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REGULATION OF ADULT STEM CELLS IN MOUSE LIVER TUMORIGENESIS

Master of Science thesis

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TARTU 2013

CONTENTS

ABBREVIATIONS

ASC – Adult Stem Cell

BSA – Bovine Serum Albumin

CCA – Cholangiocellular Carcinoma

CSC – Cancer Stem Cell

DEN – Diethylnitrosamine

EGF – Epidermal Growth Factor

EGTA – Ethylene Glycol Tetra acetic Acid

FGF19 –Fibroblast Growth Factor 19

FHCC – Fibrolamellar Hepatocellular Carcinoma

HBV – Hepatitis B Virus

HCC – Hepatocellular Carcinoma

HCV – Hepatitis C Virus

PB – Phenobarbital

PBS – Phosphate Buffer Saline

PDGF – Platelet Derived Growth Factor

PFA – Paraformaldehyde

TGF-α – Transducing Growth Factor-α

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of malignant liver cancer and the third most common cause of cancer related mortality worldwide. The main reason for this is the lack of effective treatment approaches. Although the majority of HCC cases occur in Asia, it is as well a steadily increasing problem in the Western world, where it is primarily connected to severe alcoholic abuse and viral hepatitis.

During the last several years there has been a progress in understanding the mechanisms underlying the development of liver cancer, including the role of stem/progenitor cells in tumor initiation and progression. Transformed adult stem cells (ASC) in the liver, or cancer stem cells (CSC) appear to be the cause of cancer spread and relapse in some cases. Nevertheless, the changes that precede the malignant transformation of liver cells are largely unknown, and there is very little information about the changes occurring before the onset of tumor growth. A distinctive characteristic of this study is that I observed the appearance of liver cancer as a constant process, evolving from the point of initiation to the terminal (lethal) stage, rather than dealing only with advanced tumors. This approach allowed us to build a timeframe of events, which accompany the transition from normal liver tissue to cancer and follow how liver stem cells (SC) engage in this process.

This work is part of a larger project, which investigates the role of normal liver tissue SC in the formation of liver cancer. The ultimate goal is to get a more accurate idea about the distinctive features of the cell of origin in liver cancer, which would allow to develop more effective strategies for prevention of this deadly disease.

1.BACKGROUND

1.1 Adult stem cell concept and liver maintenance

1.1.1 Adult liver stem cell concept

Adult stem cells (ASC) are generally defined as undifferentiated cells, which reside in certain tissue compartments and by differentiating along certain pathways, give rise to the major cell types, which compose a mature tissue. The most distinct characteristics of all stem cells are the ability of self-renewal and the capability to give raise to distinct cell types that make up a tissue. In case of the liver, the definition of stem cells is difficult, due to the self-renewing ability of mature biliary cells and hepatocytes (Santoni-Rugiu *et al*., 2005; Doulatov et al., 2012) (Figure 1).

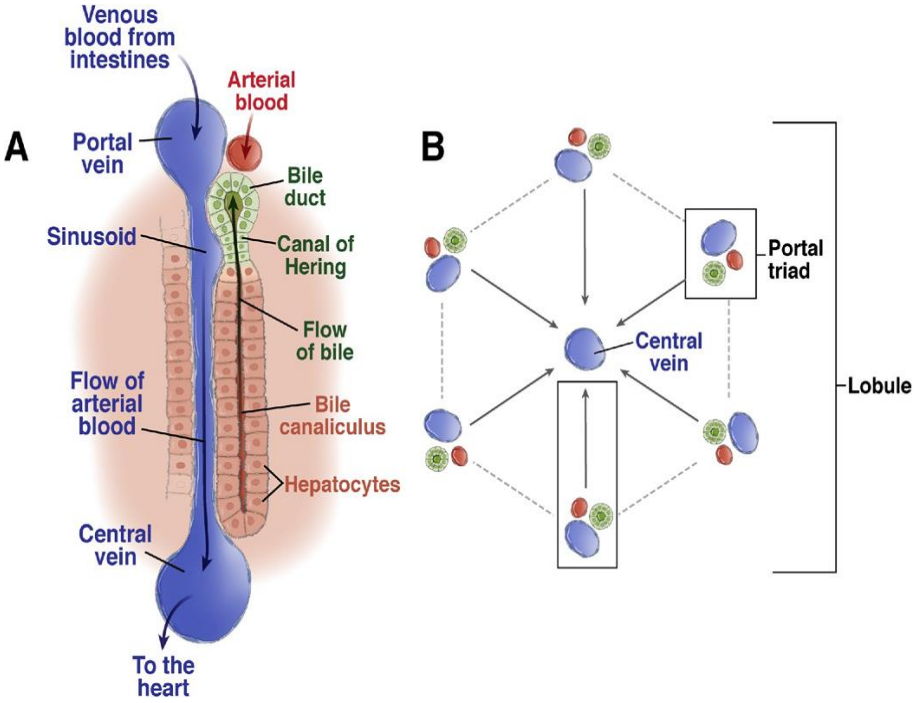


Figure 1. Functional unit of the liver. A) Structure of the portal area B) Structure of the liver lobule (Duncan et al., 2009).

1.1.2 Adult stem cells and liver maintenance

The interplay between distinct cell populations in the liver underlies its ability to maintain the size of the organ during physiological cell turnover as well as different kinds of injuries. On the one hand, the liver consists of a great number of differentiated hepatocytes and cholangiocytes that are capable of self-replication, on the other hand, a small population of cells bearing stem cell properties are present, whose role in liver homeostasis is not

completely clear, but the ability to produce liver tissue has been confirmed in transplantation studies (Schmelzer *et al.*, 2007).

It is hypothesized, that the role of these cell populations in liver regeneration and turnover might differ between rodent models and humans. Recent findings show, that in contrast to rodents, stem cells might play a role in human liver tissue turnover (Schmelzer *et al.*, 2007). According to this model new hepatocytes arise from the stem cells in the portal area and migrate in the direction of the central vein (Fellous *et al*., 2009) (Figure 1, B). This model is known as the „Flowing Liver Model“ (Zajicek *et al.*, 1985). However, most results suggest that the role of stem cells in maintenance of normal liver tissue is minor and the turnover is accomplished by the proliferation of hepatocytes and biliary cells (Malato *et al*., 2011).

Stem cells seem to play a critical role in liver regeneration when the proliferation of differentiated cells is impaired due to intoxication or chronic injury, which results in the expansion of progenitor cells, a process known as the oval cell reaction (Okabe *et al*., 2009) (Figure 2)

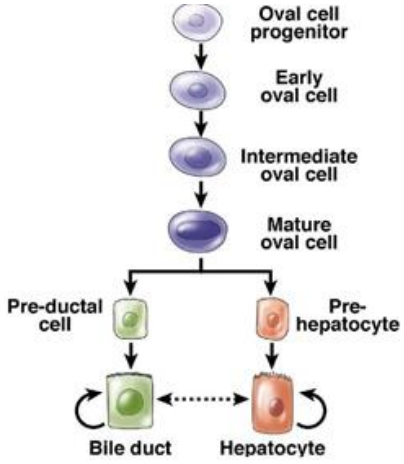


Figure 2. Liver stem/progenitor cell hierarchy. During chronic liver injury, a gradient of progenitor stem cells is generated by quiescent stem cells, residing in the biliary compartment. These cells expand into the parenchyma and regenerate the injured tissue (Duncan *et al*., 2009).

1.1.3 Characteristics of adult liver stem/progenitor cells

Most of the molecules that have been used to sort out the liver SC containing cell population are different adhesion molecules, which are expressed in the biliary epithelium. One of the surface markers that defines the liver stem cell population is the Epithelial Cell Adhesion Molecule (EPCAM), which is a characteristic for the biliary epithelium and in combination

with albumin and cytokeratin 19 expression defines the location of hepatic stem cells in both: humans and rodent models (Okabe *et al*., 2009; Schmelzer *et al.,* 2007). Another marker that has been used for the isolation of stem cells from various tissues is Prominin-1/Cd133, which is a marker used for isolation of hematopoietic (Yin *et al.,* 1997), neural (Lee *et al.,* 2005), skin (Ito *et al.,* 2006), kidney (Sagrinati *et al.,* 2006), prostate (Richardson *et al.,* 2004) and pancreatic stem cells (Oshima *et al.,* 2007). Prominin-1 also has been demonstrated to be expressed in cancer stem cells of brain (Singh *et al*., 2003), prostate (Collins *et al.,* 2005), liver (Suetsugu *et al.,* 2006), pancreas (Hermann *et al*., 2007), lung (Eramo *et al*., 2008) and colon (O'Brien *et al.,* 2007). Although the biological role of this molecule remains largely speculative; it has been shown to play some role in endocytosis and iron uptake (Bourseau-Guilmain *et al.,* 2011). The hypothetical location of stem cells in the mature liver are the canals of Hering, which connect bile collecting canaliculi to the bile duct in the portal triad (Figure 1, A) (Theise *et al*., 1999). In humans, an additional quiescent stem cell subpopulation has been shown to exist, that resembles fetal liver stem cells and is defined by the co-expression of albumin and cytokeratin 19 (Schmelzer *et al.,* 2007). The quiescent liver stem cell population is still poorly characterized and no unique markers are known, although many potential marker molecules are being investigated (Okabe *et al*., 2009). In case of injury, these quiescent stem cells are able to give rise to a second pool of stem cells, which proliferate and expand from the biliary compartment into the parenchyma and restore the injured tissue. These cells are called transit-amplifying or oval cells whose known distinctive characteristic is the expression of some surface proteins like Tumor-associated calcium signal transducer 2 (TROP2**)** or Leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) and others (Okabe *et al*., 2009; Dorrell *et al.,* 2008; Huch *et al*., 2013).

1.2 Liver carcinogenesis

1.2.1 Liver cancer classification and risk factors

Liver cancer is the third most frequent cause of cancer related death worldwide. Each year more than half a million liver cancers are diagnosed and about 90% of them are classified as Hepatocellular Carcinomas (HCC) (Jelic, 2010). In adult livers three main types of malignant liver neoplasms are distinguished:

1.2.2 Hepatocellular Carcinoma (HCC)

HCC is one of the most frequent cancers worldwide. Most cases of HCC can be attributed to chronic liver injury resulting from viral hepatitis, alcoholism, which in some cases is

accompanied by fatty liver disease. These factors together eventually lead to liver cirrhosis. Hepatitis B (HBV) infection accounts for 50% of all HCC cases, and most cases in children. Other factors that lead to elevated risk include fungal aflatoxin exposure and co-infection with Hepatitis C virus (HCV) (Hashem B. El-Serag, 2012). Males are at higher risk, which has been linked in a rodent model to higher androgen levels (Li *et al.,* 2012). The mechanism of carcinogenesis is not fully understood and some evidence suggests that HCC originates from differentiated hepatocytes (de Boer *et al.,* 1999), but a more recent hypothesis explains the cellular origin of HCC by genetically altered liver progenitor cells, that are able to generate tumors containing both hepatocytes and cholangiocytes (Rountree *et al*., 2009).

1.2.3 Cholangiocellular Carcinoma (CCA)

CCA is the second most common type of liver cancer that originates from the bile duct epithelium. Most frequently CCA develops around the seventh decade of age with a slight predisposition in males. The induction of cholangiocarcinogenesis needs several chromosomal changes, which can be triggered by prolonged inflammation of the biliary tract or mechanical injury. Resent evidence suggests that in some cases viral infection of progenitor cells might play a role in CCA initiation. In addition, several other risk factors are known, among which is exposure to carcinogens like thorium dioxide (Terraz and Becker, 2005).

1.2.4 Fibrolamellar Hepatocellular Carcinoma (FHCC)

FHCC is a distinct form of HCC with a significantly lower frequency and mean age of onset. In contrast to HCC, most cases occur in non-cirrhotic livers and no specific risk factors are known. No sex difference in frequency has been documented (Bracatelli *et al.,* 2005).

1.2.5 Potential origin of liver cancer

The origin of liver cancer still remains largely speculative. The main problem being the fact that the cellular hierarchy in liver tissue regeneration is not well described, therefore it is difficult to suggest any definitive candidate cells of origin (Yamashita and Wang, 2013). Some very recent publications have demonstrated unique features of liver regeneration, which suggest a novel explanation for the cellular heterogeneity in liver tumors. Through tracing experiments it has been made evident, that differentiated liver cells are able to transdifferentiate into biliary epithelial cells during normal tissue regeneration (Yanger *et al.,* 2013). Previously this ability of hepatocytes to give rise to CCA had been demonstrated in a genetic mouse model (Malato *et al.,* 2011). Furthermore, it was observed very recently, that

liver stem cells, lineage committed progenitors and differentiated cells are able to transform and produce a broad spectrum of tumors *in vivo.* This suggests that due to the transdifferentiation ability the same cell population may give rise to different kinds of tumors (Holczbauer *et al*., 2013).

1.3 Models of liver cancer

1.3.1 Chemical models

Induction of HCC relies in broad terms on the administration of two chemicals: a genotoxic agent that introduces the genetic alterations and a promoting agent, which allows faster expansion of cell clones with oncogenic mutations. The administration of these chemicals may be combined with the removal of ¾ of the liver, which is called partial hepatectomy (PH). The purpose of this operation is to force the resting liver cells to divide, promoting thereby tumor formation. One example of a chemical model for HCC induction is diethylnitrosoamine/phenobarbital (DEN/PB). In this model HCC is initiated with DEN, a powerful proto-oncogene whose activation relies on the activity of certain P450 cytochromes (Ye *et al.,* 2012), whose expression is highest in the centrilobular hepatocytes. PB is the promoting agent that induces chronic injury, which in turn leads to a pre-cancerous state characterized by elevated cell proliferation and chronic inflammation (Yoshie *et al.,* 1998). There are other chemical models, which rely on the administration of different chemicals, such as peroxisome proliferators, carbon tetrachloride, aflatoxin B1 and others (Heindryckx *et al.,* 2009).

1.3.2 Xenograft/allograft models

In this type of models, the cancer cells that eventually form the tumor are injected into immunodeficient mice. The cells can be injected subcutaneously or intrahepatically (Yan *et al.,* 2013; Kornek *et al.,* 2008). The intrahepatic injection model mimics due to the direct contact with liver tissue microenvironment the naturally occuring process of HCC tumor development. This allows the observation of metastasis and angiogenesis (Sun *et al.,* 1996). In case of the subcutaneous injection on the other hand the tumors do not develop normally, because they lack liver tissue contact. This model is used to evaluate the tumorigenic potential of cell lines. In both models the outcome may vary greatly depending on the cell lines used (Heindryckx *et al*., 2009; Rountree *et al*., 2011).

1.3.3 Mouse models expressing viral transgenes

Tumor formation is stimulated by the specific expression of different oncogenic proteins in liver cells of transgenic mice (Wu *et al*., 2001). *Per se*, models expressing a single viral protein are less effective than chemical models, but experimental evidence suggests that when combined with chemical tumor induction, the expression of HCV and HBV proteins can promote HCC formation (Heindryckx *et al.*, 2009).

1.3.4 Oncogene and growth factor pathway overactivation models

In this class of models, the artificial deregulation of cellular signalling pathways eventually results in highly elevated cancer risk. One example for a deregulated oncogenic pathway is overexpression of c-myc, which results in the acceleration of the cell cycle and eventually in genomic instability (Mai *et al.,* 1996). Deregulation of the β-catenin pathway is considered to be an early event in hepatocarcinogenesis, which leads to the deregulation of hepatocyte proliferation (Harada *et al.,* 2002). HCC induction occurs most effectively in transgenic models with the simultaneous deregulation of several pathways. An example of this is the combined overexpression of c-myc and a growth factor like Transducing Growth Factor-α (TGF-α) (Nicholes *et al.,* 2002), which is as effective as the chemical DEN/PB model. β-catenin pathway overactivation in combination with H-Ras mutation results in rapid formation of HCC (Harada *et al*., 2004). Other factors whose pathways are often deregulated in transgenic models are Epidermal Growth Factor (EGF) and Fibroblast Growth Factor 19 (FGF19) (Heindryckx *et al.,* 2009).

1.3.5 Transgenic models with a tumor promoting environment

One major risk factor for HCC development is liver cirrhosis and chronic liver injury. In the disease model systems these processes are induced by the overexpression of Platelet Derived Growth Factor (PDGF), which leads to liver cirrhosis and other liver pathologies (Borkham-Kamphorst *et al*., 2007; Hoyle *et al*., 1999). In another model, the tumor promoting conditions are induced by the expression of a human transport-impaired Alpha-1 antitrypsin protein, which accumulates in the liver and eventually leads to cirrhosis and cancer. Other similar models include deregulation of Transducing Growth Factor-β (TGB-β) and PTEN deletion (Heindryckx *et al.,* 2009).

2. EXPERIMENTAL PART

2.1 Aim of the project

The main goal of the project is to describe changes in adult stem cell markers of the liver during the process of tumor formation, thereby shedding light on the cell population from which liver tumors might arise.

To achieve this goal I will describe the formation of liver cancer as a constant process, evolving from the point of initiation to macroscopic and life threatening tumors, which generally do not respond to any therapy. I will investigate, which changes in liver stem cell populations precede the transformation of normal liver tissue into cancerous tissue and when these events take place. The information about the timeframe of the events leading to cancer is a prerequisite for the planning of future genetic tracing experiments and eventually might lead to the development of more effective means to prevent HCC at its initial stages, rather than dealing with consequences.

2.2 Materials and methods

2.2.1 Isolation of the non-parenchymal liver cell fraction

The first step in the isolation of liver cells is the cannulation of the inferior *V. Cava*, followed by a sequential perfusion of the liver with Krebs-Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM glucose) supplemented with 3 mM EGTA (Ethylene Glycol Tetra acetic Acid perfusion) for the first step, 1 mM $CaCl_2$ and Collagenase I (0.35 mg/ml) for the second step. The livers were perfused with each solution for approximately 10 min and after that the gall bladder was removed *in situ* and the liver was resuspended in cold PBS and passed through a 70 µm nylon mesh into a 50 ml tube (~40 ml of cell suspension). This was followed by the precipitation and depletion of the parenchymal fraction (50 g, 2 min, 4˚C, 4 times; 3 times 100 g, 1 min, 4˚C). After the parenchymal cells were depleted, non-parenchymal fraction was collected at 200 g, 10 min, 4˚C (volume ≤ 20 ml). For lysis of erythrocytes the final cell pellet was resuspended in 1 ml of BD Pharm Lyse™ Lysing Buffer (1 x) and incubated on ice for 15 min. After the incubation the suspension was diluted with cold PBS to 5 ml and centrifugated at 200 g for 5 min, 4˚C. Pellet was collected, resuspended in 90 µl of Miltenyi buffer (2 mM EDTA, 0.5% BSA in PBS) and 10 µl of Miltenyi Cd45 micro beads were added for leukocyte depletion. The cell suspension was incubated on ice for 15 min, which was followed by the

application of the cell suspension to a calibrated column. The flow fraction was collected. The depletion procedure was performed according to the manufacturer's recommendations.

2.2.2 FACS analysis

Prior to antibody staining the cells were suspended in 1% BSA/PBS solution and incubated for 1 h on ice. The cells were incubated in a 1:100 primary antibody dilution for 1 h, which was followed by 3 x 1 ml PBS wash. Analysis was performed on FACS Calibur.

2.2.3 Immunofluorescence staining and sample preparation

Fresh liver tissue was embedded in TISSUE -TEK® O.C.T. COMPOUND and shock-frozen in liquid nitrogen. Staining was performed on 6 µm thick tissue slices. Each slice was fixed with a 4% paraformaldehyde (PFA) solution in phosphate buffer saline (PBS) for 10 min followed by 2 x wash, 5 min each. Tissue samples were permeabilized (if required) with a 0.1% Triton X-100/PBS solution for 5 min at room temperature (RT), which was followed by a single wash and blocking with 4% goat serum in PBS for 1 h, RT. After blocking, the tissue samples were incubated with a dilution of the primary antibody (100 µl/sample) in the blocking solution (1 h, RT) and washed 3 times with PBS (5 min per wash). The secondary antibody was diluted in the blocking solution 1:500 and applied to the tissue samples (100 µl/sample) for 30 min, RT which was followed by 3 x wash (5 min). Mounting was performed with DAKO Fluorescent Mounting Medium (30 µl/sample).

2.2.4 Antibodies and dilutions:

Santa Cruz Biotechnology, INC. goat anti-mouse HNF-4α (IF: 1:100); Epitomics. rabbit anti-mouse Cytokeratin 19 (IF: 1:3000); eBioscience. rat anti-mouse Cd133PE (FACS 1:100; IF 1:20); eBioscience. rat anti-mouse Cd166PE (FACS 1:100; IF 1:100); eBioscience. rat anti-mouse Cd24eFluor® 450 (FACS 1:100; IF 1:100); eBioscience. rat anti-mouse Cd45 PE (FACS 1:250; IF 1:250); Biolegend. Purified. rat anti-mouse Cd325 (Epcam) (FACS 1:100; IF 1:100); BD Pharmigen. rat anti-human Cd49f-PeCy5 (IF 1:20).

2.2.5 Haematoxylin and eosin staining and sample preparation

Tissue samples were fixed overnight in a 4% PFA/PBS solution and dehydrated with a alcohol gradient ranging from 70-100 vol. over a period of four days, which was followed by incubation in xylene (2 x 1 h) and incubation in molten paraffin for embedding (first paraffin 2 h, second overnight). Staining was performed on up to 7 µm thick tissue slices. The tissue slices were dewaxed by dissolving the paraffin in xylene (2 x 15 min) and rehydrated with an

alcohol gradient starting with 100 and ending with destilled water. First the samples were stained with Mayer's haematoxylin for 5 min and rinsed in cool tab water (~1 sec) and incubated in Scott's tap water substitute (0.238M $NaHCO_3$; 0,813M $MgSO_4$ in MQ water) for up to 5 min. Counter staining with 1% eosin for 1 min and transfer into 70% alcohol for 1 min (maximum). Mounting was performed with a xylene based medium after dehydration.

2.2.6 Experimental design

Liver cancer was initiated in a group of male mice with an intraperitoneal injection of DEN in PBS (50 µg/g). Initiation was followed by the administration of PB dissolved in saline (0.5 g/l), until sacrifice. During the experiment, tissue and RNA samples were collected from several time points (Figure 3).

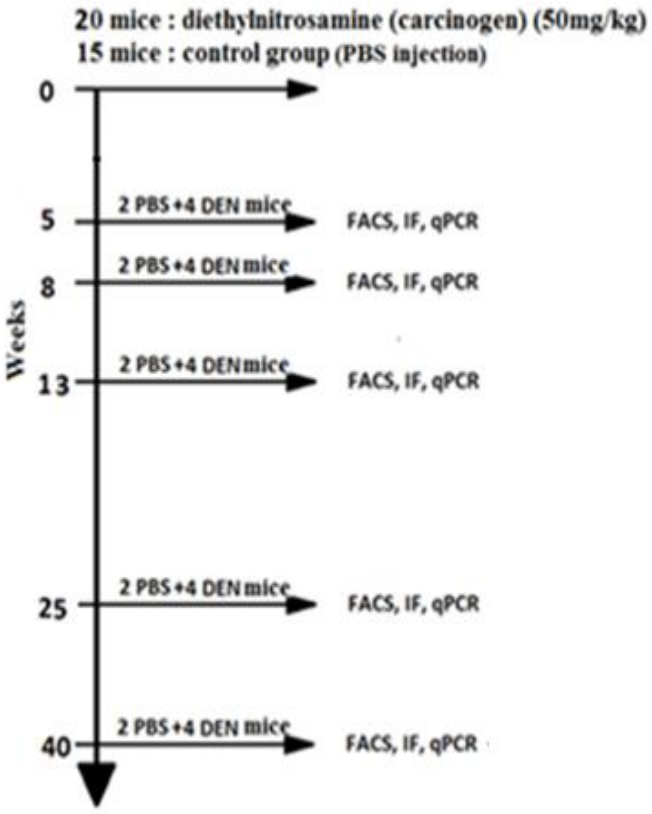


Figure 3. Scheme of the study. In the course of the experiment several kinds of samples were collected for analysis of tissue morphology (paraffin/cryo block embedded tissue), cellular composition (FACS) and gene expression (total RNA for qPCR) (5, 8, 13, 25, 40 weeks).

3. RESULTS

3.1 Histochemical analysis of liver tissue

The experimental group (30 mice) received an intraperitoneal injection of DEN in PBS. PB promotion started one week after initiation. The control group (15 mice) was injected with sterile PBS (Figure 5, a). Samples were collected 5, 8, 13, 25 and 40 weeks after initiation with DEN. From the 5th week after initiation signs of inflammation were evident in the form of basophilic infiltrates (Figure 4).

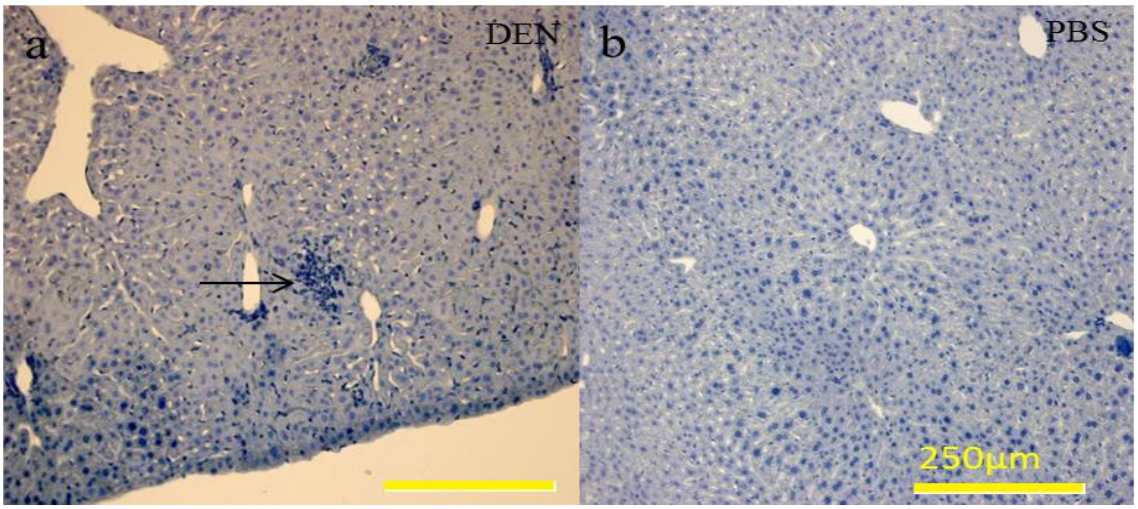


Figure 4. Haematoxylin-stained liver paraffin sections (5th week). a) Basophilic infiltrates in a DEN initiated (arrows) liver sample. b) Infiltrates were absent in the PBS-treated control samples.

The majority of these infiltrates disappear by eight week after the initiation, which indicates the end of the acute phase. At the 25th to the 40th week haematoxylin and eosin staining of the liver samples showed severe intoxication (caused by prolonged phenobarbital ingestion), which is evident from the weak eosinophilic staining of the cytoplasm (Figure 5, b) and nuclei of hepatocytes start to exhibit a greater variation in size.

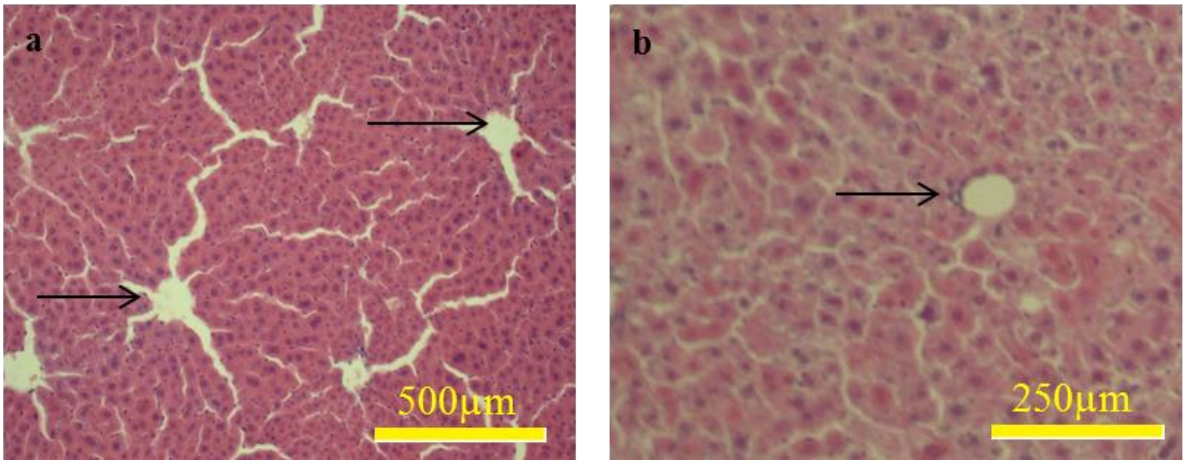


Figure 5. Haematoxylin eosin staining of liver tissue paraffin sections (arrows indicate central veins). a) Tissue sample from a control mouse (injected with PBS); b) liver tissue 25th week after initiation (DEN/PB treated), lack of staining indicates severe intoxication as a result of chronic PB ingestion.

All mice from the 40th week time point had macroscopic liver tumors, which by means of histological analysis (performed by Ivar Blank, an animal pathologist from the Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences) were identified as hepatocellular carcinomas (HCC) (Figure 6).

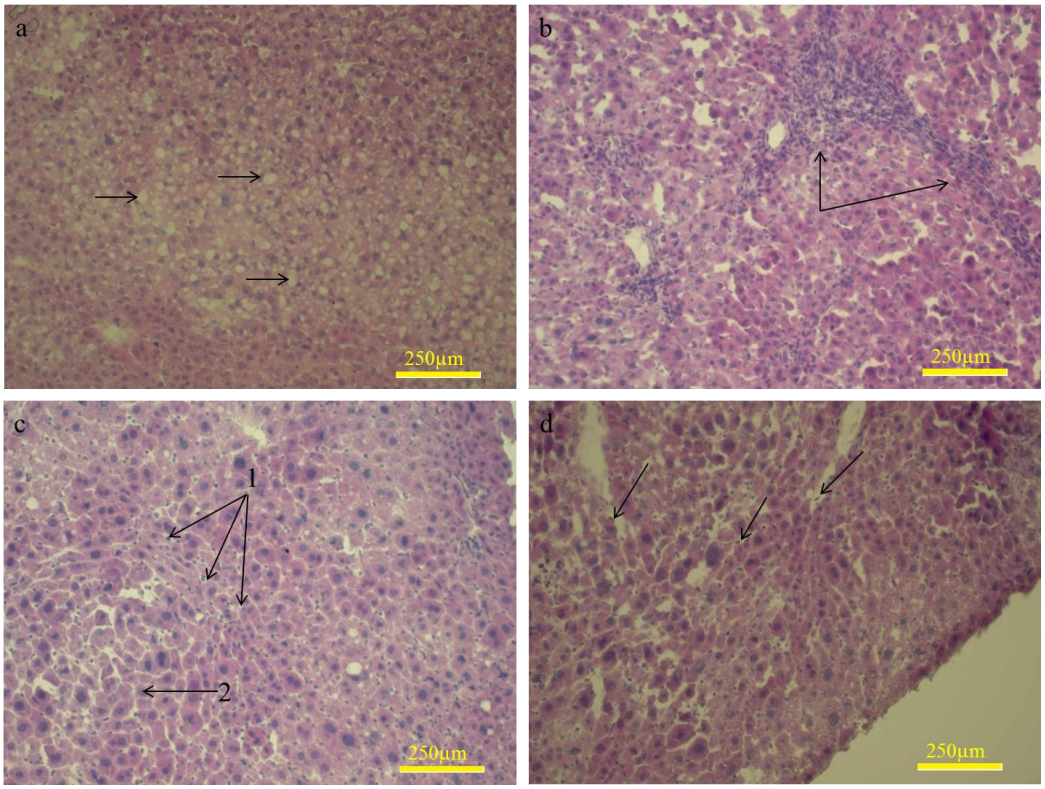


Figure 6. Haematoxylin/eosin staining of cryosections obtained from different macroscopic tumors. a) Tumor with liposis, arrows indicate fat incrustations, b) tumor containing leucocyte infiltrates (indicated by arrows), c) 1. Tissue compression caused by tumor growth, 2. HCC, d) tumor containing nuclear fragments, which indicates necrosis.

Multifocal HCC is defined by the presence of several foci composed of malignant morphology exhibiting hepatocytes. The malignant cells have and unusually small amount of cytoplasm and relatively big nuclei. In our model we did not observe this kind of HCC within macroscopic tumors, but only in one general liver tissue sample (Figure 7. a, b).

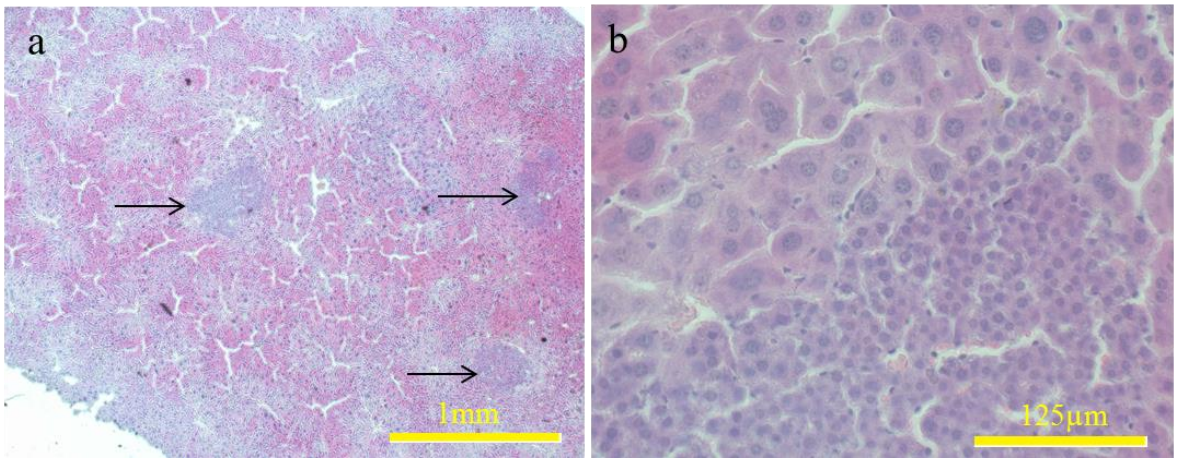


Figure 7. Hepatocellular carcinomas, paraffin section haematoxylin/eosin staining. Mouse liver sample collected from the 40th week time point (DEN and PB treated) did not contain macroscopic tumors. a) arrows indicate different HCC foci, b) HCC focus.

Histological analysis of the lungs showed the presence of neoplasms which, based on the cell morphology, were identified as liver cancer metastasis. The metastasized cell morphology resembles the liver cancer cells (Figure 8, a and b) Immunohistochemical staining of the metastasis demonstrated the absence of the hepatocyte specific marker HNF4α (data not shown) (Figure 8).

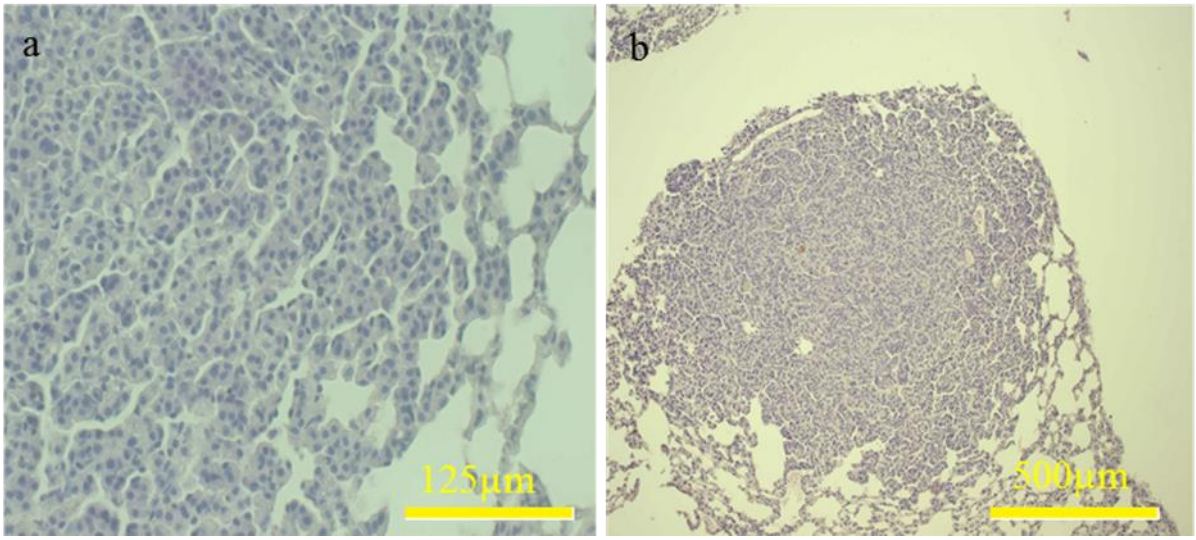


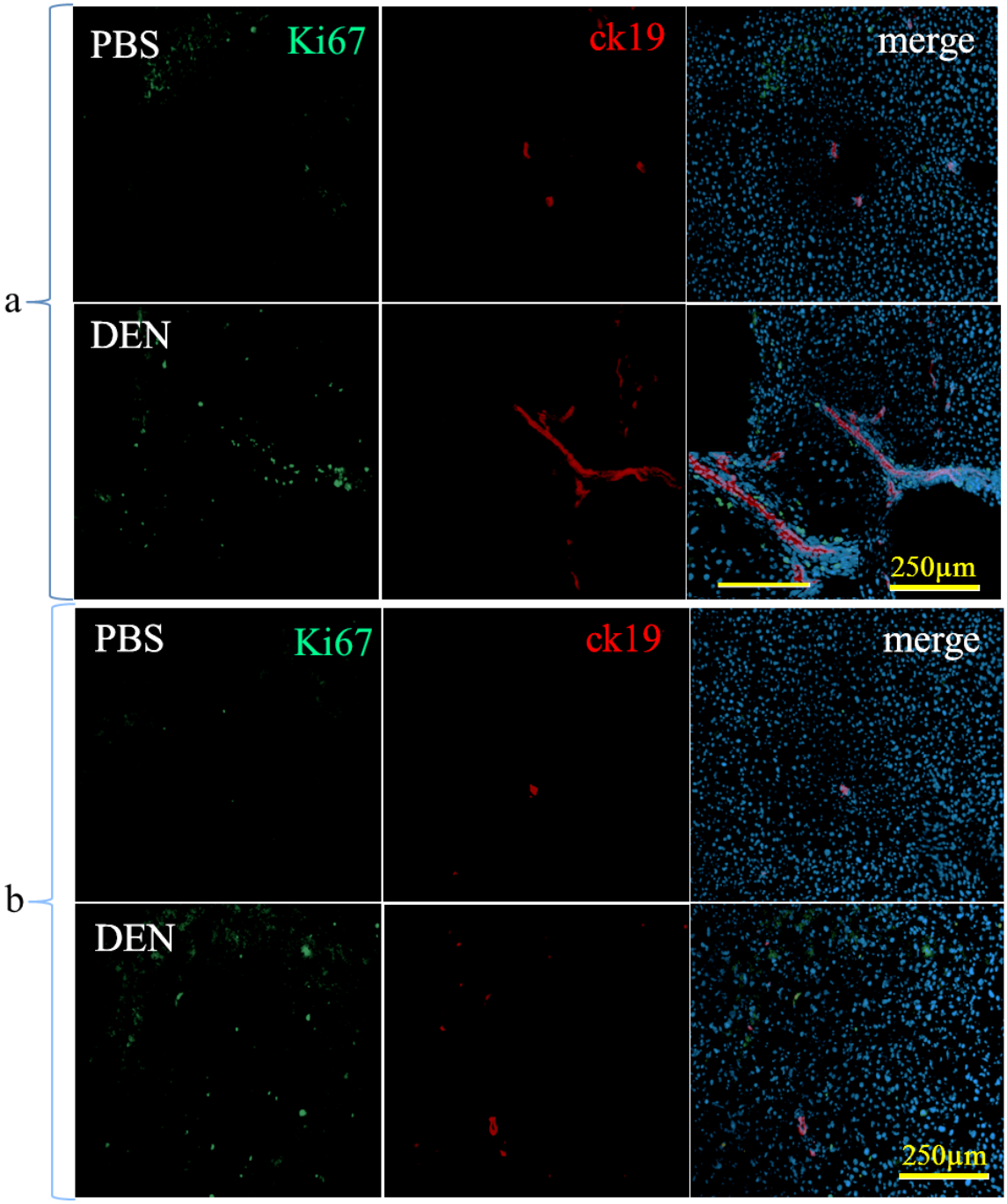
Figure 8. Metastasis to the lung. a) Cell morphology resembles liver cells. b) The metastasized cells formed clearly visible nodes on the surface of the lungs.

3.2 Analysis of changes in liver SC markers

3.2.1 Immunofluorescence analysis of stem cells markers (IF)

To describe changes in distribution of stem cell markers in the liver during the process of tumor formation I stained tissue samples from week 5 to 40 for the following stem cell markers: Epcam/Cd 326, Alcam/Cd 166), Prominin-1/Cd 133 (SC marker), Cd 24. In addition I analysed the samples for cytokeratin 19 (ck19), a biliary marker; Ki67, a proliferation marker and for Cd 45, a marker for hematopoietic cells.

Ki67 is a cell proliferation marker and is expressed in the non-injured liver in a small number of cells. Five weeks after the first DEN injection Ki67 expression is elevated compared to the PBS control (especially around K19-positive bile ducts, indicating injury in the biliary compartment; Figure 9, a - insert) and normalizes around the 13 week time point, with a small amount of positive cells dispersed over the sample (Figure 9, c).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Ki67
ck19
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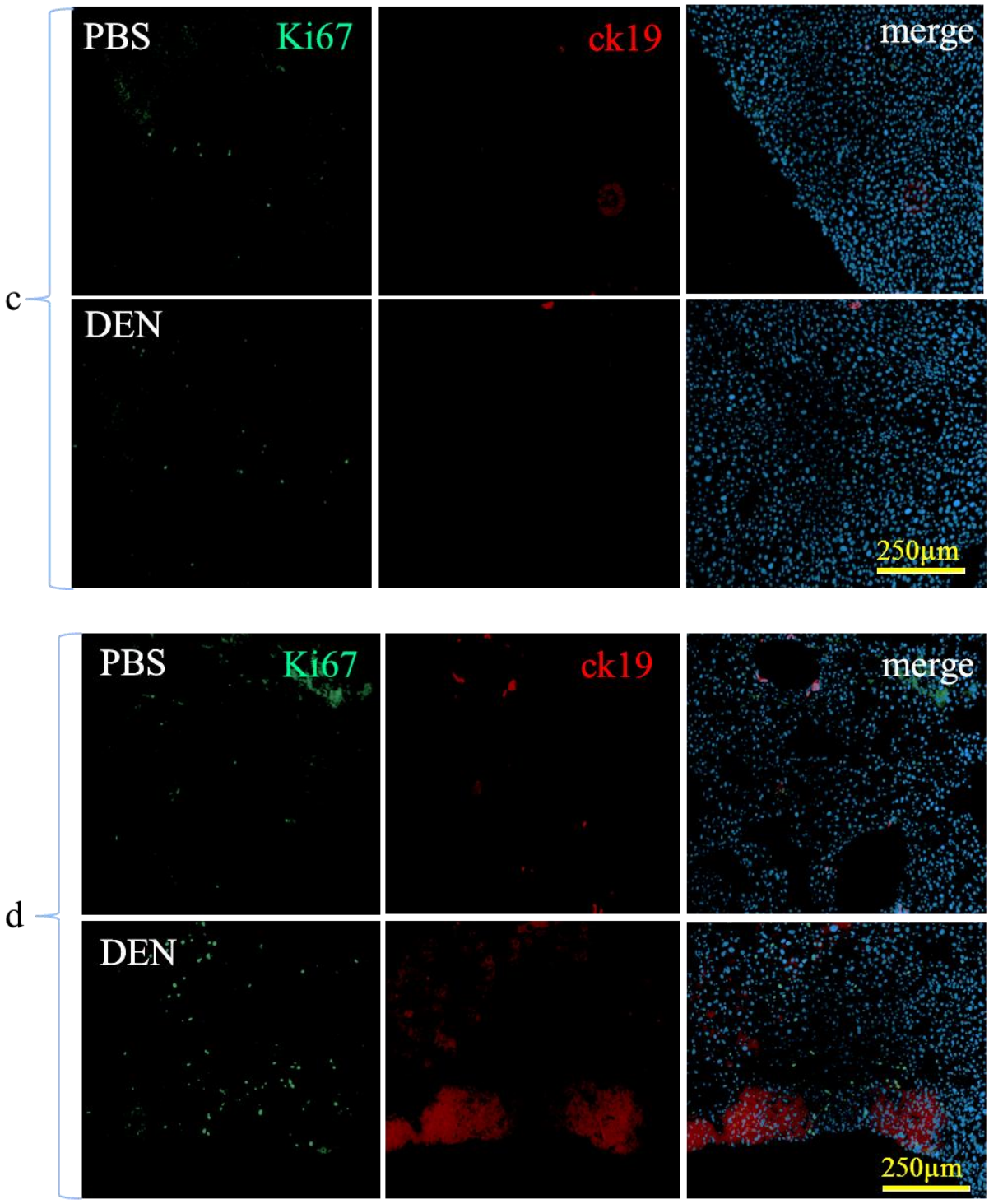
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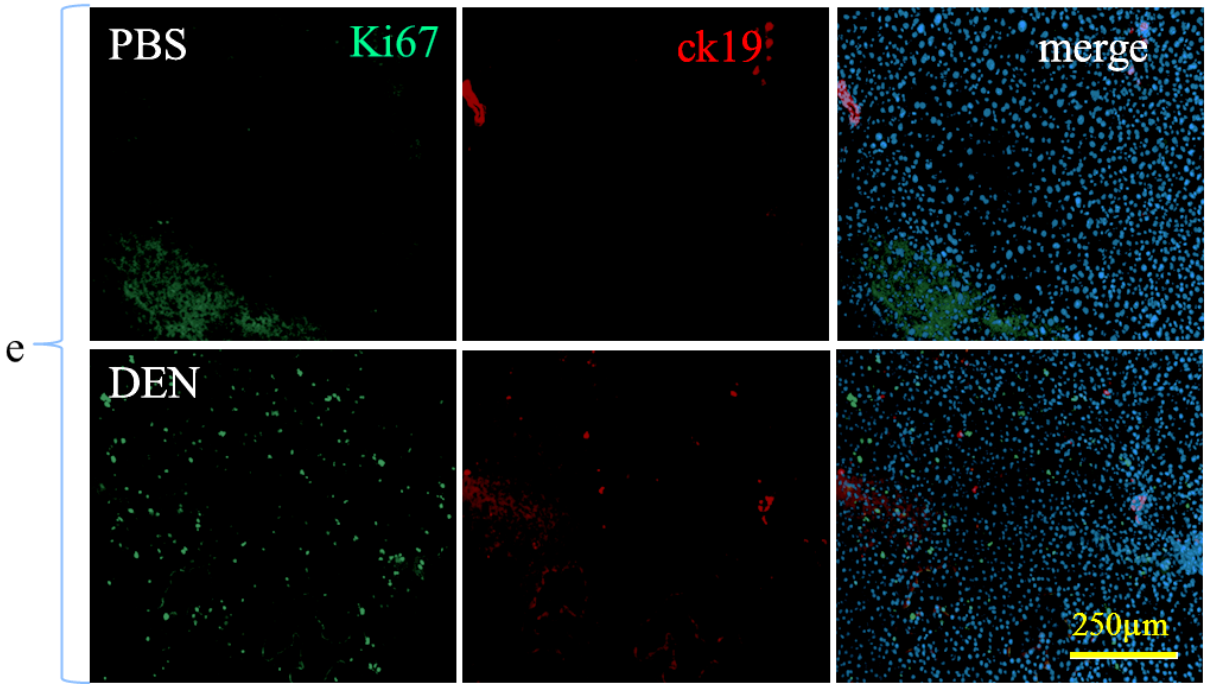

Figure 9. Cell proliferation increases after DEN injection. a) 5 weeks after (insert: scale bar 125µm) and b) eight weeks after intraperitoneal injection of the DEN/PB treatment (PBS for control mice,) I observed increased Ki67 levels in proximity of the ck19^{+} bile ducts (insert). c) By the 13th week, Ki67 levels in the initiated mouse liver samples are equal to the control samples. d) On the 25th week areas with increased levels of proliferation emerge again in the DEN/PB treated liver samples, e) which expand until the 40th week.

A second wave of proliferation occurred around the 25th week time point and continued until the 40th week (Figure 9, d and e).

Between week 13 and 25 ALCAM^{+} positive cells start expanding, producing large ALCAM positive areas. This is supported by the double staining with Ki67. (Figure 10, a and b).

At the 25 weeks an increase in overall Ki67 expression was evident, areas with increased proliferation started appearing, most of which were positive for ALCAM. This indicates that tumor formation started between the 13th and 25th week after initiation (Figure 9: c, d; 10).

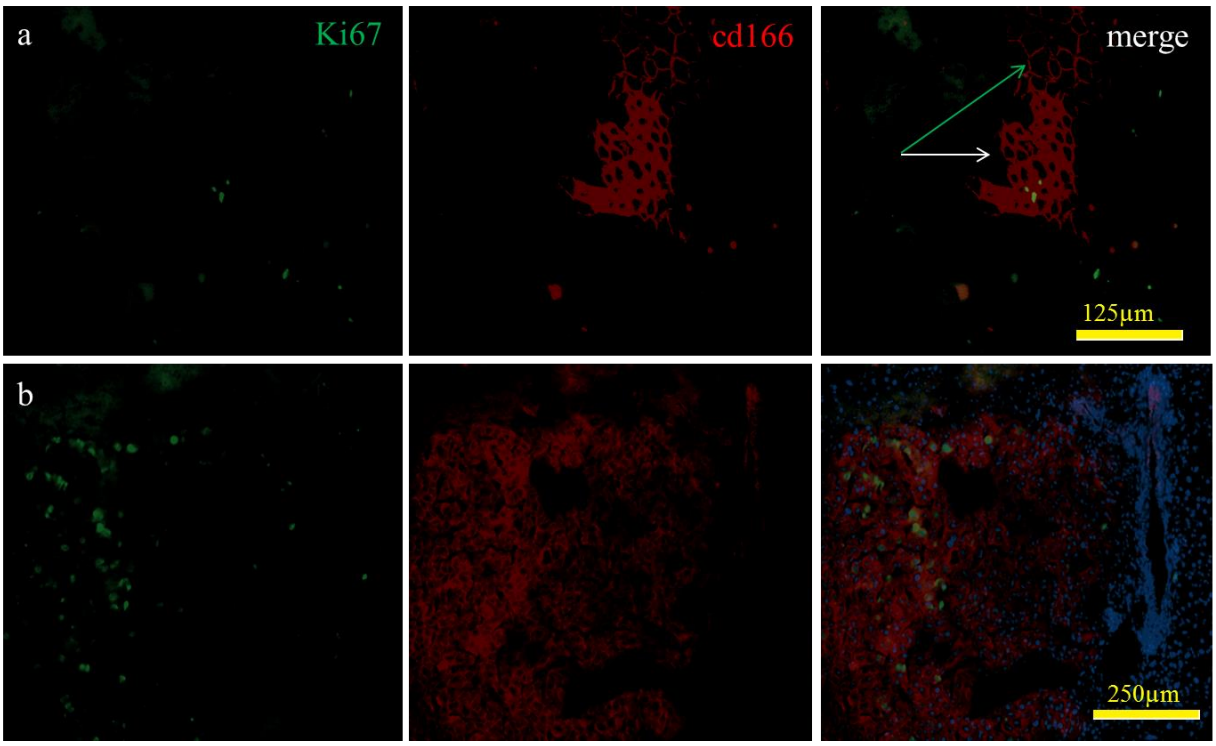

Figure 10. Proliferating Cd166^{+} cells start to form foci from the 13th week. a) Starting from the 13th week I observed the appearance of Cd166 positive foci, with an increased level of Ki67 and Cd166. Arrows indicate different levels of cd166 expression observed in the liver parenchyma of DEN/ PB treated mice. b) Proliferating focus with increased Cd166 expression. By the 25th week these foci formed large areas (microscopic tumors).

By the 40th week macroscopic tumors developed, most of which show a high level of ALCAM expression and proliferative activity (Figure 11). The ALCAM-positive areas found at the 25th week time point resemble the ALCAM/Ki67 staining pattern found in macroscopic tumors suggesting that the former present microscopic tumors formed in the liver.

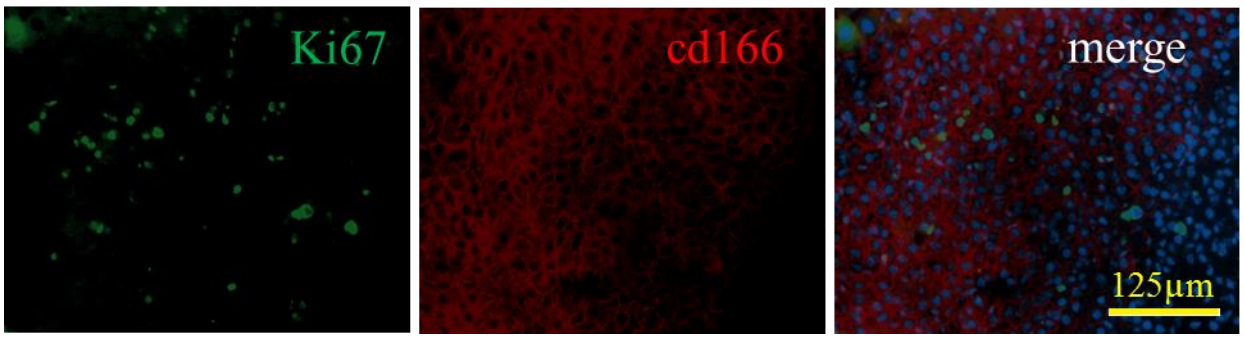

Figure 11. Tumor tissue immunofluorescence. Macroscopic tumors show elevated levels of Cd166 expression, which coincides with high areas containing cells with increased proliferation (Ki67).

In healthy liver tissue SC markers always co-localize with the biliary marker ck19 (Figure 12: a, b, c).

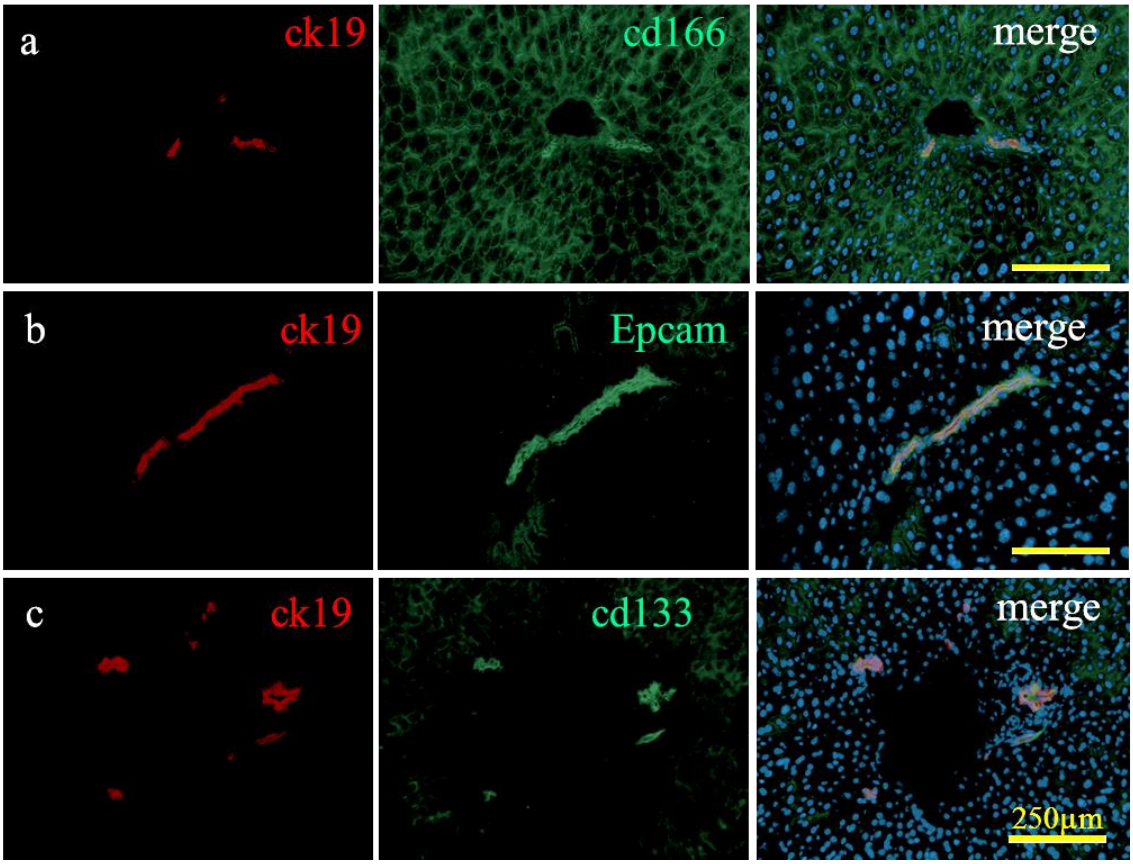

Figure 12. Distribution of SC markers in normal liver tissue. The expression level of a selection of known liver stem cell markers is highest in the biliary compartment. In uninjured tissue these markers are co-expressed with ck19 in the bile ducts. a) Cd 166 expression is highest in the biliary compartment, weak signal is present in the parenchyma, b) Epcam is localized exclusively in the biliary compartment, c) Cd133 is also expressed only in the biliary compartment.

However, in both tumor and liver tissue of DEN/PB-treated mice I observed the appearance of a novel cell phenotype that is characterised by the ck19 expression and the loss of stem cell markers (Figure 13, 14).

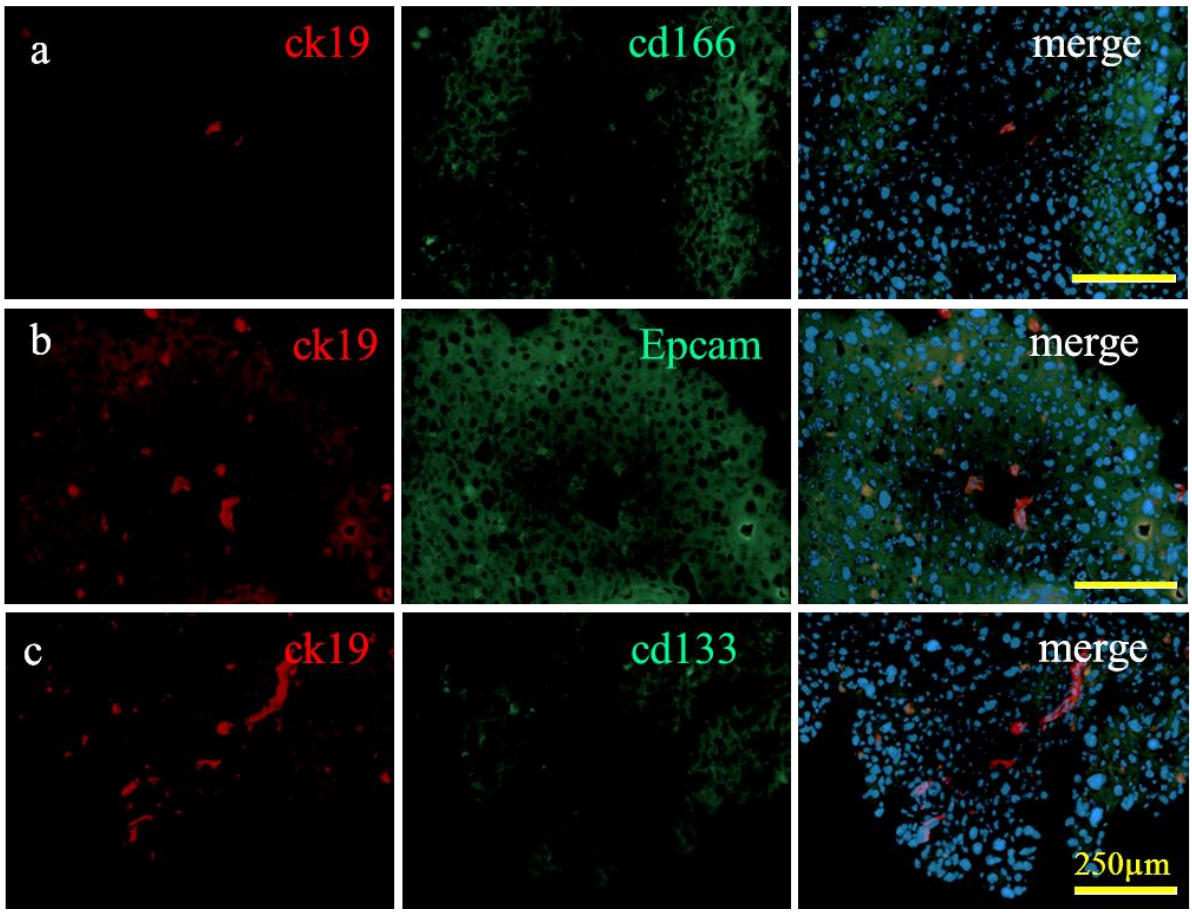

Figure 13. Distribution of stem cell markers in liver tissue samples at the 40th week time point. On the 40th week I observed the appearance of a novel ck19 single positive cell phenotype in non-cancerous liver tissue of DEN/PB-treated mice, a) ck19^{+}/Cd166^{-}, b) ck19^{+}/Epcam^{-}, c) ck19^{+}/Cd133^{-}.

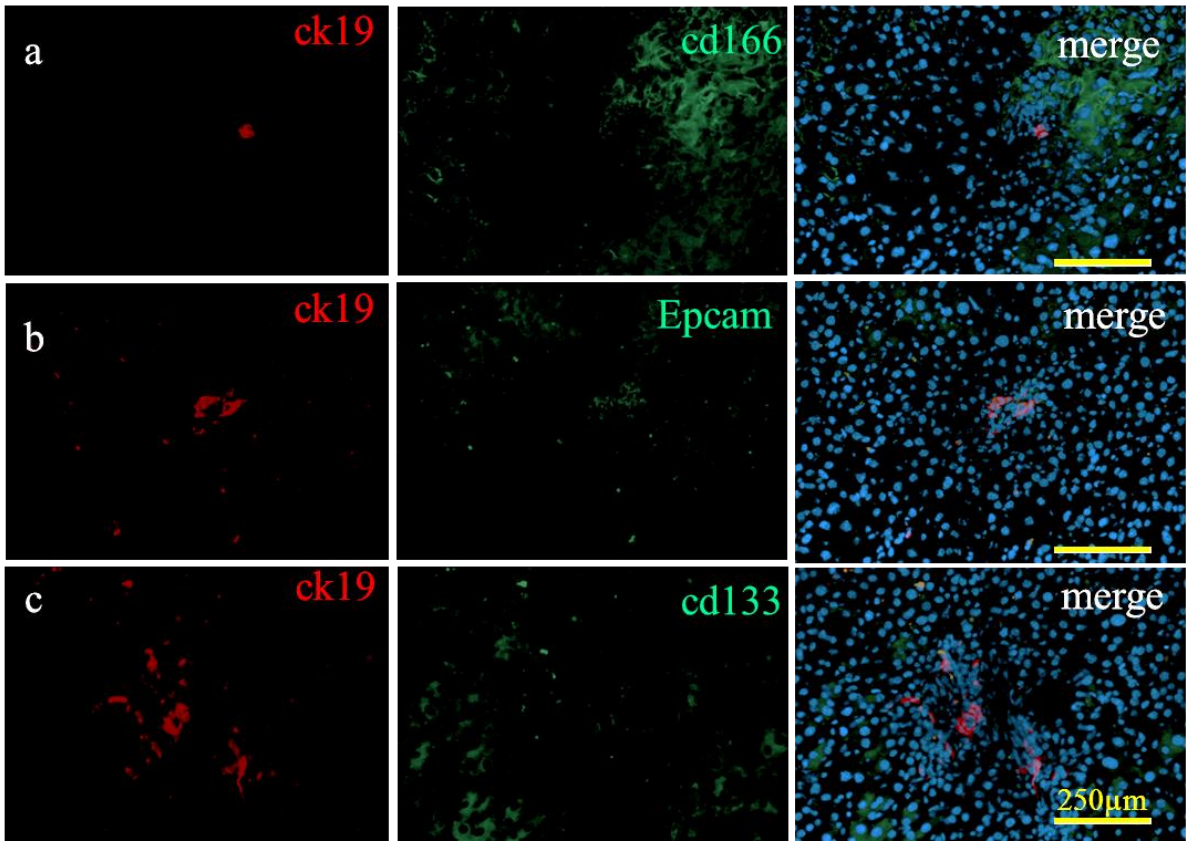

Figure 14. Distribution of SC markers in cancer tissue. a) In tumor samples ck19^{+}/Cd166^{-} single positive cells appear. Most of the Cd166^{+} cells are ck19^{-}. b) Loss of Epcam by ck19^{+} cells appears to be less extensive, but some ck19^{+}/Epcam^{-} cells can be found. c) ck19^{+}/Cd133^{-} cell population.

3.2.2 FACS analysis of liver SC markers

To study the quantitative changes in the liver stem cell marker expression I subjected the non-parenchymal fraction of liver cells, obtained at various time points during the experiment to FACS analysis of selected liver SC markers (Figure 15).

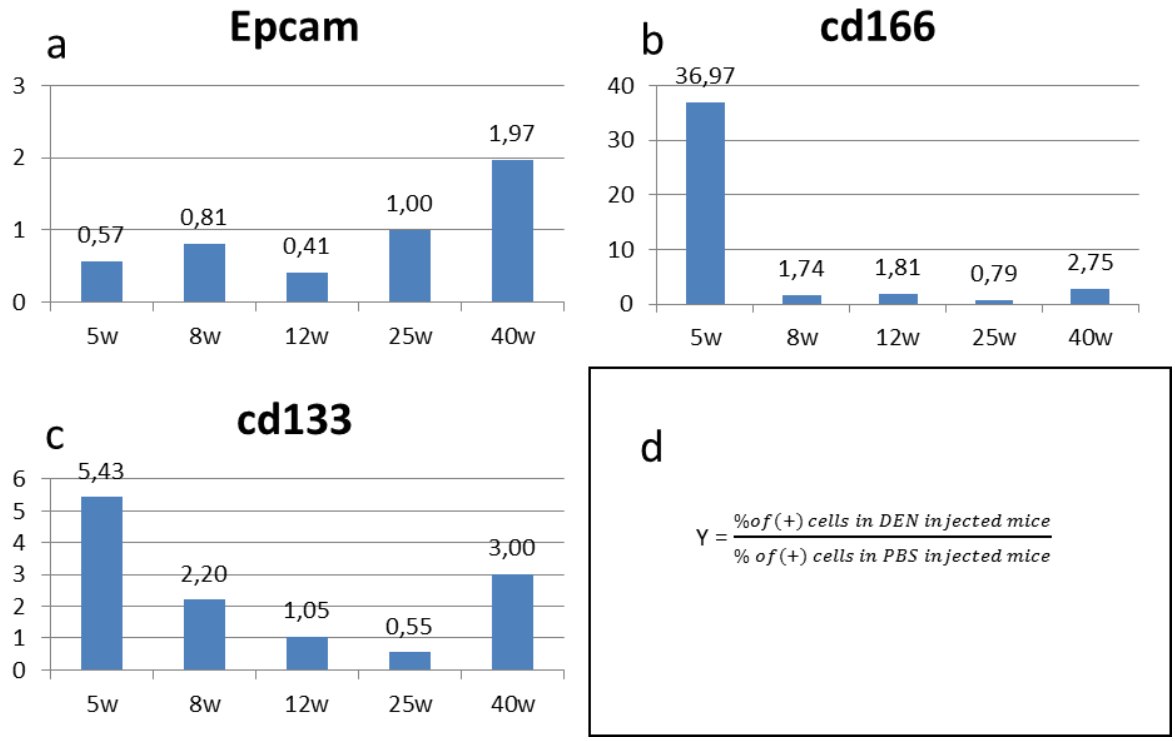

Figure 15. Summary of the FACS analysis of the non-parenchymal cell fraction. FACS analysis describes changes in the SC containing non-parenchymal fraction. Significant increase in stem cell markers is evident by the 40th week after DEN injection and start of PB promotion. a) After the DEN injection (5w) percentage of Epcam^{+} cells decreased, and it has doubled compared to the control group, by the 40th week time point. b) and c) show a similar tendency: substantial increase in Cd166^{+} and Cd133^{+} cells after the DEN injection with a threefold increase at the end of the experiment (40w). d) Value of the Y axis.

The expression level of all studied SC markers (EPCAM, ALCAM, Prominin-1) abruptly changes after initiation (DEN injection) compared to the control group, which indicates the damage of the biliary three (Figure 15).

At this initial stage (5 weeks) a significant increase in Cd133 and Cd166 can be observed, which indicates an active involvement of stem cells in the regenerative process and at the same time a twofold decrease in Epcam is evident (Figure 15). Regeneration after the acute injury (DEN injection) appears to be completed in the timespan from 13 to 25 weeks, since FACS data shows the normalization of the studied SC markers by the 25th week (DEN/PBS ration ≈ 1) (Figure 15, 25w).

DISCUSSION

In this study I aimed to shed light on the cellular events, which lead to the appearance of liver cancer. For this purpose I induced chemically liver cancers in male CBA mice and described the expression pattern of selected liver SC markers during this process.

Based on the quantitative data from FACS analysis the tumor formation process can be divided into two phases: the first phase is characterised by the regenerative response caused by the injury from the DEN injection. During this phase I noticed the increased inflammatory response and cell proliferation, especially in the peribiliary region (Figure 9; 10). The acute injury is followed by the second phase, where I identified the changes in cellular composition of the liver caused by immortalization of cells and tumor growth discussed below. Immediately after the DEN injection and start of PB (5 weeks) promotion I observed a substantial decrease in $Epcam^+$ cells, which indicates the damage of the biliary tree. First evidence of expanding cancer cells I observed at the 13 week time point when the regeneration process was over, and by the 25 week time point all DEN/PB treated mice had developed microscopic liver tumors (Figure 10). Therefore I conclude that in this model the oncogenic transformation generally occurs between the 13^{th} and 25^{th} week and the most significant cancer related changes in marker frequency must take place at this time. Those stem cell markers, which are expressed solely in the biliary compartment (Epcam, Cd133) displayed significant changes by the 40^{th} week with tumor/tissue signal ratio greater than one (Figure 15). Immunofluorescence analysis of macroscopic tumors showed the prevalence of $Cd166^+$ cells, which were ck19 negative (Figure 13). This indicates, that most of the macroscopic tumors probably originate from the immortalization of unipotent hepatocyte progenitor cells, which formed microscopic tumors as early as 13 weeks after starting the treatment (Figure 10; 11). As mentioned above, a possible explanation lies in the selective carcinogenicity of DEN (Ye *et al.,* 2012). The DEN metabolizing cytochrome P450 2E1 together with other cytochromes is considered a hepatocyte lineage marker (Golding *et al.,* 1995), which means that it is absent in bipotent or unipotent biliary lineage liver stem cells. I speculate that these are hepatic progenitors that represent the first step in the differentiation cascade of hepatic SC, which start to express the enzymes, which metabolize DEN to a carcinogenic product. Such cells might be hit preferentially by DEN and serve as cells of origin for HCC. As discussed above even differentiated cells can be the cell of origin for HCC (Holczbauer *et al.,* 2013). The use of genetically engineered mice to trace the different SC and

progenitor populations in liver HCC formation would give a definitive answer to this question.

A potential HCC metastasis to the lungs was observed at the 40 week time point. The exact origin of the cells remains speculative since the metastasized cells did not express hepatic marker HNF4α. However, this does not exclude their hepatic origin, since recent reports show, that HNF4α deletions might be one of the factors responsible for HCC promotion (Walesky *et al.,* 2013).

Another interesting observation is that in parallel to cancer progression within the liver I saw the loss of stem cell markers in the biliary compartment. One possible explanation would be that the loss of adherence molecules would facilitate the detachment of cells from their niche, which is a prerequisite for metastasizing and epithelial-mesenchymal transition. The latter is considered an important step in cancer progression (Brabletz *et al.,* 2001;Thiery *et al.,* 2009).

The origin of multifocal HCC (Figure 7) remains unclear, since I did not find any markers specific for this type of HCC. At the same time the metastasized cells observed in the lungs show close morphological resemblance to the cells comprising these HCC foci.

I observed also the presence of ck19-expressing cells in the DEN/PB treated group at the 40th week, which were devoid of other SC/biliary markers. The appearance of such cells is probably the result of chronic intoxication with PB, since they were present both in non-cancerous tissue as well as in tumor samples.

Taken together, the changes in SC/biliary marker expression during the initial phase (~first 13 weeks) occur most likely due to the presence of the regenerative processes in response to the acute injury imposed by DEN injection. The results suggest that the increase in the number of cells expressing Epcam and Cd133 that was observed after the 25 week time point is related to tumor growth, since after this time point I observed the appearance of macroscopic tumors. I also found that the changes arising in the SC compartment in the DEN/PB liver cancer model are too modest to be detected using immunofluorescence analysis, but are readily identified using FACS analysis (Figure 15). A further comparison of marker expression at the mRNA level in tumors and bulk tissue samples is needed to validate my observations.

The research addressing the events preceding the onset of cancer expands our knowledge about liver cancer formation and is important for the development of any new treatment or preventive approaches of liver cancer path way for the identification of novel drug targets for HCC treatment.

SUMMARY

Hepatocellular carcinoma is the most common form of malignant liver cancer and the third most common cause of cancer related mortality worldwide. The main reason for this is the lack of effective treatment approaches.

Transformed adult stem cells in the liver, or cancer stem cells appear to be the cause of cancer spread and relapse in some cases. Nevertheless, the changes that precede the malignant transformation of liver cells are largely unknown, and there is very little information about the changes occurring before the onset of tumor growth. The goal of this study was to describe the appearance of liver cancer as a constant process, evolving from the point of initiation to the terminal (lethal) stage, rather than dealing only with advanced tumors.

Therefore, I used a chemical model of liver cancer, which relies on the administration of a the carcinogen diethylnitrosamine (DEN), and phenobarbital (PB), to induce liver cancer in a group of male CBA mice. The control group consisted of non-initiated CBA mice. Liver samples from both groups were collected 5, 8, 12, 25 and 40 weeks after administration of DEN.

Analysis revealed increased inflammatory response and proliferation during the first 13 weeks after initiation, caused by acute injury. In the timespan from the 13^{th} to 25^{th} weeks I observed the appearance of $Cd166^{+}$ expressing microtumors.

By the 40^{th} week all DEN/PB treated mice developed macroscopic tumors, which were classified as hepatocellular carcinomas (HCC). The majority of HCC tumors showed an increased expression level of Cd166. I also observed increase in the cancer-/stem cell markers Cd133 and Epcam, which appears to be related to tumor growth.

This approach allowed us to build a timeframe of events, which accompany the transition from normal liver tissue to cancer and how liver stem cells engage in this process.

REGULATION OF ADULT STEM CELLS IN MOUSE LIVER TUMORIGENESIS

Denis Belitškin

RESÜMEE

Käesoleva magistritöö eesmärgiks oli uurida maksa täiskasvanud tüvirakkude osalust maksa kasvajate tekkeprotsessis. Selleks kasutati DEN/PB manustamisel põhinevat keemilist maksa kasvajate indutseerimise mudelit.

Kirjanduse ülevaates anti kokkuvõte maksa tüvirakkudest ja nende rollist maksa koe taastamises erinevates olukordades. Samuti tutvustati ka kasvajatüvirakke ja nende seost täiskasvanud tüvirakkude ja vähiga. Lisaks sellele kirjeldati peamisi kasutuses olevaid maksa vähi mudeleid.

Töö raames indutseeriti isastel CBA katsehiirtel maksa kasvajate teke, mille järel võeti proovid maksa koest 5, 8, 13, 25 ja 40 nädalat pärast kasvajate initsieerimist. Kontrollgrupi moodustasid sama vanad normaalsed isased CBA hiired. Leiti, et alates 13. nädalast algab Cd166 ekspresseerivate rakkude paljunemine ja 25. nädalaks on moodustunud mikroskoopilised kasvajad, mis on eristatavad kõrgenenud Cd166 ekspressioonitaseme tõttu.

40. nädalaks arenesid kõigil DEN/PB initsieeritud hiirtel maksarakulised kasvajad. Edasine analüüs näitas, et Cd166 ekspressioonitase makroskoopilistes kasvajates oli oluliselt kõrgem kui normaalse koe proovides, millest võib järeldada, et nende tuumorite formeerumine algas umbes 13 nädalat pärast initsieerimist. Kuna need rakud ei ekspresseerinud sapijuha markereid, võib spekuleerida, et need on suure tõenäosusega immortaliseerunud parenhümaalse hepatotsüüdi järeltulijad. Lisaks sellele leidsime ka teiste tüviraku ja kasvajatüviraku markerite tõusu (Epcam, Cd133), mille olemust tuleb selgitada edasiste uuringute käigus.

Antud katse ülesehitus erineb paljudest teistest, kus vaadeldakse maksa vähki kui produkti. Antud projektis rakendatud lähenemine võimaldab kirjeldada vähi tekkele eelnevaid sündmusi, mis omakorda võib viia uute maksa vähi teraapia ja ennetusstrateegiate väljatöötamisele.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Viljar Jaks for constant guidance and patience, Ivar Blank (Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences) for his expertise in the field of histopathology and Janeli Viil for the assistance during my experiments.

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Lihtlitsents lõputöö reprodutseerimiseks ja lõputöö üldsusele kättesaadavaks tegemiseks

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REGULATION OF ADULT STEM CELLS IN MOUSE LIVER TUMORIGENESIS,
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mille juhendaja on PhD VILJAR JAKS,
(*juhendaja nimi*)

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Tartus 26.05.2013 (*kuupäev*)