excessive exposure to active thyroid hormone. For instance, in the fetal brain, D3 expression is highly regulated in different regions, protecting the brain from excessive T3 until differentiation is required (3).

In contrast to the high D3 activities in the fetus, its activities are normally undetectable in most adult tissues. This is probably why D3 has long been neglected in studies on the regulation of thyroid hormone bioavailability in the adult. However, recent studies have revealed the reexpression of D3 in different pathophysiological conditions,

among which cancer, cardiac hypertrophy, myocardial infarction, chronic inflammation and critical illness (4-9). Just like fetal growth, most of these are processes in which proliferation or cell growth take place.

It is generally believed that T3 stimulates the differentiation of cells and decreases their proliferation. Hernandez et al. recently showed that differentiation of precursor cells to adipocytes was associated with a decrease in D3 expression (10), and Dentice et al. demonstrated that D3 is increased in proliferating keratinocytes (11). Also these findings confirm the concept that in general T3 stimulates cellular differentiation, and suggest a role for D3-mediated low intracellular T3 concentrations in cellular proliferation.

Liver regeneration is also a process dependent on cellular proliferation. Following hepatectomy of 70% of the liver, liver cells switch from a quiescent state to a proliferative state and re-enter the cell cycle (12). In rodents, during the first 4 hours after partial hepatectomy (PH), quiescent hepatocytes enter the cell cycle (G1 phase). This proliferative phase is rather short in rats, but is prolonged in mice, the peak of DNA synthesis being 40-44 h after PH (12, 13). During these processes, many fetal genes, which are not expressed in normal adult liver, are reactivated (12, 14). In the present study we investigated the possible reexpression of type III deiodinase in regenerating liver, by determining deiodinase activities and mRNA expression in rat and mouse liver after PH.

Materials and Methods

Materials

[3'-^{125}I]T3 and [3',5'-^{125}I]T4 were obtained from GE Healthcare (Amersham, UK); and [3',5'-^{125}I]rT3 was prepared by radioiodination of 3,3'-T2 as described previously (15). Non-radioactive iodothyronines were purchased from Henning Berlin GmbH (Berlin, Germany); dithiothreitol (DTT) from ICN Biochemicals Inc. (Costa Mesa, CA). Real-time PCR probes and primers were obtained from from Sigma (St. Louis, MO).

Partial hepatectomy model

Wistar rats were obtained from Hubrecht Laboratory, Utrecht, The Netherlands. Mice on the FVB background were obtained from Harlan, Horst, The Netherlands The animals were allowed free access to food and water. Mice and rats were anesthesized by 2% Isoflurane supplemented with buprenorphine analgesia, and then underwent PH by removing 70% of the liver, or only opening of the abdominal skin in the case of sham-treated control animals. Serum and liver tissues were collected at PH (t=0) and at different time periods after PH. The same region of the liver was isolated from sham-treated controls. All liver samples were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis; serum was stored at –20 °C. All procedures performed on the animals were approved by the Committee on Animal Experiments of the Utrecht University (protocols 102648-1, 102648-2).

Determination of BrdU incorporation

Two hours before ending the experiment animals received an intraperitoneal injection with BrdU in PBS, using 30 µg/gram body weight BrdU for mice or 50 µg/gram body weight BrdU for rats.After the animals were sacrificed liver tissues were fixated in 10% formalin and imbedded in paraffin.Slides were stained overnight with primary mouse anti-BrdU antibody M0744, clone Bu 20a (Dako, Heverlee, Belgium), diluted 1:50 for rats and 1:100 for mice. Subsequently, peroxidase-conjugated rabbit anti-mouse antibody was applied and immunoreactivity was detected using the DakoCytomation Envision system, HRP (Dako, Heverlee, Belgium) according to the manufacturer's guidelines. Slides were counterstained with Haematoxylin for 30 seconds and mounted in Eukitt mounting medium. Percentages of BrdU positive cells per slide were calculated after counting 1000 nuclei per animal.

Determination of T4 and T3 concentrations in serum and liver samples

Serum T4 and T3 were measured by Vitros ECI technology (Immunodiagnostic System, Ortho-Clinical Diagnostics, Beerse, Belgium). Because of small tissue volumes liver tissues of three different rats per time period after hepatectomy were pooled for the determination of liver T4 and T3. The iodothyronine levels were determined by highly sensitive and specific radio immuno assays after extraction and purification of the iodothyronines from the liver, as previously described (16).

Deiodinase activity assays

Tissues were homogenized on ice in 10 volumes 0.1 M phosphate (pH 7.2), 2 mM EDTA, containing 1 mm DTT, using a Polytron (Kinematica, Lucerne, Switzerland). The tissue homogenates were stored at -80 °C until further analysis. Protein concentrations were determined using the method of Bradford (17), using BSA as standard. D1 activities were determined by incubation of 0.1 µM rT3 (including 200,000 cpm [3',5'-^{125}I]rt3) for 30 min at 37 °C with 10 µg protein/mL tissue homogenate in 0.1 ml 0.1 M phosphate (pH 7.2), 2 mM EDTA, 10 mm DTT (PED10). D2 and D3 activities were determined by incubation of 1 nM [3',5'-^{125}I]T4 (200,000 cpm, D2) or 1 nM T3 (including 200,000 cpm [3'-^{125}I]T3, D3) for 60 min at 37 °C with 5 mg protein/mL tissue homogenate in 0.1 M PED10. Reactions were stopped by the addition of 0.1 ml ice-cold methanol. After centrifugation, 0.1 ml supernatant was mixed with 0.1 ml 0.02 M ammonium acetate (pH 4.0), and 0.1 ml of the mixture was applied to a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands), and eluted with a gradient of acetonitrile in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 mL/min. The proportion of acetonitrile was increased linearly from 30 to 44% in 10 min. The radioactivity in the eluate was determined using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

RNA isolation and quantitative RT-PCR

RNA from rat liver was isolated from 25 mg liver tissue using the high pure RNA tissue isolation kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's guidelines. RNA from mouse liver was isolated using Trizol (Invitrogen,

Breda, The Netherlands). Subsequently, 10 µg RNA was treated with 10 U DNAse (Promega, Leiden, The Netherlands) for 30 min at 37 °C, and purified using the RNeasy mini kit RNA cleanup protocol (Qiagen). 500 ng RNA was used for cDNA synthesis using TaqMan RT reagents (Roche Diagnostics). RNA samples were verified to be free from genomic DNA by performing negative control cDNA synthesis reactions of 500 ng total RNA using Taqman RT reagents without reverse transcriptase.

Quantitative real time PCR was carried out using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Reactions were performed in 25 µl Taqman universal PCR master mix (Roche), containing 20 ng cDNA. mRNA levels are expressed relative to the total RNA content. Per reaction 200 nM of primers and probe were used for D3,D1 and for the housekeeping gene β-actin. Real time PCR primers and probe for the house keeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were provided as preoptimized control system (Applied Biosystems). Table 1 shows the sequences of the different primers and probes. Real time PCR reactions were done for 2 minutes at 50 ^{0}C and for 10 minutes at 95 ^{0}C , followed by 40 cycles of 15 sec at 95 ^{0}C and for 1 min at 60 ^{0}C. Cycle at threshold (Ct) values represent the cycle numbers at which probe-derived absorbance reaches the calculated threshold value. ΔCt represents the Ct value of the housekeeping gene minus the Ct value of the target gene. Data are expressed as $2^{(n-Ct)}$ and as $2^{\Delta Ct}$xn (relative number of mRNA copies).

Results

Effects of PH on liver deiodinase expression and BrdU incorporation

We first tested deiodinase activity in rat liver at different time periods after PH (t=0). Figure 1A shows that D3 activity was increased 10-fold at 20 h and 3-fold at 48 h after PH, whereas no significant effect on D1 activity was found. D2 activity was absent in all rat liver samples. Next, we determined rat liver deiodinase mRNA levels, GAPDH and β-actin levels. We found not only upregulation of D3, but also of the house keeping genes GAPDH and β-actin (results not shown). Also other reports show that GAPDH and β-actin are strongly upregulated after PH (18) or under other conditions of cell proliferation (19, 20). Therefore, we expressed deiodinase mRNA levels relative to total RNA content. D3 mRNA expressed deiodinase mRNA expression tended to increase at 20 hours, where as D1 mRNA expression tended to decrease (Fig.1B).

We also studied the effects of PH on deiodinase activity and mRNA levels in mice. In this model, D3 activity was increased 5-fold at 12 h, 8-fold at 24 hour, 40-fold at 36 h, 15-fold at 48 h, and 7-fold at 72 h after PH compared to t=0, whereas SHAM-treated animals show only a 4-fold increase at 48 h after PH (Fig. 2A). D1 activity decreased 2-fold compared to t=0, but was not affected compared to SHAM-treated animals 48 h after PH (Fig.2A). In all mouse livers D2 activity was absent. Figure 2B shows a 6-fold increase of D3 mRNA at 36 h after PH compared to t=0, and no significance in D1 mRNA levels between time periods after PH. GAPDH and β-actin mRNA levels were not affected by PH in mice (21, data not shown). Figure 3 depicts the levels of BrdU

incorporation as a measure of cellular proliferation status at different time periods after PH. In correlation with D3 activity, the BrdU level peaked at 20 h to 24 h in rats (data not shown), and at 36 h after PH in mice (Fig.3).

Effects of PH on systemic and local thyroid hormone levels

Finally, we analyzed local and systemic thyroid hormone levels at PH and at different time periods thereafter. In rats, liver and serum T3 and T4 levels decreased markedly, with maximum 2 to 3-fold decreases at 20 h after PH (Figs. 4A and 4B). This correlated well with the peak in D3 activity at 20 h after PH in rats (Fig.4C). In mice, serum T3 and T4 decreased until minimum levels at 36 h after PH (Fig. 5), coinciding with the peak in D3 activity and BrdU incorporation at 36 h after PH.

Discussion

D3 is highly expressed in tissues of the feto-placental unit, and has therefore long been considered to be mainly important in the regulation of the fetal thyroid hormone status. By inactivating the active thyroid hormone T3 and the prohormone T4, D3 is thought to protect developing organs against excessive exposure to thyroid hormone (22). Although D3 is not expressed in most normal adult tissues, recent studies have demonstrated the reexpression of the enzyme in various disease states. For instance, D3 is induced in liver and skeletal muscle of critically ill patients (6), and high levels of D3 are found in vascular tumors (4, 5). Furthermore, in different animal models, D3 was found to be induced in cardiac hypertrophy, myocardial infarction and chronic inflammation (7-9). The finding that in some of these conditions the accelerated

degradation of thyroid hormone causes low serum T3 (6) or even severe hypothyroidism (4, 5) indicates the clinical importance of D3 in the regulation of local and systemic thyroid hormone status.

Not only fetal growth but also the above-mentioned pathophysiological conditions are processes in which cellular growth or proliferation take place. Therefore, we hypothesized that D3-mediated low intracellular T3 levels are needed for cellular proliferation. Interestingly, Hernandez et al. recently showed high D3 expression in proliferating preadipocytes from brown adipose tissue, and a marked decrease in D3 expression when the cells differentiate into mature adipocytes (10). Additionally, by knocking down D3 in basal cell carcinomas Dentice et al. recently proved that D3 promotes the proliferation of malignant keratinocytes by inhibiting the differentiating action of T3 (11). Liver regeneration is also dependent on cellular proliferation and involves induction of various fetal genes (14). Therefore, in the present study we investigated deiodinase expression, local and systemic thyroid hormone status in rat and mouse liver after PH. We found a 10 to 40- fold induction of hepatic D3 activity after PH which was associated with an increased cellular proliferation and decreased local and circulating T3 and T4 levels.

It should be noted that D1 activity was 2-fold decreased and D3 activity was 4-fold increased in SHAM-treated mice compared to t=0. Therefore, although D1 activity after PH is 2-fold decreased compared to t=0, it is not significantly altered compared to SHAM-treated controls. However, when D3 activity after PH is compared to SHAM-treated controls, it remains highly induced after PH. The mechanisms involved in the

decrease of D1, and the increase of D3 activity after SHAM-treatment is unknown. The combined upregulation of D3 and downregulation of D1 by the SHAM operation may be caused by illness (6) and/or inflammation (9). Also the possible influence of the used anesthetics on deiodinase expression should be considered, since it is well known that some anesthetics alter thyroid function. We used the opiate buprenorphine. Baumgartner et al. showed an acute downregulation of D2 and upregulation of D3 in rat brain after administration of the mu-opiate agonist etonitazene (23).

We measured tissue and serum T3 and T4 levels, and found a significant decrease of local and systemic thyroid hormone levels at 20 h after PH in rat and at 36 h after PH in mice, which correlated well with the increase in D3 at these time periods. Tien et al. also studied rT3 levels in mouse liver after PH, and reported on a significant increase in the rT3 level after PH compared to SHAM-treated controls at 36 h after PH, which was correlated to a significant decrease in the T3-regulated genes tyrosine aminotransferase (TAT) and basic transcription element binding protein (BTEB) (21). In agreement with our data, they found 50% decreased D1 at 36 h after PH compared to SHAM-treated controls. However, because of nearly undetectable D3 mRNA expression levels, they did not determine D3 activities (21).

The molecular mechanism responsible for the reactivation of D3 is still poorly understood. In the proliferating skin hedgehog signalling is involved in the induction of Dio3 expression (11). However, gene expression microarray analyses of regenerating liver after PH do not indicate that the sonic hedgehog/gli protein pathway is involved in this process (24-27). Another possibility is the TGFβ/Smad signalling pathway, which

has been shown to induce Dio3 gene transcription in different cell types (28). Actually, TGFβ1 expression does increase after PH, with a peak level 24-48 h after operation (29-31). However, this increase in TGFβ is accompanied by a downregulation of the TGFβ receptors and by increase of the transcriptional repressors SnoN and Ski (29-31), and TGFβ does not increase but suppresses hepatocyte proliferation through direct effects on the hepatocytes (32). Interestingly, Simonides et al. recently showed that hypoxia induces D3 expression via a hypoxia inducible factor (HIF)-dependent pathway (32). Another mechanism involved in the induction of D3 after PH might be cellular hypoxia. Interestingly, Simonides et al. recently showed that hypoxia induces D3 expression via a hypoxia inducible factor (HIF)-dependent pathway (33). Since PH is well recognized as a hypoxic-ischemic injury, and HIF-1α is induced after PH (34), the HIF-dependent pathway may well contribute to the induction of D3 after PH. However, the effect of HIF-1α on D3 expression is at the transcriptional level, whereas our data suggest that in the regenerating liver also post-transcriptional mechanisms are involved in D3 induction.

Regulation of D3 expression is complex, because the *Dio3* gene is imprinted, with preferential expression from the paternal allele (35). The gene is located in an imprinted region on human chromosome 14, mouse chromosome 12 and rat chromosome 6. One of the other imprinted genes in this region is called *Dio3os*, and is transcribed from the opposite DNA strand compared to *Dio3* (35). Interestingly, Hernandez et al. demonstrated an inverse correlation of the paternally imprinted *Dio3* gene with the maternally imprinted *Dio3os* gene (10). This *Dio3os* gene partially overlaps the coding

region of the *Dio3* gene, and may thus interfere with the translation of the Dio3 transcript. We are currently investigating the influence of Dio3os transcripts on D3 mRNA expression and translation of Dio3 transcripts.

In summary, we found a large induction of D3 expression in regenerating liver which was correlated with an increased cellular proliferation and decreased local and systemic T3 and T4 levels. Future research should elucidate the role of *Dio3os* in the regulation of Dio3 transcription and translation. Our findings support the concept that D3-mediated low cellular T3 is needed for cellular proliferation. Future studies, e.g. using conditional D3 knock-out mice, should further unravel the role of D3 in this process.

Acknowledgements

This work was supported by by NWO grant 916-56-186.

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Legends

Fig.1. Effect of partial hepatectomy in rats on D1 and D3 activity (A) and mRNA level relative to total RNA content (B). Reaction conditions for the D3 activity assay were 1 nM 125I-labeled T3, 5 mg protein/ml tissue homogenate, and 1 h incubation. Reaction conditions for the D1 assay were 100nM 125I-labeled rT3, 10 µg protein/ml tissue homogenate, and 1 h incubation. Results are the means±SEM (n=2-4). Significance of differences is indicated as follows: ***: $P<0.001$ *vs.* control (0); **: $P<0.01$ *vs.* control (0)

Fig. 2. Effect of partial hepatectomy in mice on D1 and D3 activity (A) and mRNA level corrected for total RNA content (B). Reaction conditions for the D3 activity assay were 1 nM 125I-labeled T3, 5 mg protein/ml tissue homogenate, and 1 h incubation. Reaction conditions for the D1 activity assay were 100 nM 125I-labeled rT3, 10 µg protein/ml tissue homogenate, and 1 h incubation. Results are the means±SEM (n=3). Significance of differences is indicated as follows: ***: $P<0.001$ *vs.* control (0); **: $P<0.01$ *vs.* control (0); ++: $P<0.01$ *vs.* control (48 SHAM); +: $P<0.05$ *vs.* control (48 SHAM).

Fig. 3. Effect of partial hepatectomy on BrdU incorporation in mice. Results are the means±SEM (n=3). Significance of differences is indicated as follows: ***: $P<0.001$ *vs.* control (0).

Fig. 4. Effect of partial hepatectomy on liver T3 and T4 levels in rats (A) or on serum T3 and T4 levels in rats (B), or on D3 activity and liver and serum T3 levels in rats (C). Results are single determinations from pools of two to four tissues per time period (A) or means±SEM (n=2-5). In Fig. B significance of differences is indicated as follows: **: $P<0.01$ *vs.* control. (0); *: $P<0.05$ *vs.* control (0).

Fig. 5. Effect of partial hepatectomy on serum T3 and T4 levels in mice. Results are the means±SEM (n=3-5). Significance of differences is indicated as follows: ***: $P<0.001$ *vs.* control (0); **: $P<0.01$ *vs.* control. (0); *: $P<0.05$ *vs.* control (0).

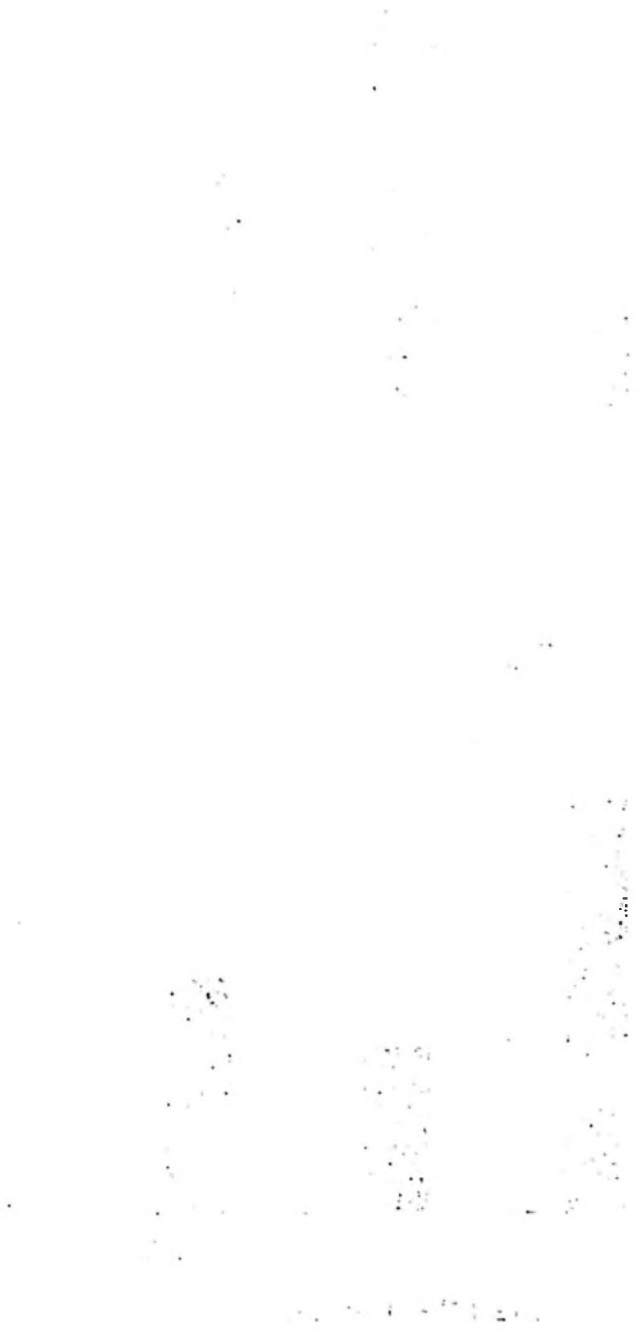


Fig. 1A

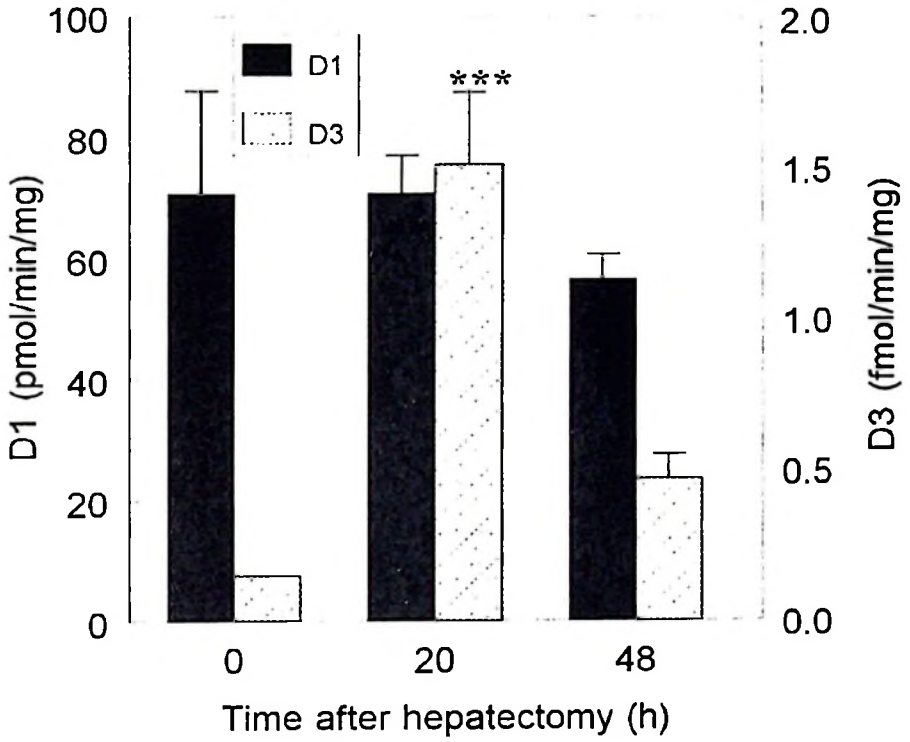
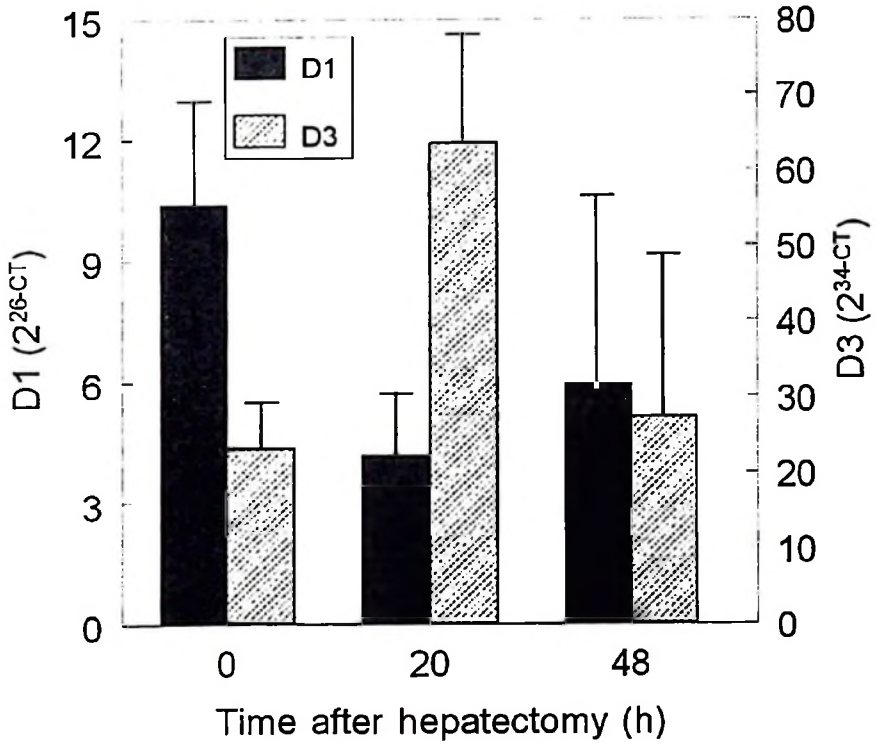

100
80
60
40
20
0
D1 (pmol/min/mg)
D1
D3
***
2.0
1.5
1.0
0.5
0.0
D3 (fmol/min/mg)
0
20
48
Time after hepatectomy (h)

Fig. 1B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12
9
6
3
0
D1 (2^26-CT)
D1
D3
80
70
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30
20
10
0
D3 (2^34-CT)
0
20
48
Time after hepatectomy (h)

Fig. 2A

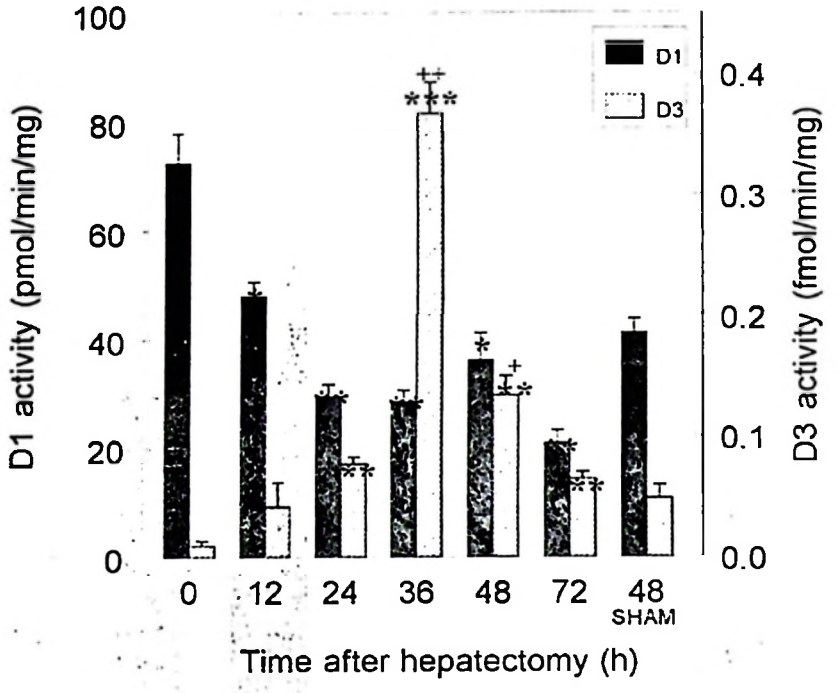
D1
D3
D1 activity (pmol/min/mg)
D3 activity (fmol/min/mg)
100
80
60
40
20
0
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
72
48
SHAM
Time after hepatectomy (h)

Fig. 2B

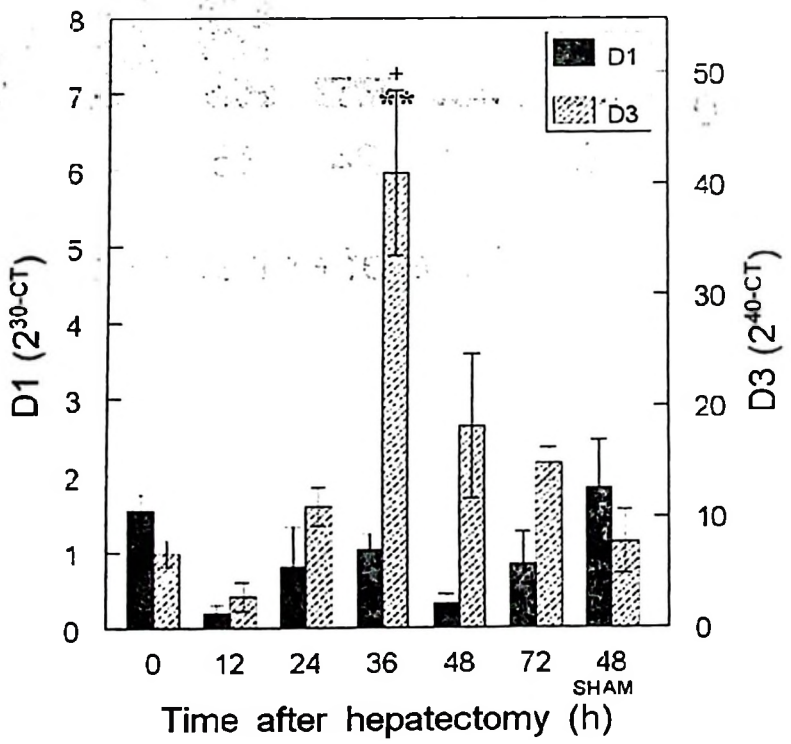
D1
D3
D1 (2^30-CT)
D3 (2^40-CT)
8
7
6
5
4
3
2
1
0
50
40
30
20
10
0
0
12
24
36
48
72
48
SHAM
Time after hepatectomy (h)

Fig. 3

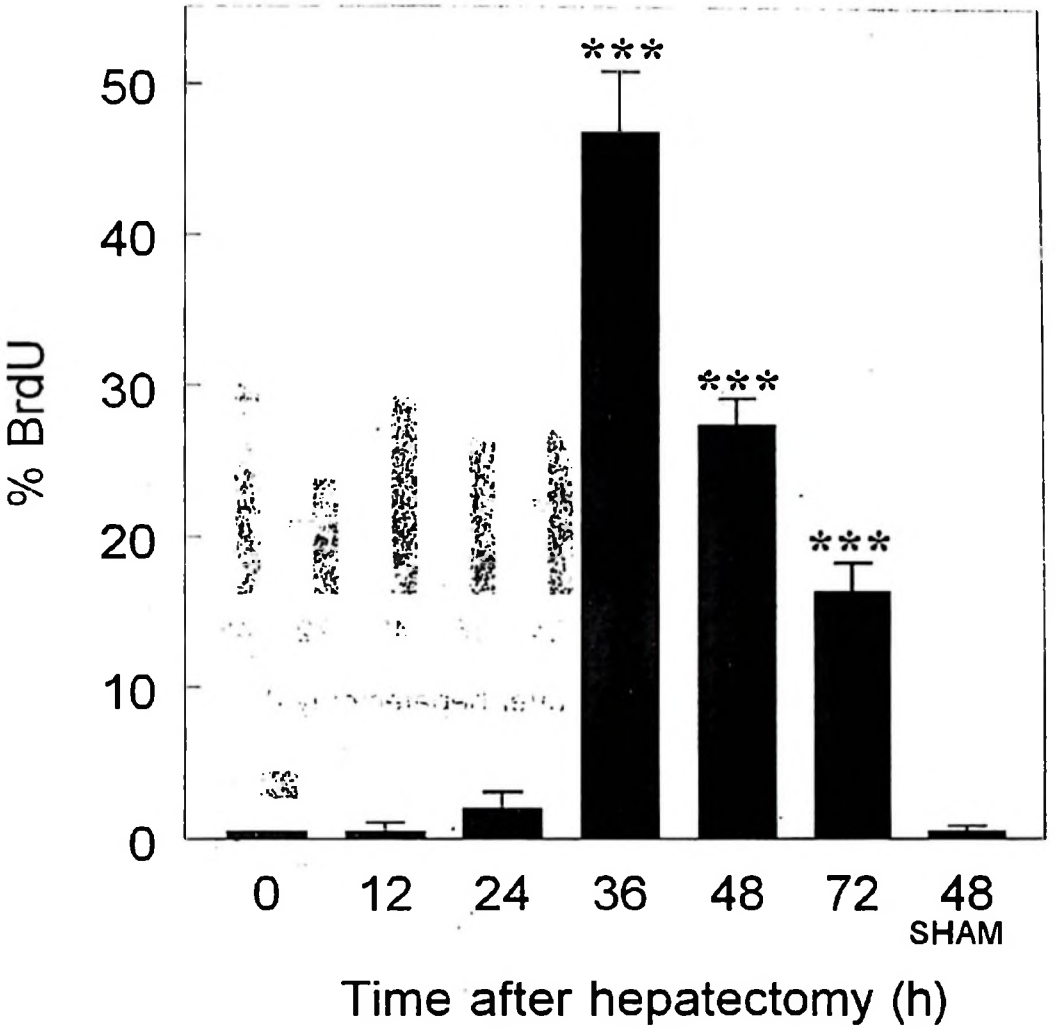
% BrdU
50
40
30
20
10
0
***
***
***
0
12
24
36
48
72
48
SHAM
Time after hepatectomy (h)

Fig. 4A

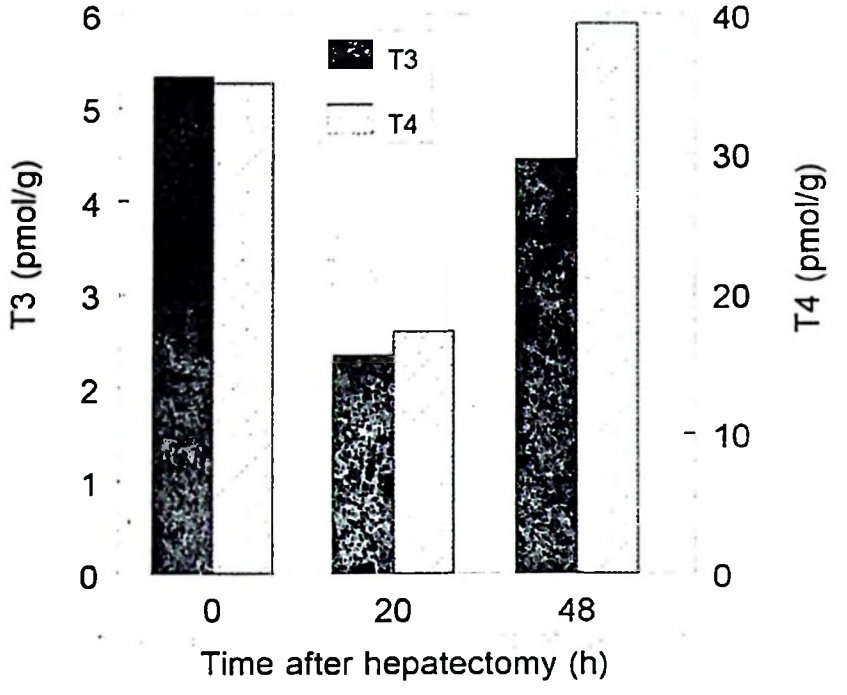
T3
T4
T3 (pmol/g)
T4 (pmol/g)
0
1
2
3
4
5
6
0
10
20
30
40
0
20
48
Time after hepatectomy (h)

Fig. 4B

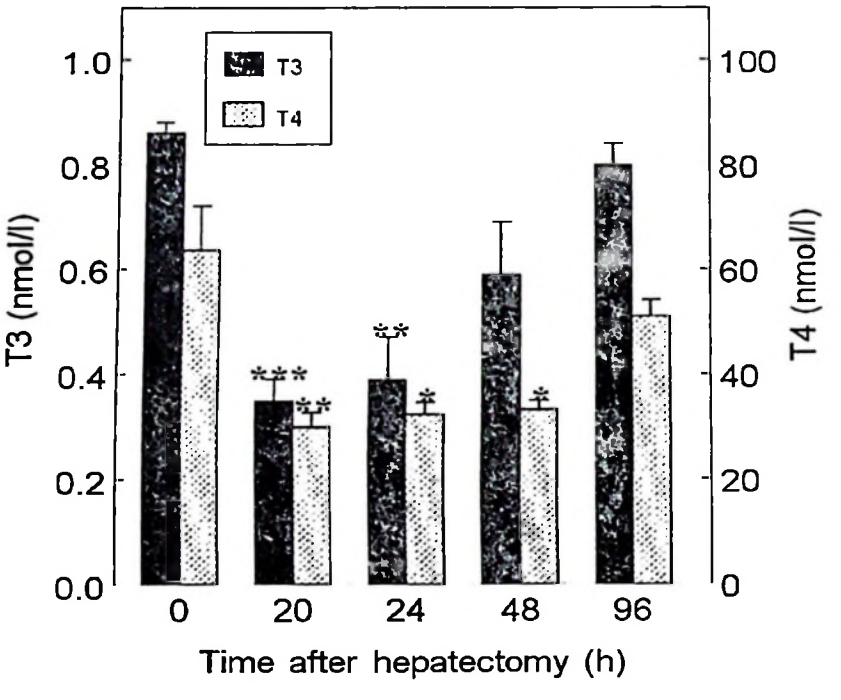
T3
T4
T3 (nmol/l)
T4 (nmol/l)
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
***
**
*
*
0
20
24
48
96
Time after hepatectomy (h)

Fig. 4C

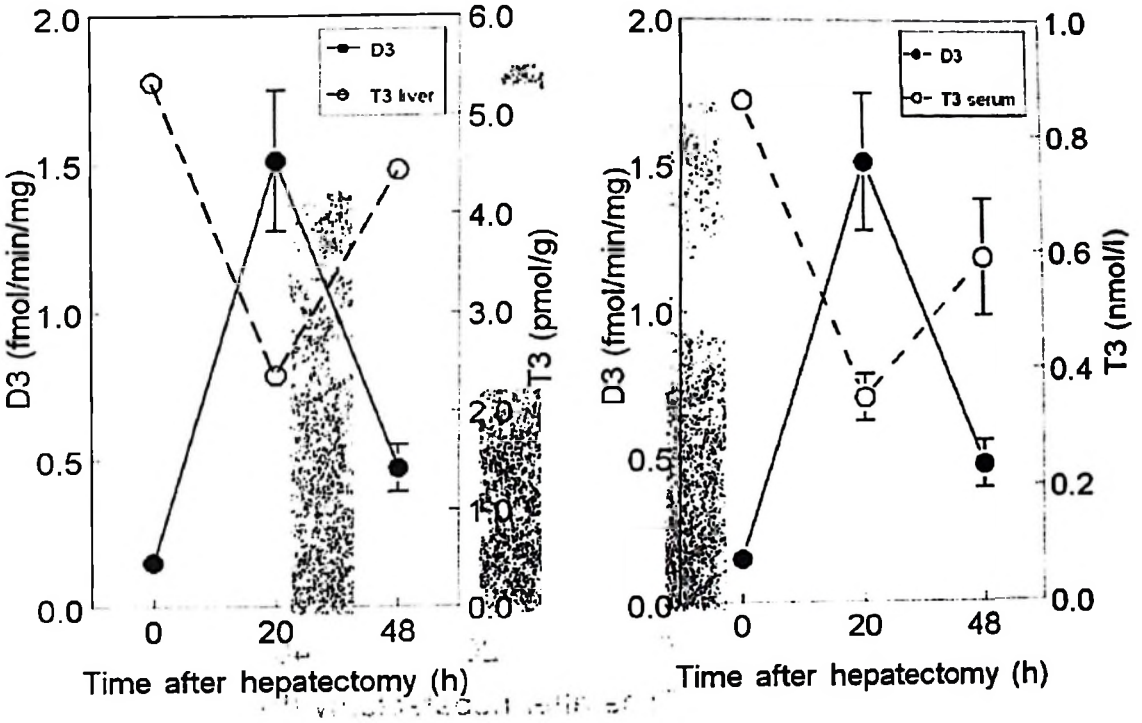
D3
T3 liver
D3 (fmol/min/mg)
T3 (pmol/g)
Time after hepatectomy (h)
D3
T3 serum
D3 (fmol/min/mg)
T3 (nmol/l)
Time after hepatectomy (h)

Fig. 5

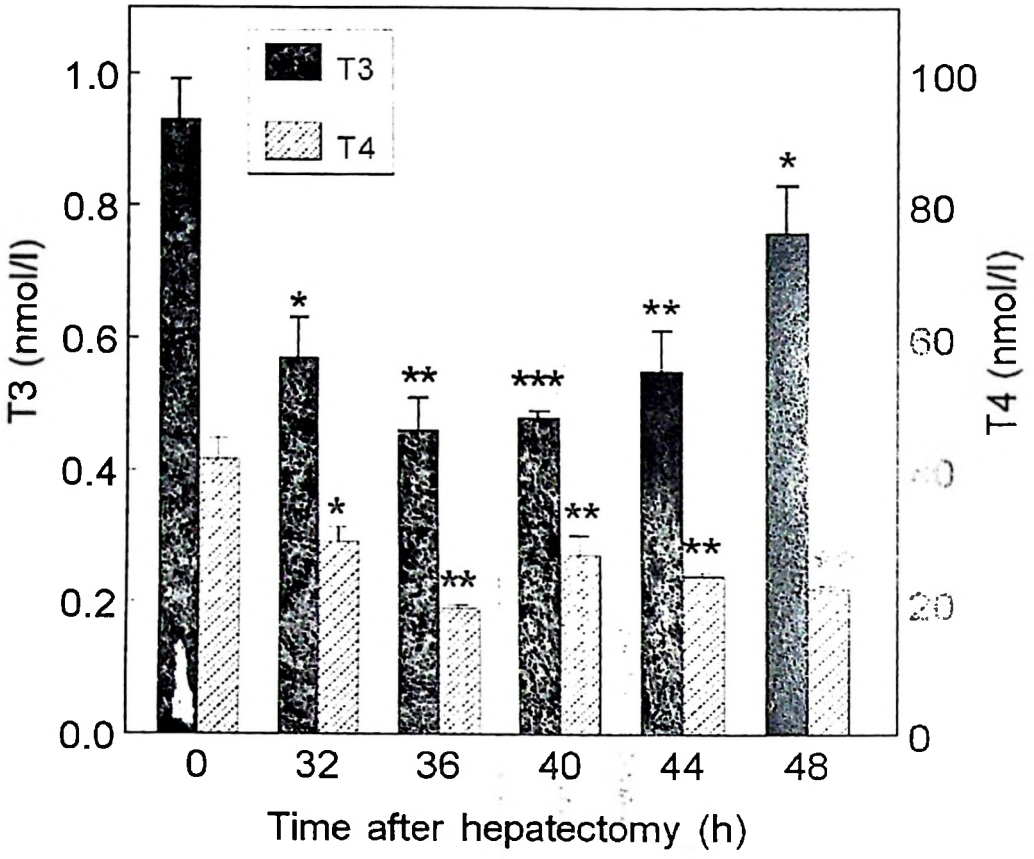
T3
T4
1.0
0.8
0.6
0.4
0.2
0.0
T3 (nmol/l)
100
80
60
40
20
0
T4 (nmol/l)
*
*
**
***
**
**
*
**
0
32
36
40
44
48
Time after hepatectomy (h)

Table 1. Oligonucleotide primers used for real-time PCR.

Gene	Sense primer	Antisense primer	Probe
D3	5'-TTCATGGCGCGGATGAG-3'	5'-GATGATAAGGAAGTCAACGTCGC-3	'5'-FAM-TTCCAGCGCCTGGTCACCAAGTACC-TAMRA-3'
D1	5'-ATTTGACCAGTTCAAGAGACTCGTAG-3'	5'-CCACGTTGTTCTTAAAAGCCCA-3'	5'-FAM-TCATTTACATTGAAGAAGCTCACGCCACAGA-3'
β-actin	5'-TGACCCAGATCATGTTTGA-3'	5'-TAATGTCACGCACGATTTC-3'	5'-FAM-ACCGAGCGTGGCTACAGCTT-TAMRA-3'

CHAPTER 5

Summary

Retinoblastoma (Rb) and p53 tumour suppressor genes are frequently mutated or inactivated in human hepatocellular carcinomas.The fact that simultaneous deletion of these genes in the mouse liver did not induce liver cancer signal the complexity of liver carcinogenic process and further indicates that other factors are required. Liver regeneration is known to be among the factors involved in the development of liver cancer. Many cases of human HCC involving chronic liver injury and regeneration has been described, in most cases involving viral infections or alcoholic liver injury. Also recurrence after treatment of colorectal liver cancer by surgical resection is common and usually accompanied with metastasis to other organs. Understanding the actual role of liver regeneration in carcinogenesis may be important not only in the understanding the pathogenesis of liver cancer but also may be helpful in determining effective treatment of cancer patients, which is currently lacking.

Like in the liver cancer, the process of liver regeneration which occurs as a result of decreasing liver mass is complex, and many biological and pathological processes have been found to be involved in the liver regeneration process. Among them includes hepatic necrosis, apoptosis, surgical resection, hepatocyte proliferation or activation of hepatic progenitor cells. Activation of hepatic progenitor cells occurs in situation where hepatic and biliary cells are not able to replicate or in case these can replicate but they are not sufficient.

Irrespective of the cell type involved in the liver regeneration process, the process is known to be initiated by cytokines triggered by liver injury. This early phase is considered to be important as these cytokines confers the cells ability to respond to subsequent growth stimuli from various growth factors. Growth factors are therefore considered to play a secondary role, stimulating primed hepatocyte and biliary cells to proliferate. Many growth factors have been described; among them include platelet derived serotonin (5HT) and thyroid hormone (T3). These factors are known to induce expression of cyclins, important for cell cycle entry.

5HT is synthesized in the enterochromaffin cells of the gastro intestinal tract and neurons. More than 95% of circulating 5HT is stored platelets with the help of Setotonin transporter (SERT). We have recently shown that, platelets in rats lacking SERT lose the ability to take in 5HT for storage. Also other investigators have reported that lack of SERT does not affect 5HT synthesis.

Thyroid hormone, another growth factor involved in liver regeneration is secreted from thyroid gland as inactive T4. Activation of T4 to T3, the form which is capable of activating thyroid hormone receptors, requires de-iodination of T4 to T3.This process is catalysed by deiodinase enzymes wheres as type 1 (D1) and type 2 (D2) catalysyses activation of T4 to T3 and type 3 (D3) catalyses inactivation of T3 and T4. D3 is known to be crucial in controlling T3 and T4 bioavaillability and is highly expressed during fetal development as well as in vascular tumours.

The aims of this thesis were to investigate the role of liver regeneration in liver cancer following two serial partial hepatectomy (PH) at 70 days interval, the role of platelet derived serotonin in rat liver regeneration, and to evaluate deiodinase activities and mRNA expression in rat and mouse liver using one time 70% PH model.

In chapter 2 we performed two PH at 70 days interval in mice with liver specific conditional deletion of p53 and Rb. We show that more than 60% of mice deficient of p53 and Rb in the liver developed undifferentiated hepatocellular carcinomas 4-5 weeks after second partial hepatectomy. We also show that these cancers develop at one anatomical location and some metastasize to other organs including the regional lymph nodes and surrounding tissues, and histologically neoplastic cells resemble oval cells. Surrounding non neoplastic liver regions show increased proliferation of oval cells around the portal triad. In addition neoplastic liver and surrounding liver show elevated expression of alpha-fetoprotein, an oval cell marker along with elevation of E2F1 transcription factor, an downstream partner of Rb. These findings show that, oval cell proliferation and undifferentiated hepatocellular carcinomas develop in mice deficient of p53 and Rb upon PH.

In chapter 3 we performed 70% PH in SERT proficient and SERT deficient rats and study BrdU incorporation at 20, 24, 48 and 96 hours after PH. SERT deficient rats show marked decrease in liver and blood 5HT associated with prolonged bleeding. However, we show that these rats exhibit normal liver regeneration after PH. The results show that a release of high level of 5HT from platelets is not required for liver regeneration, and that low level of 5HT in blood and/or liver are sufficient for liver regeneration.

In the last chapter, we evaluated the deiodinase activities after 70% PH in rats and mice. D3 enzyme activities increased 10 fold, while mRNA increased 2-3 folds at 20hours after PH along with 2-3 fold decrease of serum and liver T3 and T4. D3 activity increased 5-fold at 12, 8-fold at 24, 40-fold at 36, 15-fold at 48, and 7-fold at 72hours after PH. Expression of D3 mRNA was observed to be highest (6-fold) at 36hours after PH, correlating with high activity of D3. Our results show that large induction of hepatic D3 expression after PH correlated with an increased cellular proliferation, suggesting that D3 is important in the modulation of thyroid hormone levels in the regenerating liver, in which a decrease in T3 permits an an increase in cellular proliferation.

Acknowledgments:

I would like to extend my thanks to NUFFIC (Netherlands organization for international cooperation in higher education) for granting me fellowship for my stay and study in the Netherlands. Also I would like to express my thanks and gratitude to Sokoine University of Agriculture for financial assistance, and granting me permission to pursue this study.

I would like to thank my supervisor Prof Alain de Bruin for his tireless supervision and guidance throughout my research and study.

I am grateful to Prof Andrea Grone and members of Pathobiology Department who participated in teaching pathology courses. I would like to thank former and current fellow members of *in vivo* study, in the Tissue Repair group, Hilda Toussaint, Peter Tooten, Carine Punt, Ranitha Vongpromek, and Sushil Pandit. Also I would like to thank Judith Homberg and other Members of Edwin Cuppen group (Hubrecht Laboratory), and group of Theo J. Visser (Erasmus Medical Centre, Rotterdam) for the assistance and/or their valuable contribution in this thesis.

I also extend my thanks to Peter Cornelissen, Tony Ultee and all members in the histology and Immunohistochemistry laboratory, all former and current members of Tissue repair group. Thanks to Martin van Leeuwen (Small animal haematology laboratory); Hans Vernooij (Department farm Animal health) for his statitistical guidance, my fellow students and all staffs in the Department of Pathobiology. I sincerely appreciate the support and assistance I received from the office of international cooperation throughout my application for this course and my stay in the Netherlands. The same apply to all members of the Faculty of Veterinary Medicine and University of Utrecht who are not mentioned here but have contributed in one way or another in this thesis.