MEDICAL UNIVERSITY OF VIENNA

Phenobarbital and Cyproterone acetate, two prototypical non-genotoxic carcinogens and their impact on the hepatic mesenchyme

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy

Submitted by

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Vienna 12/01/2020

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List of abbreviations

AFB1	Aflatoxin B1
AP	Alkaline phosphatase
APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
COX2	Cyclooxygenase 2
CPA	Cyproterone acetate
CYP2E1	Cytochrome P450 2E1
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EC-F	Endothelial cell fraction
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ErbB1	Erythroblastic leukemia viral oncogene homolog 1
ERK	Extracellular signal regulated kinase
EtOH	Ethanol
FA	Formaldehyde
FCS	Fetal calf serum
Foxa1/2	Forkhead box protein A1/2
GC	Genotoxic carcinogen
GFP	Green fluorescent protein
GO	Gene Ontology
GSEA	Gene set enrichment analysis
GSTp	Glutathione S-transferase Pi

H/E	Hematoxylin and eosin
HB-EGF	Heparin-binding EGF-like growth factor
HBV	Hepatitis B virus
HBx	Hepatitis B viral protein X
HC	Hepatocyte
HCC	Hepatocellular carcinoma
HC-F	Hepatocyte fraction
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
IFN-β	Interferon beta
IGF-2	Insulin-like growth factor 2
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
InCroMAP	Integrated analysis of Cross-platform MicroArray and Pathway data
IRF-3	Interferon regulatory factor 3
JNK	c-Jun N-terminal kinase
KC	Kupffer cell fraction
KC	Kupffer cell
KEGG	Kyoto Encyclopedia of Genes and Genomes
KGF	Keratinocyte growth factor
LOOH	Peroxidized linolic acid
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Mc	Monoclonal
MC	Mesenchymal cell
MC-F	Mesenchymal cell fraction
MEM	Minimum essential medium
MET	Hepatocyte growth factor receptor
mRNA	Messenger RNA
Myd88	Myeloid differentiation primary response gene 88

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBT	Nitro blue tetrazolium chloride
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGC	Non-genotoxic carcinogen
NNM	N-Nitrosomorpholine
NS3	Non-structural 3
NS4	Non-structural 4
NS5A	Non-structural protein 5A
NSAID	Non-steroidal anti-inflammatory drug
p16	Cyclin-dependent kinase inhibitor 2A
P450	Cytochrome P450
p53	Tumor protein 53
PNL	Pre-neoplastic lesion
PB	Phenobarbital
PBS	Phosphate-buffered saline
Pc	Polyclonal
PCR	Polymerase chain reaction
PXR	Pregnane X receptor
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SC	Stellate cell
SDS	Sodium dodecyl sulfate
STAT3	Signal transducer and activator of transcription 3
T3	Triiodothyronine
TBE	Tris/borate/EDTA
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine

TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

Publications arising from this thesis:

1) **MESENCHYME-DERIVED FACTORS ENHANCE PRENEOPLASTIC GROWTH BY NON-GENOTOXIC CARCINOGENS IN RAT LIVER**

   Marzieh Nejabat[1$], Teresa Riegler[1$], Tabea Reitinger[1], Sandra Subosits[1], Michael Römer[2], Johannes Eichner[2], Martin Bilban[3], Andreas Zell[2], Wolfgang W. Huber[1], Rolf Schulte-Hermann[1], Bettina GraslKraupp[1#]

2) **PRO-INFLAMMATORY MESENCHYMAL EFFECTS OF THE NONGENOTOXIC HEPATOCARCINOGEN PHENOBARBITAL: A NOVEL MECHANISM OF ANTI-APOPTOSIS AND TUMOR PROMOTION**

   Teresa Riegler1, Marzieh Nejabat1 , Johannes Eichner2 , Melanie Stiebellehner1 , Sandra Subosits1 , Martin Bilban3 , Andreas Zell2 , Wolfgang W. Huber1 , Rolf Schulte-Hermann1 and Bettina Grasl-Kraupp

English abstract:

Introduction: Hepatocellular carcinoma is the second most common cause of cancer-related death worldwide. Multi-step carcinogenesis consisting of initiation, promotion, and progression is an accepted theory for explaining the underlying mechanism of chemically induced hepato-carcinogenesis [1]. Non-genotoxic carcinogens (NGCs) are important in the cancer promotion step [2]. The mechanism of interaction between mesenchymal cells and hepatocytes that leads to cancer promotion is not completely clear.

Purpose: In this study, we attempted to find important biomarkers and/or cellular cascades that are induced by NGCs and promote the initiated cells. In particular, we focused on the mode of action (MOA) of two important NGCs with regard to cooperation and intercommunications between the treated normal and initiated hepatocytes (HCs) and mesenchymal cells (MCs).

Materials and Methods: We treated Wistar rats *in vivo* and the rat liver cells *in vitro* with phenobarbital (PB) or cyproterone acetate (CPA) as representatives of two groups of NGCs (a PXR-ligand and a CAR –ligand). Liver cells were separated at different time points to obtain two cell-fraction HCs (hepatocytes) and MCs (consisting of Kuppfer cells, stellate cells, and endothelial cells).

Oligo-array and real-time PCR (polymerase chain reaction), as well as DNA replication assays and different staining methods, were used to clarify the cross-talk between HCs and MCs.

Results and Discussion: PB induces genomic alterations in the mesenchyme more than in the parenchyme, and for CPA, this was the opposite. The number and types of genes whose expression have been changed after *in vivo* treatment with CPA/PB is greater than those whose expression changed in *in vitro* treated HCs, showing that interaction between MCs and HCs in the body altered not only the number of affected

genes, but also the type of activated genes. After CPA treatment, the majority of activated genes belong to growth cascades, whereas treatment with PB leads to the activation of pro-inflammatory genes, as well as survival factors.

Conclusion: The NGCs we studied induced a significant, compound, specific cell alteration in hepatocytes and in mesenchymal cells. Activation of the mesenchyme caused the release of several growth factors, as well as supporting survival factors, which play an important role in promoting pre-neoplastic lesions. Further studies relevant to humans should be performed to identify the NGC characteristics in hepato-carcinogenesis.

German Abstract:

Einleitung: Hepatozelluläres Karzinom ist die zweithäufigste Todesursache bei Krebserkrankungen weltweit. Die mehrstufige Karzinogenese, bestehend aus Initiierung, Promotion und Progression, ist eine anerkannte Theorie zur Erklärung des zugrundeliegenden Mechanismus der chemisch induzierten Hepatokarzinogenese [1]. Nicht-genotoxische Karzinogene (NGCs) sind im Krebsförderungsschritt von Bedeutung [2]. Der Mechanismus der Wechselwirkung zwischen Mesenchymzellen und Hepatozyten, der zur Krebsförderung führt, ist nicht vollständig geklärt.

Zweck: In dieser Studie haben wir versucht, wichtige Biomarker und / oder Zellkaskaden zu finden, die durch NGCs induziert werden und die Initiierung der Zellen fördern. Insbesondere haben wir uns auf die Wirkungsweise (MOA) von zwei wichtigen NGCs im Hinblick auf die Kooperation und Interkommunikation zwischen den initiierten Hepatozyten (HCs) und mesenchymalen Zellen (MCs) konzentriert.

Material und Methoden: Wir behandelten Wistar-Ratten in vivo und die Rattenleberzellen in vitro mit Phenobarbital (PB) oder Cyproteronacetat (CPA) als Vertreter von zwei Gruppen von NGCs (PXR-Ligand und CAR-Ligand). Es wurde eine Trennung der Leberzellen zu verschiedenen Zeitpunkten durchgeführt, um die zwei Zellfraktionen HCs (Hepatocaytes) und MCs (bestehend aus Kuppfer-Zellen, Sternzellen und Endothelzellen) zu erhalten.

Oligo-Array- und Echtzeit-PCR (Polymerasekettenreaktion) sowie DNA-Replikationstests und verschiedene Färbemethoden wurden verwendet, um die

Interaktion zwischen HCs und MCs zu klären.
Ergebnisse und Diskussion: PB induziert bei Mesenchym mehr genomische Veränderungen als bei Parenchym; bei CPA war es umgekehrt. Die Anzahl und Art der Gene, deren Expression sich nach einer in-vivo-Behandlung mit CPA / PB geändert hat, ist höher als bei den in-vitro-behandelten HCs. Dies zeigt, dass die Wechselwirkung zwischen MCs und HCs im Körper nicht nur die Anzahl und Art der betroffenen Gene, sondern auch die Anzahl der HCs verändert hat. Nach der CPA-Behandlung werden mehrheitlich Gene der Wachstumskaskaden aktiviert, während unter Behandlung mit PB es zur Aktivierung von proinflammatorischen Genen sowie von Überlebensfaktoren kommt.

Schlussfolgerung: Die untersuchten NGCs induzieren eine signifikante, verbindungsspezifische Zellveränderung in den Hepatozyten und Mesenchymzellen. Die Aktivierung von Mesenchym verursacht die Freisetzung mehrerer Wachstumsfaktoren sowie unterstützende Überlebensfaktoren, die eine wichtige Rolle bei der Promotion von präneoplastischen Läsionen spielen. Weitere für den Menschen relevante Studien sollten durchgeführt werden, um die NGC-Merkmale bei der Hepatokarzinogenese zu identifizieren.

Acknowledgments:

- I thank sincerely to Ass.Prof. Dr. Alexander Haug for supporting me and accepting my supervision

- Thanks to Prof. Dr. Mag. Wolfgang Mikulits who helped me so much as the coordinator of 'malignant disease group' to finish my PhD.

- Thanks to the funding and funding of the PhD thesis goes to Prof. Bettina Grasl-Kraupp. I would like to thank for the scientific discourse and learning laboratory work as a general physician.

- Thanks to my colleagues in this project: Istvan Paraszti, Florian Kellner and Theresa Riegler who worked with me on a good team. Many thanks to othe laboratory colleages Waltraud Schrottmeier, Mario Kuttke, Melanie Stiblellehner, Bettie Wingelhofer, Christiane Maier, Lisa Nika, and Jakob Paur. Good conversations and a pleasant atmosphere brought joy to work.

- Thanks to our kind laboratory-assistant Birgit Mir-Karner who was always available to help specially by rat perfusion days. Helga Koudelka was happy to share her impressive experience with immunostaining. For that - and for help with many other small challenges - thank you!

- I want to express my deepest thank to my husband, Seyed Mohammad Sadegh, I could always rely on you, as well as to my dear son Mohammad Ali. I would like to thank my gorgeous family for their love and spiritual support especially my father who encouraged me and empowered me all the time for finishing this way..

- Above all I want to thank God from the depths of my heart, for having made everything possible by granting me strength and courage to undertake this work and keep me going...

1 CHAPTER ONE: INTRODUCTION

1.1 General introduction

1.2 Principles of chemical hepato-carcinogenesis

Chemical carcinogens are organic or inorganic substances that are directly involved in causing cancer [3]. Generally, they can be categorized as either genotoxic (GTX) or non-genotoxic (NGTX) based on their mode of action. GTX substances or their metabolites interact directly with genomic DNA or its spindle and induce specific mutations or chromosome aberrations that lead to persistent damage in DNA, which can cause tumours [4]. Poly-aromatics and hydrocarbons are examples of GTX chemicals.

Genotoxic carcinogens can be detected by a battery of *in vivo* and *in vitro* genotoxicity tests, such as the Ames test, the chromosomal aberrations test, and the micro nucleus test.

In contrast to GTX compounds, NGTX substances represent chemicals that are capable of producing tumorigenesis, but with secondary mechanisms that are not directly related to DNA damage [5]. Such drugs/chemicals show negative results in DNA mutation tests. In recent years, continuous exposure to many natural and /or manmade chemicals has become a major concern in humans. Therefore, much effort has focused on finding a way to distinguish between non-genotoxic tumorigenic and non-tumorigenic substances.

Because the liver is an end-target organ or the metabolic location of many drugs/chemicals, the hepato-carcinogenicity of several chemical substances has been studied during the past decades. Nevertheless, the exact mode of action of hepato-carcinogenicity–especially of NGC (non-genotoxic carcinogens)—is still poorly understood.

1.2.1 Multi-stage concept of hepato-carcinogenesis

It is well known that the development of HCC is a long, multi-step process with at least three distinct stages: “initiation;” “promotion;” and “progression.”

“Initiation” begins with one or more mutations in the genome of the parenchymal cells, which is caused spontaneously or by genotoxic carcinogens. Such mutations are sustained and even heritable. This can lead to formation of dysplastic nodules, but there is no clear evidence for progression of all these lesions to HCC [6] . The mechanism of initiation may vary with different initiators in the same tissue, but the basis of an “irreversible mutation” is the same in all tissue types and various initiators in the same tissue [7].

Unlike initiators, tumour promoters do not bind directly to the DNA, but alter cell proliferation, DNA repair, DNA methylation, and cell signalling, which increases the likelihood of promotion of the initiated cells. Promotion is defined as a process by which initiated cells are selectively stimulated to develop a visible tumour that is often a benign lesion and the pathological changes are reversible. In the liver, the result of promotion is the expansion of pre-neoplastic foci and adenomas from the initiated cells [8].

During ‘progression,’ the benign tumours progress to malignant cancers. Early epigenetic aberrations have been proposed to contribute to the transformed phenotype by promoting the expansion of pre-malignant cells, which then lead to malignant tumours. Different cell culture studies done with chemical carcinogens have reported that multiple phenotypic changes in tumour cells are required for a progressive process. Promotion and progression can be distinguished with morphological evidence and even from the response to a certain chemical treatment [9]. In addition, despite ‘promotion,’ ‘progression’ consists of a process by which the first persistent hepatocyte nodules develop and undergo a series of changes that lead to cancer, including invasion and metastasis [10]. These persistent nodules are the first neoplastic step, as autonomous overgrowth has been observed in such cell populations.

1.2.2 Genotoxic hepato-carcinogens and their impact on tumour initiation; mode of action

As mentioned previously, GTX substances can interact with or damage DNA. That is why mutations are the critical biomarker for cancer risk assessment in GTX. They can also affect other cellular mechanisms that are important in DNA replication or DNA repair processes. The result of DNA impairment by GTXs produces various forms of DNA lesions, such as DNA adducts, base mismatches, DNA cross-links, etc. [11]. These DNA lesions in the nuclei lead to chromosomal damage or at least gene mutation, which is considered to be the original event in cancer 'initiation' [12]. The mutated DNA requires two cell proliferation cycles to be permanent as a mutation and to become heritable. The accumulation of these changes appears to be critical for "initiation" (Figure 1) [13]. The initiated hepatocytes are defined as cell populations that can be stimulated under special situation or treatment regimens . Therefore, not all mutagenic/genotoxic substances are carcinogenic, as there are chemicals that are highly mutagenic, but do not induce cell proliferation [7]. Genotoxic carcinogens are chemicals that cause mutation in critical regulatory genes, which leads to abnormal proliferation or an imbalance in cell loss [14].

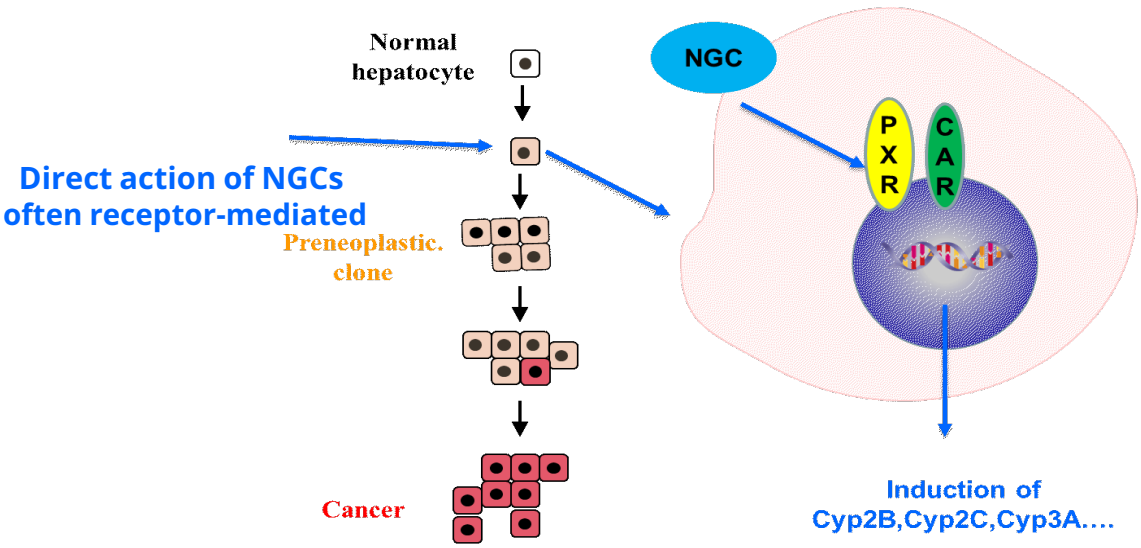

Figure 1 Schematic view of direct effect of NGCs on initiated hepatocytes that are modulated via NRs. Binding to NRs triggers changes in receptor configuration, leading to initiation of transcription in a specific manner.

GTXs substances can initiate the cells via different modes of action:

1. Mutation of proto-oncogenes and accumulation of mutated hot spots lead to permanent activation of these genes (like the κ-Ras proto-oncogene in rodents), resulting in an acceleration of the cell division cycle and neoplastic cell transformation [15]; and
2. Loss of function of mutated tumour suppressor genes like TP53 leads to a reduction of cell apoptosis and uncontrolled cell growth [16, 17].

As mentioned previously, genotoxic carcinogens cause structural genomic mutations which may alter the gene expression patterns that affect cellular pathways. Some pathways, such as Wnt/β-catenin, TGF-β/IGF-2R, and IL-6/IL-6R, have been shown to be activated in HCC stem cells and / or HCC rat liver tissue [8]. These pathways play a pivotal role in cell growth, differentiation, migration, and cell survival [18, 19].

The result of such mutations is impairment of liver cell homeostasis in such a way that cell proliferation and/or cell growth cascades become activated and/or cell apoptosis cascades are prohibited.

Some of the known genotoxic substances, such as aflatoxin B1 (AFB1), N-nitrosodimethyamine (NNDM), N-nitroso-morpholine (NNM), and their mode of action are described below.

1.2.2.1 Aflatoxin B1

Aflatoxins are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus.* These fungi can colonize a variety of food commodities like maize or nuts in tropical and sub-tropical areas. Aflatoxins consist of approximately 20 related fungal metabolites.

The four major aflatoxins are B1, B2, G1, and G2. AFB1 is considered a naturally potent carcinogen. In the liver, it is converted by the cytochrome P450 system into an ultimate carcinogenic agent. Aflatoxin B1 intoxication is considered to be a non-cirrhotic cause of liver cancer and is quite common in areas with inappropriate food storage conditions. It has been confirmed that, in such areas, the incidence of HCC is high due to chronic exposure to aflatoxins [20]. More often, the toxic effects of

AFB1 presents in persons with chronic HBV infection [21]. Also, it appears to have a synergistic effect on HCV-induced liver cancer [22] [23] [24]. About 4.5-5.5 billion people in the world are at risk of exposure to these toxins (mainly in Africa, Eastern Asia, and South America) [25, 26].

It has been assumed that the two main mechanisms by which AFB1 may trigger the process of carcinogenesis are as follows:

1) AFB 1, as a genotoxic carcinogen, can be metabolized to an epoxide, which further may form DNA adducts, and consequently, DNA strand breaks or GC-TA mutation (e.g., leads to a mutation in the TP 53 gene) ADDIN EN.CITE [27-29]; and
2) Induction of liver cell necrosis and involvement of oxidative stress cytokines and enzymes, including superoxide dismutase ADDIN EN.CITE [30-32].

1.2.2.2 Dimethylnitrosamine (DMN)

The substance belongs to a large class of chemical compounds called N-nitrosamines. This class of compounds is known to be highly toxic, especially hepatotoxic. Based on carcinogenicity studies, DMN can also generate different types of epithelial tumours in different animal species, even at low concentrations [33]. DNM currently is a used for liver carcinogenic studies because it is water soluble and can be used in a single dose or as multiple doses in a short time for the initiation of the carcinogenic process in the liver of experimental models, such as the rat [34].

The acute hepatotoxic effect of DMN begins shortly after application, with impairment of the protein insertion into hepatocytes. DMN can also interfere with the respiratory cycle in the hepatocytes and induce a necrotizing process [35]. The most common pattern of necrosis after acute and chronic poisoning is a centrilobular haemorrhagic necrosis that is similar to carbontetrachloride (CCl4). DMN is no longer used in any industry, but can still be produced as an offshoot of different industrial products, such as dyes, rubber tires, and pesticides. It also can be released as a result of physiologic /photogenic processes in the water or air. It is not active by itself, but may be metabolized to a known methylating agent that is

responsible for the toxic and carcinogenic effects. Carcinogenic effects appear after one or multiple applications of DMN. Animal experiment models have shown that DMN metabolites affect RNA and lead to RNA breakdown in messenger RNA [36]. DNM-induced liver tumours are mainly anaplastic and originate from hyperplastic nodules, not biliary duct cells.

1.2.2.3 N-Nitrosomorpholine (NNM)

NNM also belongs to the nitrosamine class and is a known, strong hepato-carcinogen that is often used in animal studies to initiate chemically induced hepato -carcinogenesis. It has been found primarily in products of the rubber industry (in rubber nipples for baby bottles). It has also been found in different cheeses, vegetables, and alcoholic beverages as result of the exposure of plants with industrial-waste-released water [37, 38]. Subsequently, NNM was considered a human carcinogen (group 2B), based on multiple animal exposure studies. Animal studies have reported effects on tumour formation in the liver, lung, nasal cavity, and kidney after oral exposure [33]. Even chronic inhalation of NNM can induce neoplastic nodules, as well as carcinoma, in the liver [39]. There are no epidemiological studies that have evaluated human exposure to NNM.

Regarding the mode of action, it has been observed that NNM treatment triggered deregulation of genes involved in tumour-suppressor P53-mediated DNA repair pathways [40]. The impairment of tumour suppressor genes and apoptosis genes leads to 'initiation.' If the liver is exposed chronically to NNM or cancer-promoting agents like phenobarbital, the initiated cells develop into pre-neoplastic foci and further progress to carcinoma. Therefore, there are known treatment protocols using NNM for chemical hepato-carcinogen studies in laboratory animals, especially rodents [41].

1.2.3 The role of non-genotoxic hepato-carcinogens for tumour promotion

NGC compounds commonly require chronic exposure to produce tumours. These agents also display dose-response relationships between exposure and tumour

formation, and, because of that, most of the time, a threshold can be observed.

NGCs may induce tumour 'promotion' by disturbing the balance between cell replication and cell death through the interaction with molecules involved in the regulation of both processes. These interactions can occur through a wide variety of mechanisms. Some recently discovered mechanisms are: receptor-mediated endocrine disruptors;

non-receptor-mediated endocrine disruptors; xenobiotic receptor activation; peroxisome proliferation; oxidative stress; inhibition of the intracellular gap-junction; induction of an inflammatory response; and interaction of the mesenchyme with parenchymal cells [42].

As mentioned before, tumour-promoting activities—unlike initiation—are largely reversible and require continuous exposure to a toxic dose of NGCs. As an example, phenobarbital inhibits tumorigenesis in the liver when administered simultaneously with special carcinogens. Nevertheless, it can promote carcinogenic activity when given after the same carcinogens [43].

One common effect of NGC compounds is the induction of cell proliferation. This proliferation can be the result of two mechanisms in the liver: either inhibition of apoptosis and interaction with some cellular receptors, or, through modulation of growth factors and cell growth regulatory cascades, which leads to increased DNA synthesis and mitosis [44]. There are also chemicals like chloroform that induce hyperplasia.

The altered expression of tumour suppressors and oxidative stress genes –often with the production of radical oxygen species (ROS)—are common sub-mechanisms enhanced by several non-genotoxic carcinogens and are considered critical in the process of NGC-driven carcinogenesis [45]. Reactive oxygen species can be metabolically activated by cytochrome P450, which has been shown to be related to DNA damage, and to the activation of oncogenes and hyperplasia.

Different modes of action, tissue specificity, and also the lack of genotoxicity, make NGC identification a challenging task. In recent years, rodent bio-assays are considered the best available method for detecting such carcinogens.

It is now widely accepted that chemical NGCs have different modes of action, with some of them acting upon hepatocytes via different membranes and nuclear receptors, while others induce cancer promotion by affecting mesenchymal cells. However, the underlying mechanism leading to cancer promotion in both directions is not completely understood.

There is evidence showing that the microenvironment, and especially, mesenchymal-parenchymal interactions, play a crucial role in HCC induction. For example, HCC may serve as a paradigm for inflammation-induced cancer [46, 47]; however, how chronic inflammation is linked to HCC at the molecular level is not completely clear. Some studies indicate that some part of the inflammatory mediators produced by mesenchymal cells, such as Interleukin 6 (IL-6) or tumour necrotizing factor alpha (TNF-α), may promote carcinogenesis by inducing compensatory proliferation and regeneration in the aberrant cells (Figure 2) ADDIN EN.CITE [48-50].

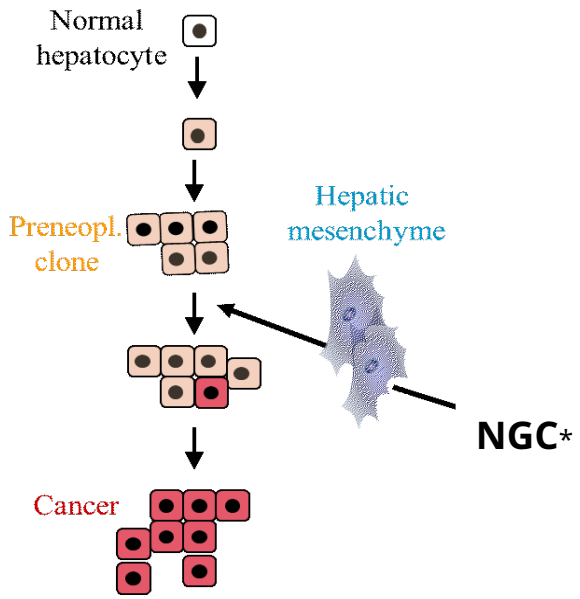

Figure 2: Schematic view of the presumed effect of mesenchymal cells on parenchymal cells and consequent cancer promotion. (*): NGC: Non-genotoxic carcinogen

Nuclear factor kappa B (NF-ƙB) is one of the most activated transcription factors in the immune cells within the innate immune response, as well as within the secondary immune response [51, 52]. It has been shown that NF-ƙB has a crucial role in epithelial tissues to fight infectious agents and maintain barrier function in such cells. It is well established that activation of NF-ƙB activates TNF-α transcription that can induce proliferation of liver cells ADDIN EN.CITE [53, 54]. In mammals, NF-ƙB comprises five members: NF-ƙB-1; NF-ƙB-2; NF-ƙB-3; Rel; and Rel A. In the normal non-stimulated cells, all of these five proteins are inactive via the regulatory domain, which binds with specific NF-ƙB inhibitors known as IƙB proteins. Phosphorylation of IƙB with IƙB kinase (IKK) leads to activation of NF-ƙB.

NF-ƙB is activated in a large number of tumours, but the underlying cause of this activation of NF-ƙB can be different. In the classical form, the NF-ƙB signalling pathway is activated by IL-1, LPS, or TNF-□□σ. This pathway plays a pivotal role in innate immunity, inflammation, and inhibition of apoptosis. The other alternative pathway is activated with nuclear translocation of Rel-B –p52 dimers and is important in the adaptive immune response and humoral immunity [55].

Activation of the classic pathway and inhibition of apoptosis (programmed cell death) is considered an important concept in tumour promotion. The second theory for the observed activated NF-ƙB in malignant cells involves the mutations in genes that encode NF-ƙB sub-units or mutations that affect the components of signalling pathways that their upstream is activation of proliferation [56].

Briefly, chronic inflammation (which can be induced by chemical or physical injury) is associated with tumour promotion and aberrant compensatory proliferation, and on the other hand, with inhibition of apoptosis [43] [57].

Wnt signalling, transforming growth factor β (TGFβ) signalling, and the PDGF signalling pathway, again, as mentioned previously, are activated in the process of carcinogenesis, but the underlying mechanism at the cellular level is not completely understood. These cascades can be initiated by mesenchymal cell stimulation [58, 59].The intra-tumoural density of all mesenchymal cells—lymphocytes, vascular endothelial cells, as well as fibroblasts—has been shown to have prognostic

significance in different solid tumours, including HCC ADDIN EN.CITE [60-62].

1.2.3.1 Receptor-mediated effects

During the past few decades, several concerns have developed about describing the mode of action of chemically induced hepato-carcinogens. In rodents, exposure to the NGTX substances repeatedly leads to upregulation /induction of cytochrome P450 enzymes [63]. There are numerous types of enzymes involved in the hepatic P450 cytochrome and these induction responses are normally mediated via a receptor-mediated mechanism that finally leads to enhancement of gene transcription in the liver cells [64]. Some of these important receptors are arylhydrocarbon receptor (AHR) and nuclear receptors, such as CXR, CAR, and PPAR (Figure 1). Many NGCs are ligands of nuclear receptors, such as CAR, PPAR alpha, PXR, etc. [2].

1.1.3.2. Importance of nuclear receptors in NGC-induced hepato-carcinogenesis

Nuclear receptors (NRs) are transcription factors that respond to synthetic and natural ligands, including steroid hormones, fatty acids, bile acids, and vitamins. The NR family is the largest group of transcriptional regulators, consisting of 48 members [65].

The glucocorticoid receptors and estrogenic receptor were the first NRs cloned in 1985 and 1986 [66].

Together with other steroid hormone receptors, the thyroid hormone receptor and receptors for Vitamin A and D, these high affinity receptors belong to the classic endocrine NRs. With few exceptions, they have a common domain-based structure consisting of four fundamental domains: the A/B domain with the ligand independent activation; the DNA binding domain (C domain); a hinge region (the D domain); and the E/F domain, which governs the ligand-dependent function [67] . According to the sequence homology of endocrine receptors, numerous NRs have been cloned subsequently, but the natural function of many of them was initially unknown. This group is called “Orphan-NRs.” The natural ligands of some

NRs in this group have now been identified. Meanwhile, in this group, a class of NRs have been identified that regulate lipid, glucose, and bile acid homeostasis. This class is one of the most promising and investigated drug-targeted NRs. A subgroup of this class, which is called “enigmatic” adapted orphans, are connected to the liver metabolism and have considerable potential as chemical and pharmacological targets.

In addition, NRs interact with classic pro-inflammatory transcription factors, such as nuclear factor kappa B (NFkB). These NRs regulate the transcription of genes that play a key role in normal physiology and in the development of the liver. NRs have also been implicated in the process of hepatocyte priming after injury, in proliferation during liver regeneration, in chronic hepatitis, and in the development of HCC [68]..

Many drugs, chemicals, and toxins, including NGCs, are known to act via these receptors (Figure1). Studies have shown that agents that act through selected NRs are associated with the ability to regulate cell proliferation and / or survival and apoptosis. Also, such agents can promote overgrowth in cells with genetic damage (mutated cells) into pre-neoplastic lesions, and thus, under special situations, progression to cancer development.

1.1.3.3. PXR and CAR

Based on the recent classification of NRs, the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) both belong to the NR II family [69].

The first identified gene regulated by CAR was CYP2B, which belongs to cytochrome P450 enzymes. PXR is also known as a steroid and xenobiotic receptor and induces transcription of phase I drug metabolizing enzymes ADDIN EN.CITE [69-71]. These receptors are also involved in the regulation of phase II drug metabolism and excretion of drugs and chemicals, including uridine diphosphate glucose UDP-Glu [72].

Studies in wild-type mice and rats show that activation of CAR by special ligands (like benzene) leads to increased cellular proliferation [73]. In addition, anti-apoptotic factors from the BCL-II family that repress P53 have been suggested,

which are CAR-dependent [74]. It has been shown that CAR plays a role in tumour promotion [74].

Unlike CAR, the PXR receptor does not have significant constitutive activity, and therefore, has to be activated by ligands to exert its effect [75].

PXR has widespread expression in the tissues of humans and rodents. It is highly expressed in the liver, small intestine, and colon, and plays an essential role in the metabolism of xenobiotics [76].

Unlike other NRs, the number of genes shown to be regulated by PXR is growing rapidly. The major transcriptional targets of PXR are CYP3A4 and MDR1, which can affect xenobiotic, as well as endobiotic, metabolism [77]. It has been shown that the activation of PXR via some ligands, like rifampicin, can attenuate NFkB proteins. Moreover, the activation of NFkB was shown to inhibit PXR and vice versa [78].

It is now becoming apparent that these receptors "cross-talk" with other endogenous stimuli (for example, inflammatory/growth factors produced by mesenchymal cells) to regulate various aspects of liver physiology, such as cell growth, tumour development, and hormone homeostasis.

1.3 Epithelial-mesenchymal cross-talk and its role in hepato-carcinogenesis

The role of the microenvironment in the pathogenesis of malignant disease is still not completely understood. Several studies have demonstrated that the phenotype of pre-neoplastic, as well as neoplastic lesions in some malignancies, can be modulated by external triggers from the surrounding microenvironment ADDIN EN.CITE [79-82].

It has been documented that specific gene expression profiles in the cancerous tissue surrounding hepatocellular carcinoma (HCC) can predict the survival and /or recurrence of the disease ADDIN EN.CITE [83, 84].

Consistent with this, recent studies from our laboratory indicate that the cross-talk between chemically initiated hepatocytes with mesenchymal cells in the liver plays

a critical role in tumour formation [85], again, pointing to the importance of the microenvironment for the initial stages of hepato-carcinogenesis.

There are studies showing that a growth-constraining or senescent tissue environment is capable of generating a powerful force for the selective expansion of pre-neoplastic lesions in the liver, subsequently leading to HCC [86].

1.3.1 Liver mesenchymal cell types and their function

Hepatocytes are the main parenchymal cells in the liver. In addition, there are different non-parenchymal cells (mesenchymal cells) in the hepatic tissue, which make up a complex microenvironment consisting mainly of hepatic stellate cells, endothelial cells, and immune cells. Macrophages are the most important immune cells in the liver. It has been established that two sets of macrophages coexist in the normal rat liver. They can be distinguished via histochemistry: small kupffer cells (KC) that have an ED-1 antigen on the surface and completely mature KC—or simply KCs—that can be recognized by ED-2 monoclonal antibody. Kupffer cells are composed of about 30% sinusoidal cells [87, 88].

The differentiated KCs are mostly located in the sinusoidal lumen, in close contact with endothelial cells ADDIN EN.CITE [89-91]. KCs play a crucial role in the initiation of immune responses in the liver and, by producing several cytokines, functionally cross-talk with hepatocytes, as well as other mesenchymal cells. It has also been shown that the KC population expanded in response to different stimulation mechanisms [92, 93].

The morphological characteristics and varying activities of these two types of macrophages have been studied using immunohistochemistry *in vitro* and *in vivo* [94, 95]. KCs, because of their strategic position, are the determinants for the removal of particulates, such as organisms, as well as for the removal of soluble materials from portal blood.

Hepatic dendritic cells (DCs) are also important immune cells in the liver, which normally are present in the sinusoidal space. They have multiple sub-group

populations, but the major function of DCs is to work as natural killer cells.

Hepatic stellate cells (HSC) are another important component of mesenchymal cells. In normal liver tissue, they are characterized by a lipid droplet containing vitamin A. HSCs are important in extracellular matrix modelling after liver injury [96].

It is also well known that activated HSC infiltrate HCC stroma and peri-tumoral tissue and are localized in the sinusoid, fibrous septae, and tumour capsule [97, 98]. A schematic view of different non-parenchymal cells and their assumed position in normal liver tissue is shown in Figure 3.

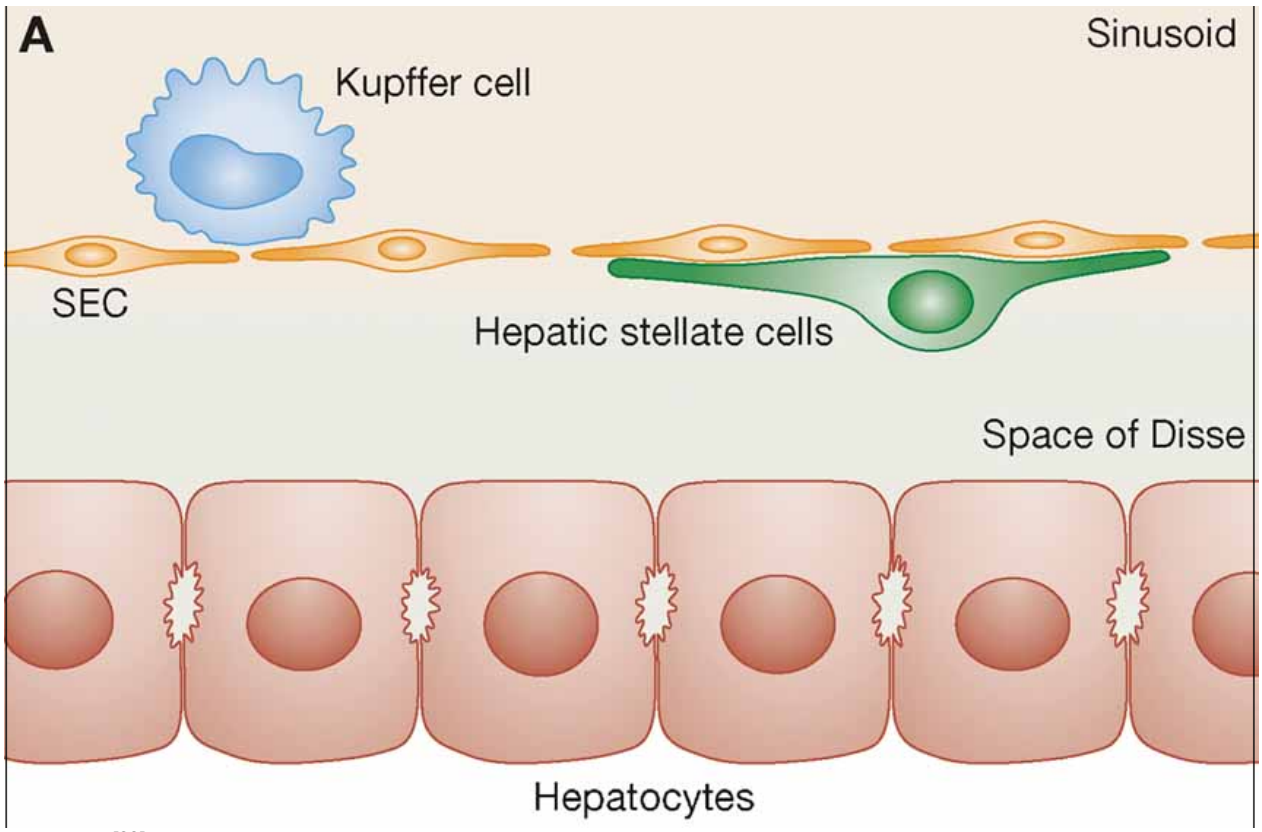

Figure 3 [99].

Figure 3 **Schematic morphology of different mesenchymal cells in the liver (SEC: sinusoid endothelial cell)**

1.3.2 Growth regulation in the liver

As mentioned previously, the liver plays an important role in the detoxification of drugs and chemicals before they reach the systemic circulation. The metabolic reactions and mechanisms that are responsible for detoxifying chemicals—phase two enzyme bio-activation—have many overlaps with growth modulatory systems in the liver. As an example, activation of the P-450 system results in the provocation of enzymes that are involved in the production of oxygen radicals and/ or induction/inhibition of apoptosis, necrosis, or cell proliferation. Thus, liver injury and regeneration of the hepatocytes after acute and chronic injury is an important task. The liver has the unique capability of compensatory regeneration after acute toxic damage or surgical resection, but this specialty seems to be insufficient in chronic liver injuries, such as alcohol abuse or HBV/HCV infection or in long-time exposure to some chemicals [99]. Such situations can lead to dysregulation of growth/apoptosis pathways and even the development of liver cancer. Therefore, a good understanding of growth regulation mechanisms and important growth regulatory factors in the liver can help to improve therapeutic strategies in liver cancer and provide a biomarker for the early detection of high-risk carcinogenic chemicals.

Initially partial hepatectomy (PH) has been used as a useful model with which to understand the mechanism of liver growth, as it does not lead to extensive cellular necrosis, but rather, only a systemic inflammatory response due to surgical removal. In contrast, subsequent models were developed using chemicals such as CCl4 that induce centrilobular necrosis. In these chemical models, the sequence of regeneration pathways is dominated by acute inflammatory pathways.

Although the initiation mechanisms of liver cells after stimulation with different types of liver injuries share many similarities, at the molecular level, there are increasing data indicating that there is a detectable, compound-specific mechanism, which will be discussed later [100, 101].

Biochemical studies on the *in vitro* cultured HCs show that hepatocyte growth factor (HGF), epidermal growth factor (EGF), and transforming growth factor alpha (TGF α) are the most important factors that reveal a direct mitogenic effect on HCs. Other

factors, such as tumor necrosis factor alpha (TNF-α) or prostaglandins, can indirectly induce hepatocyte replication. It has been shown that TNF-α in low concentrations induces DNA synthesis in the normal rat liver. TNF-α and IL-1 can also induce decarboxylase, which is involved in cell proliferation [102] [103]. Some of these factors are produced by the HC itself and have paracrine in addition to autocrine effects, such as like FGF1 and FGF 2 (fibroblast growth factor).

Not only is the origin of the production of such factors different, but also the underlying cascades that become active vary depending on the underlying cause of injury. For example, a viral infection in the liver activates cytotoxic T lymphocytes and this leads to production of pro-apoptotic cytokines, such as TNF-α, TGF- β, and p53. But, in liver transplantation models, the expression of apoptosis regulatory proteins, like fas, perforin, and granzyme B, are considered a rejection marker. TNF -α induces proliferation only when it activates the NF-κB cascade; otherwise, it leads to apoptosis [104].

However, there are factors and pathways involved in the control and correction of inappropriate cell proliferation. TGF β1 has been reported to inhibit HC-proliferation [105].

To summarize, the balance between apoptosis and proliferation after each type of liver injury is important for the survival of liver cells. Chronic damage leads to dysregulation, which accelerates multiple cellular diversifications in HCs, together with the surrounding microenvironment, including the mesenchyme.

1.3.2.1 Mesenchyme-derived growth factors are crucial for liver growth regulation

As studied so far, the regulation of cell replication and apoptosis cycles in HCs is controlled by an abundant number of cytokines and growth factors, mainly produced by the activated microenvironment. During the process of regeneration, mesenchymal cells also proliferate to preserve the intercellular communication. Furthermore, they produce growth factors that are involved in proliferation and /or the inhibition of regeneration. During the past decade, many *in vivo* and, especially, *in vitro* studies focused on the role of each cell population in the liver mesenchyme.

Based on their function in the liver (mentioned in Chapter 1.2.1), they can induce both stimulatory and/or inhibitory effects after liver damage. However, the dominance of each effect—stimulation or inhibition—depends on etiology and the type of injury [106]. As an illustration, TNF-α and IL-6 are the most important growth factors and TGFβ and IL-1-α are known as counter-regulators for cell proliferation, which are produced and released by KCs [107]. Another important pathway that is activated by KCs after liver injury is the INF-ɣ pathway, which may also trigger the carcinogenesis process. However, it has been shown that in the absence of a receptor, the INF-ɣ pathway can suppress carcinogenesis [108].

Hepatic DCs, as an additional support for liver immune cells, also can produce TNF -α in the course of liver injury. Studies show that hepatic DCs are more active in immune suppression than stimulation, although, after liver damage, they can produce IL-1 and IL-10, which block the NF-κB pathway, and, as mentioned previously, leads to cell death.

Endothelial cells can produce endothelial growth factor (VEGF) that works potentially as a cell proliferator [109, 110].

The role of hepatic stellate cells (SCs) in liver growth is somewhat complex: SCs, after stimulation with different types of chemicals, can produce IL-6, which induces the proliferation of EDs, KCs, and SCs, as they themselves have a receptor for IL-6 (probably with an autocrine effect). In addition, SCs can synthesize HGF, PDGF, and fibroblast growth factor 1 and 2 (FGF1 and FGF2), which have a direct proliferatory effect on HCs.

1.4 Effects of NGCs on mesenchyme-parenchymal cross-talk

This short characterization of different parenchymal cells shows that there are many overlapping and possibly interfering pathways between different parenchymal cells that makes the understanding of the mesenchyme-parenchymal axis somewhat complex. In our study, we tried to find determinant, connective pathways between the mesenchyme and the parenchyma after stimulation with NGCs.

1.3.1 Two prototypical NGC: PB and CPA

As noted above, NGCs serve as chemicals that are able promote tumour formation via secondary mechanisms which are not directly related to the gene damage [5]. But, there are still no clear-cut criteria for the detection of NGCs due to the lack of information about the mode of action and subcellular mechanisms that lead to carcinogenesis by such substances. Phenobarbital and cyproterone acetate are two known representatives of PXR/CAR trans-activating substances that have been widely used in carcinogenicity studies.

1.3.1.1. Phenobarbital

Phenobarbital (PB) is a long-acting barbiturate, still widely used as an anticonvulsant drug, often in combination with phenytoin and other therapeutic agents. It is a well-established, non-genotoxic carcinogen in rodents [111]. PB and related agents are not genotoxic, but they work as a tumour promoter, increasing the incidence of spontaneously and chemically induced pre-neoplastic lesions in the liver of selected strains of mice and rats ADDIN EN.CITE [111-114].

Chronic exposure to PB (0, 05% w/v in drinking water for 12 months) results in significant promotion of hepatic tumours in rodents [114, 115].

The precise mechanism of tumour-promoting activity by PB in rodents is not completely clear. The experimental evidence proposed the mode of action (MOA) of PB in rodents to be partly via activation of the nuclear receptors CAR, PXR, and peroxisome proliferator-activated receptor alpha (PPAR α) ADDIN EN.CITE [16, 116-118].

As mentioned previously, CAR can be activated by numerous chemicals and/or medications and is required for gene expression changes, hepatomegaly, and tumour formation ADDIN EN.CITE [119-121].

CAR plays a crucial role in the tumour-promoting activity of PB, because the appearance of tumours can no longer be induced by PB in CAR-deficient mice [122].

A second mechanism, likely involved in PB-mediated tumour promotion is the selection of hepatocytes carrying the activated mutant and constitutively activated version of transcription factor ß-catenin [123]. The conditional knock-out of the relevant gene in mice (Ctnnb1) leads to the eradication of PB-induced tumour promotion [124].

It seems that, in therapeutic doses in men (1-4 mg/kg body weight), the impact was too low to make a significant change in the susceptibility to liver tumour development, even after prolonged drug administration with daily dose levels of >80 mg/kg body weight. The proliferative response of cultured hepatocytes and/or the inhibition of apoptosis followed by PB treatment in rodents have, thus far, not been observed in comparable cultures of human primary hepatocytes ADDIN EN.CITE [125-127]. Recently developed humanised PXR/CAR mice models displayed the induction of

P450s and the induction of HC hypertrophy, but did not show hepatocyte proliferation [128]. In another study, long-term treatment with PB induced a highly similar hepatic transcriptional program in wild-type and humanised CAR/PXR mice [115]. This transcriptional response included the upregulation of some cell cycle genes, as well as proliferation markers like Ki67. In transgenic models that only express human CAR in the liver (hCAR mice), treatment with PB for one week resulted in relative increases in liver weight and cell proliferation, but significantly less than that in wild-type controls [129]. It seems that, despite similarities in the affected transcriptional program between mice and humans, human CAR and PXR do not support tumour promotion by PB in humans as strongly as in mice [122].

In human epidemiological studies, anti-convulsant drugs, and particularly PB, are known to increase liver enzymes related to cytochrome P450 and consequently—under selected situations—may activate and /or detoxify carcinogens [130, 131]. Epidemiological data on liver cancer and PB are limited, but indicate an absence of any specific association between HCC and PB in humans. The data indicate that, in epileptic patients, factors other than anticonvulsant treatment—specifically, PB-like factors that are associated with an increased risk of cirrhosis—are responsible for

the earlier appearance of liver cancer observed in epileptic patients [132].

1.3.1.2 Cyproterone acetate (CPA)

Cyproterone acetate (CPA) is a synthetic progesterone with anti-androgenic activity, which has been widely administered, especially during the past few decades for the treatment of female hirsutism, acne, alopecia, and, more importantly, as a part of contraceptive pills. For many years, “Diane” was a popular contraceptive drug that contained CPA in combination with ethinyl-estradiol. It was also prescribed as hormone therapy for prostate carcinoma in men because of its progestational and anti-gonadotropic effect. CPA binds to androgen receptors and blocks androgenic activity via competitive inhibition of androgen receptors.

It has been known for years that CPA produces liver tumours in rats after administration of very high doses [133].

In pre-clinical tests in mammalian cells, mutation frequencies were not increased at dose levels between 5-80 µg/ml [134]. Also, CPA did not cause increased chromosome damage/aberrations at concentrations from 5-150 µg/ml in a cryptogenic study with human cultured lymphocytes [135]. Further studies showed that CPA increased cell proliferation in the rat liver (*in vivo* and *in vitro*) and the tumorigenic potential was likely attributable to the growth-promoting effect [133, 136].

In contrast, there are published studies that have shown that CPA produces DNA adducts and induces DNA repair in primary hepatocytes of female rats and humans of both genders, as well as in the liver of intact rats ADDIN EN.CITE [137-139].

The level of adduct was dose-dependent in a range of 1 to 10 µM, but about 20-fold higher in female hepatocytes compared to those in to male HCs. The genotoxicity of CPA was also investigated in cultures of human liver cells with the induction of DNA repair synthesis [138]. Interestingly, in contrast to rat liver, in these cell culture studies, no significant sex dependence was observed.

To summarize, a series of clinical studies have been done in the last several decades indicating that CPA has the potency to act not only as the promoting agent, but also as an initiating agent in hepato-carcinogenesis.

In epidemiological studies in humans, there was no evidence of any significant association with long-term intake of combined contraceptive pills containing CPA and HCC ADDIN EN.CITE [140-142].

In our laboratory, we focused on phenobarbital and cyproterone acetate as two well-established chemical hepato-carcinogens in rodents to clarify the cancer-promoting mechanisms. We focused on mesenchyme-parenchymal interactions of these two non-genotoxic carcinogens. We used various methods, such as gene expression analysis, omics technology, and histopathological examination.

1.5 Aims of this thesis

1) To study the tumour-promoting mechanism of phenobarbital in the rat liver, its influence on hepatocytes as well as mesenchymal cells, and the interactions between mesenchymal and parenchymal cells in the process of phenobarbital-induced cancer promotion.
2) To understand the receptor-mediated, tumour-promoting effect of cyproterone acetate in primary rat hepatocytes and the interactions between mesenchymal cells and hepatocytes after *in vivo* and *ex vivo* treatment.
3) To study the process of liver inflammation triggered by NGCs and its possible role in hepato-carcinogenesis.

2 CHAPTER TWO: RESULTS

2.1 Prologue

To fulfil the aim of the thesis, (see Chapter 1.5), and as part of the MARCAR project, the overall aim of our work package was to elucidate the interactions between the mesenchyme and the parenchyma, which might provide a better understanding of non-genotoxic carcinogenesis and important applications in the

development of markers to predict the action of NGCs.

At first, we investigated the NGC-induced effects in the rat liver mesenchyme and the parenchyma. For this purpose, HC and MC were separated from livers that had been treated with the NGC phenobarbital for 14 days. Gene expression analysis and proteome analysis of the secretome of the MC and HC revealed that PB treatment *in vivo* led to a marked pro-inflammatory reaction in the MC. Further, we focused on the significance of the mesenchyme in the induction of NGC-driven outgrowth of pre-neoplastic lesions (PNL). A unique cell culture model of the initial stages of hepatocarcinogenesis was applied. A few days after treatment with various genotoxic carcinogens, single initiated hepatocytes appeared that were detectable by their selective immune activity against placental glutathione S-transferase (GSTP-positive initiated cells) [143]. A considerable fraction of GSTP-positive cells developed into PNL. When we isolated these cells, and transferred them into culture, they showed an inherent growth advantage and overresponse to known growth stimulators or suppressors and to various NGC, such as cyproterone acetate (CPA), in a manner identical to that in the *in vivo* situation [143]. In this culture model, the addition of the MC supernatant (secrotome) to the treated and untreated HCs was also investigated. The first publication was designed to show the drug-specific reaction of HCs to the secretome of *in vivo* treated MC. The differences between the secretome obtained from MC treated with PB or CPA helped to better understand the mode of action (MOA) of each drug.

2.2 PDF of first paper:

Archives of Toxicology

GENOTOXICITY AND CARCINOGENICITY

(2018)
92:953–966
https://doi.org/10.1007/s002
04-017-2080-0

Mesenchyme-derived factors enhance preneoplastic growth by non-genotoxic

carcinogens in rat liver

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Received: 11 August 2017 / Accepted: 21 September 2017 / Published online: 21 December 2017

Abstract

Many frequently prescribed drugs are non-genotoxic carcinogens (NGC) in rodent liver. Their mode of action and health risks for humans remain to be elucidated. Here, we investigated the impact of two model NGC, the anti-epileptic drug phe- nobarbital (PB) and the contraceptive cyproterone acetate (CPA), on intrahepatic epithelial–mesenchymal crosstalk and on growth of first stages of hepatocarcinogenesis. Unaltered hepatocytes (HC) and preneoplastic HC (HC_{PREN}) were isolated from rat liver for primary culture. DNA replication of HC and HC_{PREN} was increased by in vitro treatment with 10 µM CPA, but not 1 mM PB. Next, mesenchymal cells (MC) obtained from liver of rats treated with either PB (50 mg/kg bw/day) or CPA (100 mg/kg bw/day), were cultured. Supernatants from both types of MC raised DNA synthesis of HC and HC_{PREN}. This indicates that PB induces replication of HC and HC_{PREN} only indirectly, via growth factors secreted by MC. CPA, however, acts on HC and HC_{PREN} directly as well as indirectly via mesenchymal factors. Transcriptomics and bio-informatics revealed that PB and CPA induce extensive changes in the expression profile of MC affecting many growth factors and pathways. MC from PB-treated rats produced and secreted enhanced levels of HBEGF and GDF15, factors found to suppress apoptosis and/or induce DNA synthesis in cultured HC and HC_{PREN}. MC from CPA-treated animals showed enhanced expression and secretion of HGF, which strongly raised DNA replication of HC and HC_{PREN}. In conclusion, our findings reveal profound effects of two prototypical NGC on the hepatic mesenchyme. The resulting release of factors, which suppress apoptosis and/ or enhance cell replication preferentially in cancer prestages, appears to be crucial for tumor promotion by NGC in the liver.

Keywords Hepatocarcinogenesis · Liver mesenchyme · Non-genotoxic hepatocarcinogenesis · Phenobarbital · Cyproterone acetate

Abbreviations

BW	Body weight
CAR	Constitutive androstane receptor
CO	Untreated or solvent treated control rats or cells
COX2	Cyclooxygenase 2
CPA	Cyproterone acetate
CTGF	Connective tissue growth factor
EC	Endothelial cell

Marzieh Nejabat and Teresa Riegler contributed equally.

Electronic supplementary material The online version of this article (doi:10.1007/s00204-017-2080-0) contains supplementary material, which is available to authorized users.

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EM	Exsudative monocyte
GDF15	Growth and differentiation factor 15
(r)HBEGF	(recombinant) heparin-binding EGF- like growth factor
HC	Hepatocyte
HC_{PREN}	Initiated/preneoplastic hepatocyte
HGF	Hepatocyte growth factor
IL6	Interleukin 6
KC	Kupffer cells
LI	Labeling index
MC	Mesenchymal liver cells

MC-CO	MC obtained from a solvent-treated or untreated control rat
MC-CPA	MC obtained from of a CPA-treated rat
MC-PB	MC obtained from a PB-treated rat
mPR	Membrane located progesterone receptor
NGC	Non-genotoxic carcinogen
nHBEGF	HBEGF-neutralizing antibodies
nHGF	HGF-neutralizing antibodies
NNM	N-nitrosomorpholine
NOS2	Nitric oxide synthase 2
PB	Phenobarbital
PBS	Phosphate-buffered saline
PPAR-alpha	Peroxisome proliferator-activated receptor alpha
PGRMC1	Progesterone receptor membrane component 1
PXR	Pregnane X receptor
SN	Supernatant
SN/MC-CO	Supernatant of MC obtained from untreated or solvent-treated rats
SN/MC-CPA	Supernatant of MC obtained from CPA treated rats
SN/MC-PB	Supernatant of MC obtained from PB-treated rats
LI labelling index	% Cells replicating DNA

Introduction

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Many risk factors for this disease have been identified so far, such as chronic inflammation secondary to hepatitis-virus infection, chronic ethanol consumption or metabolic disorders (Llovet et al. 2016). The liver is also exposed to a great variety of environmental pollutants, synthetic steroid hormones or other drugs, which act as NGC in rodent bioassays. Considering the worldwide application of such compounds, thorough knowledge on the mode of NGC action is prerequisite to better estimate the possible liver cancer risk in exposed humans (Jacobs et al. 2016; Luch 2005).

According to current concepts, the first stage of hepatocarcinogenesis may be induced by genotoxic carcinogens, causing mutations in critical (growth) regulatory genes and leading to the formation of initiated cells. NGC, however, do not affect or bind directly to DNA but alter proliferation and survival of cells, DNA methylation, transcriptome patterns and/or cell signalling, which increase the likelihood of promotion of initiated cells. These effects are largely reversible and require continuous exposure to a sufficient dose to be

sustained (Ellinger-Ziegelbauer et al. 2008; Pogribny and Rusyn 2013; Schulte-Hermann et al. 1990).

For the present study, we chose two prototypical NGC: (i) the barbiturate PB, a drug used to treat certain forms ofepilepsia (Trinka and Kälviäinen 2017). PB is not considered to be DNA-reactive. In rats and mice chronic administration of PB causes liver tumor formation (Elcombe et al. 2014). (ii) CPA, a steroidal synthetic progestagen and anti-androgen, is applied as chemotherapy of androgen-dependent prostate cancer and is frequently prescribed as birth control pills for women suffering from hyperandrogenic conditions, such as acne or hirsutism (Azziz 2007; Bastide et al. 2013). CPA is known for years to produce liver tumors in rats and mice (Kasper 2001).

With regard to the molecular mode of action in the liver, PB activates the nuclear receptor constitutive androstane receptor (CAR), while CPA is a ligand preferentially for pregnane X receptor (PXR) or steroid hormone receptors (Elcombe et al. 2014; Kasper 2001; Schuetz et al. 1998). These receptor interactions mediate hepatic growth and adaptive increases of specific enzyme groups or organelles. Induction of liver growth by NGC is a self-limited and tightly controlled process, which is per se not carcinogenic. However, initiated/preneoplastic HC (HC_{PREN}) and their successors show an altered response to PB or CPA, including excessive proliferation and insufficient elimination by apoptosis. Withdrawal of PB or CPA increases dramatically the apoptotic activity in the preneoplastic lesions, reversing the process of hepatocarcinogenesis (Schulte-Hermann et al. 1990; Grasl-Kraupp et al. 1997). However, the growth factors driving these PB- or CPA-induced shifts between cell renewal and cell death remain to be identified.

Increasing evidence attributes an important role to the hepatic stroma, driving tumor development by a complex pattern of bidirectional signaling between epithelium and microenvironment and involving a plethora of growth stimulatory and inhibitory factors. Thus, hepatocarcinogenesis may largely result from alterations in the normal stromal-epithelial dialogue. We and others have shown that several NGC activate mesenchymal liver cells (MC), resulting in secretion of pro-inflammatory cytokines and reactive oxygen species (Riegler et al. 2015; Roberts et al. 2007). Furthermore, PB caused an elevated secretion of the mesenchymal pro-inflammatory cytokine TNFα. This induced nuclear translocation of NFkB and suppression of the apoptotic activity in HC, an effect being crucial for tumor promotion by this NGC (Riegler et al. 2015). However, it remained to be elucidated whether NGC alter the intrahepatic stro- mal-epithelial dialogue and form paracrine growth loops to enhance growth of cancer prestages.

Considering the daily intake of PB, CPA, or other NGC by millions of humans and in view of the lack of adequate systems to test the impact of these compounds on human

liver, we applied a unique cell culture model for epithelial–mesenchymal interactions and growth control of the very first stages of hepatocarcinogenesis. A few days after treatment of rats with the genotoxic hepatocarcinogen *N*-nitrosomorpholine (NNM), single HC_{PREN} appear, which are detectable by their selective immunoreactivity for placental glutathione-*S*-transferase (GSTp) (Grasl-Kraupp et al. 2000). A considerable fraction of these cells develops into preneoplastic lesions. In primary culture, HC_{PREN} show an inherent growth advantage and overresponse to known growth stimulators or suppressors, as observed in vivo (Löw-Baselli et al. 2000). Here, we investigated whether MC are involved in NGC-driven hepatocarcinogenesis. A combination of transcriptomics, bioinformatic analyses and subsequent experimentation enabled to identify growth factors which are released from PB- or CPA-treated MC and which suppress apoptosis and/or enhance cell replication preferentially in the first stages of hepatocarcinogenesis. Thus, NGC-induced alterations in the liver stroma appear crucial for the action of NGC and have to be considered for a better mechanistic understanding of non-genotoxic hepatocarcinogenesis.

Materials and methods

Animals and treatment

Male and female Han-Wistar rats, 6–8 weeks old, were obtained from Charles River (FRG). They were kept under standardized SPF-conditions. Male rats were treated with PB (5-ethyl-5-phenylbarbituric acid sodium salt; Sigma, St. Louis, MO) at 50 mg/1000 g body weight as single gavage or via drinking water for a period of 7 or 14 days. Controls received tap water only. Female SPF Wistar rats were treated with CPA (Sigma, St. Louis, MO) at 100 mg/10 ml corn oil/1000 g body weight by single gavage on one day or on 6 consecutive days. Oil-treated rats served as control.

Separately, NNM (Sigma, St. Louis, MO), dissolved in phosphate-buffered saline (PBS, pH 7.4), was applied as a single dose (250 mg/10 ml PBS/1000 g body weight) by gavage to 3–5 week old rats. All experiments were approved by the "Committee of Animal Protection" of the Austrian government and performed according to Austrian regulations.

Separation of liver cells and primary cultures

Livers of untreated or treated rats were perfused with collagenase (Worthington, Lakewood, NJ). The cell suspension obtained was used to separate MC from HC by low-speed centrifugation in percoll-gradients. MC were further separated by selective cell adherence into an endothelial cell (EC)-enriched fraction and a fraction consisting mainly of cd68 +/ED1 + exsudative monocytes/macrophages (EM)

and liver-resident cd163 +/ED2 + Kupffer cells (KC). The purities of cell fractions were determined to be: 98.4 ± 0.6 for HC, 99.9 ± 0.1 for MC, 80 ± 19.4 for KC/EM, and 94.5 ± 2.8 for EC. For details on culture conditions, procedures and purities of the cell fractions see Böhm et al. (2013). Stock solutions were prepared for PB and CPA and aliquots were added to the medium to provide final concentrations (Table S1). Treatment of cells commenced 2 h after plating (time point 0).

Determination of DNA replication

^{3}H-thymidine was added 24 h before cell harvest. Autoradiography served to determine the percentage of nuclei with incorporated ^{3}H-thymidine (LI %). In each of the experiments 2000 HC nuclei and 600 HC_{PREN} nuclei were counted. Further details see Löw-Baselli et al. (2000).

Determination of apoptosis

HC were fixed with 4% formaldehyde for 60 min at room temperature and washed twice with PBS. DNA was stained with Hoechst (benzimide H33258; 8 µg/ml PBS) for 5 min. After washing steps and drying, cells were mounted in Kaiser´s glycerol gelatine. Apoptoses (evaluated by chromatin condensation and fragmentation) were counted by fluorescence microscopy. Overall, 1000 HC per dish were analyzed (Riegler et al. 2015).

Whole-genome gene expression analysis

To perform whole genome expression analyses, RNA was extracted. The quality of RNA was tested by the 2100 Bioanalyzer-System (Agilent, St.Clara, CA). Complementary RNA targets were prepared and hybridized according to the manufacturer's procedures on high-density oligonucleotide microarrays (Affymetrix RAT 230 2.0 GeneChip). The microarrays were processed as described before (Riegler et al. 2015).

Following the import of Affymetrix raw data (CEL files) into the R programming language and environment for statistical computing, sufficient quality of the raw data was checked by various metrics and statistical analyses provided by the package ArrayQualityMetrics (Kauffmann et al. 2009). With the help of the RMA method data were normalized and probesets were mapped to gene symbols and Entrez IDs using the appropriate metadata packages ofBioconductor (Gentleman et al. 2004; Irizarry 2003). Open access to data is provided at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qjyjgcqqxpavler&acc=GSE68111.

To detect differential gene expression, a moderated *t* test (implemented in LIMMA package for R/Bioconductor) was

applied combined with subsequent Benjamini–Hochberg correction for multiple testing to ensure a false discovery rate of < 0.05 (Smyth 2004). Fold-change cutoffs of ≥ 2 and ≤ 0.5 determined upregulated and downregulated genes, respectively. For validation of transcriptome data by quantitative real-time PCR (RT-qPCR), see Fig. S1.

Gene expression analyses by RT-qPCR

The extracted mRNA was processed and measured by the ABI-Prism/7500 Sequence Detection System (Applied-Biosystems, Foster City/CA) using TaqMan-based assays (Applied-Biosystems). For primers and assays see Table S1.

Immunodetection

Antisera and ELISA-kits were used, see Table S1. ELISAs were performed according to the manufacturer´s instructions. Material not included in the kit: Tetramethylbenzidine-peroxidase-solution for colour development (Thermo-Scientific); microtiter plates (Costar, Corning, NY). For the protocol of the HBEGF-ELISA see electronic supplementary material.

Results

Effect of PB and CPA on replication of unaltered and preneoplastic hepatocytes in primary culture and the role of mesenchymal growth factors

According to our model, first stages of hepatocarcinogenesis were generated in rats by a single NNM application (Grasl-Kraupp et al. 2000). Twenty-one days later liver cells were isolated. Then, HC_{PREN} and unaltered HC were co-cultured in the same system (Fig. 1A). HC_{PREN}, identified by GSTp expression, showed a higher probability for DNA replication than normal appearing GSTp-negative HC, resulting in an inherent growth advantage of HC_{PREN}, as described before (Fig. 1B: CO; Löw-Baselli et al. 2000).

We tested whether PB or CPA elevate DNA replication in HC and HC_{PREN}, when applied directly to the culture. Interestingly, PB treatment tended to lower the replication rate of HC and HC_{PREN} (Fig. 1B). This was evident also at a lower dose of PB (Fig. S2). However, addition of CPA to the medium induced DNA synthesis considerably in HC and HC_{PREN} (Fig. 1B). Due to the high basal frequency of replicating HC_{PREN}, the additional stimulation by CPA caused that every sixth HC_{PREN} started to cycle, which is remarkably high for HC in primary culture.

Next we applied PB or CPA in vivo and studied the role of the hepatic mesenchyme. To be specific, rats received a

single dose of either compound, and MC were isolated and kept in culture for 24 h. The supernatant (SN), conditioned by the MC (SN/MC-PB, SN/MC-CPA), was transferred to cultured HC and HC_{PREN} from NNM-treated animals. MC- supernatants from solvent-treated rats (SN/MC-CO) served as control (Fig. 1C). SN/MC-CO elevated DNA replica- tion in both, HC and HC_{PREN}, indicating that even without treatment MC produce and release growth factors acting on unaltered HC as well as on first stages of hepatocarcinogen- esis. Application of SN/MC-PB or SN/MC-CPA elevated DNA replication in HC and HC_{PREN} even further, more pro- nounced with SN/MC-CPA than with SN/MC-PB (Fig. 1C). These data suggest that in cultured HC and HC_{PREN} (i) supernatant of untreated MC enhances cell replication, (ii) elevation of HC replication by PB needs mesenchymal growth factors and (iii) CPA exerts—in addition to its direct action—also an indirect effect, mediated by MC-derived factors.

Identification of mesenchyme-derived growth factors supporting DNA replication of hepatocytes

To identify growth factors from untreated hepatic mesenchyme which enhance the replication of HC, we tested numerous candidates including the recombinant form of several erbb- or FGFR-ligands, HGF, some cytokines and also prostaglandins, and compared their DNA replication-inducing potential in HC. Most of the recombinant erbb- and FGFR-ligands, rHGF, rGDF15, and some prostaglandins elevated DNA replication (Fig. 2A).

In parallel, we investigated the intrahepatic epithelial–mesenchymal distribution of the factors (Fig. 2B). Cells were isolated from the liver and separated into HC and MC. By selective cell adherence MC were further divided into an endothelial cell (EC)-enriched fraction and into a KC/EM-enriched fraction. HBEGF, FGF2, FGF4, FGF7, FGF18, IL1ß, TNFα, HGF, IL6 and COX2, the key enzyme of the prostaglandin biosynthesis, were expressed at higher levels in EC- and/or KC/EM-enriched fractions when compared to HC (Fig. 2B). Thus, the growth-inducing effect of SN/ MC-CO might be due to a complex mixture of HBEGF, HGF, FGF7, GDF15, TNFα, IL6, prostaglandins and prob- ably other factors.

We developed an ELISA for HBEGF and found that EC release considerably more HBEGF than KC/EM cells into the supernatant (Fig. 2C). For further clarification we added neutralizing anti-HBEGF (nHBEGF) to SN/ MC-CO and found partial ablation of the growth stimu- lating effect of the supernatant on HC (Fig. 2D). Also HGF was secreted by MC into the supernatant and appli- cation of neutralizing anti-HGF (nHGF) largely blocked the stimulating effect of SN/MC-CO on DNA replication of HC (Fig. 2D). This indicates that HBEGF and HGF are

ography as black spots over nucleus; magnification: ×200. **B** Direct effects of PB or CPA: cells were treated with 1 mM of PB or 10 µM of CPA; treatment was renewed at 48 h and lasted for 72 h. CO, sol- vent controls. **C** Indirect effects of PB or CPA: after a single dose of

the most important components in SN/MC-CO contributing to the paracrine growth stimulation of HC.

PB and CPA induce pronounced alterations in the transcriptome profile of mesenchymal liver cells

Next, we asked which mesenchymal factors mediate the growth-inducing capacity of PB or CPA on HC and HC $_{PREN}$. Rats were treated with PB once or for 14 days or

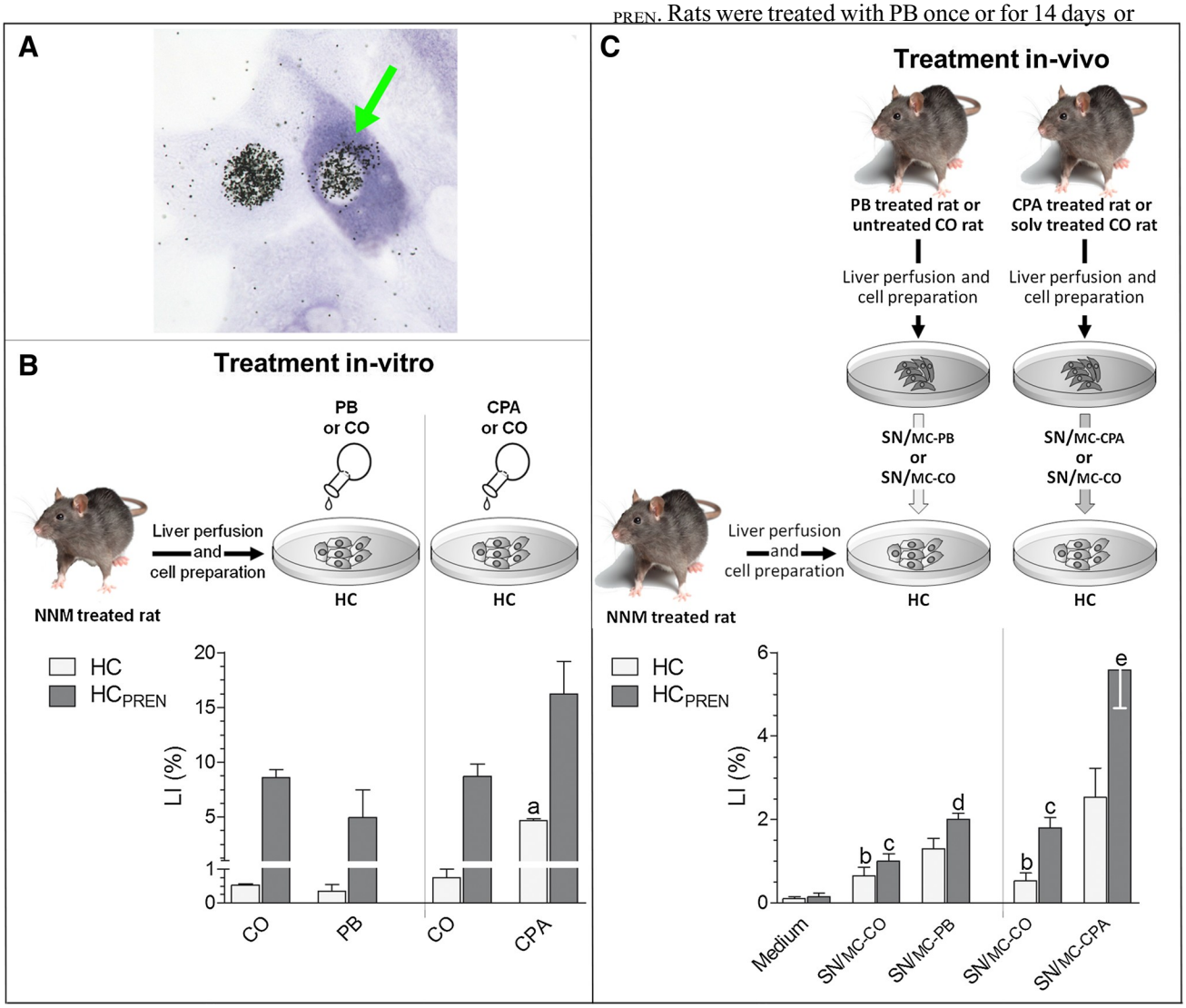

Fig. 1 Direct and/or indirect, MC-mediated effects of PB or CPA on cell replication of first stages of hepatocarcinogenesis. NNM was applied to induce the formation of HC$_{PREN}$; 21 days later HC and HC $_{PREN}$ were isolated and cultured. **A** HC$_{PREN}$ (green arrow) in cul- ture: ^{3}H-thymidine incorporated into DNA is visualized by autoradi-

PB, CPA or solvent in vivo, MC were separated and cultured for 24 h to condition the supernatants, termed SN/MC-PB, SN/MC-CPA, or SN/MC-CO. Supernatants were added to cultured HC and HC_{PREN} for 48 h. **B**, **C** ^{3}H-thymidine was added 24 h before cell harvest; autoradiography served to determine LI. Data are mean ± SEM from 3 in **B** and 5 independent experiments in **C**. Statistics by unpaired *t* test in **B** for control vs. CPA: a) $p < 0.01$; in **C** for control medium vs. SN/ MC-CO: b) $p < 0.05$; c) $p < 0.01$; for SN/MC-CO vs. SN/MC-PB: d) $p < 0.01$; for SN/MC-CO vs. SN/MC-CPA: e) $p < 0.5$

received CPA by gavage on 1 or on 6 consecutive days. Thereafter cells were isolated from the liver and separated into HC and MC. HC and MC from solvent-treated rats served as control. Oligonucleotide arrays revealed that a single dose of PB de-regulated considerably more genes in MC than in HC, an effect even more pronounced after 14 days of PB treatment (Fig. 3). In contrast, single and repeated treatment with CPA induced massive alterations in the gene expression profile of HC and less in MC. To conclude, the data provide clear evidence that the

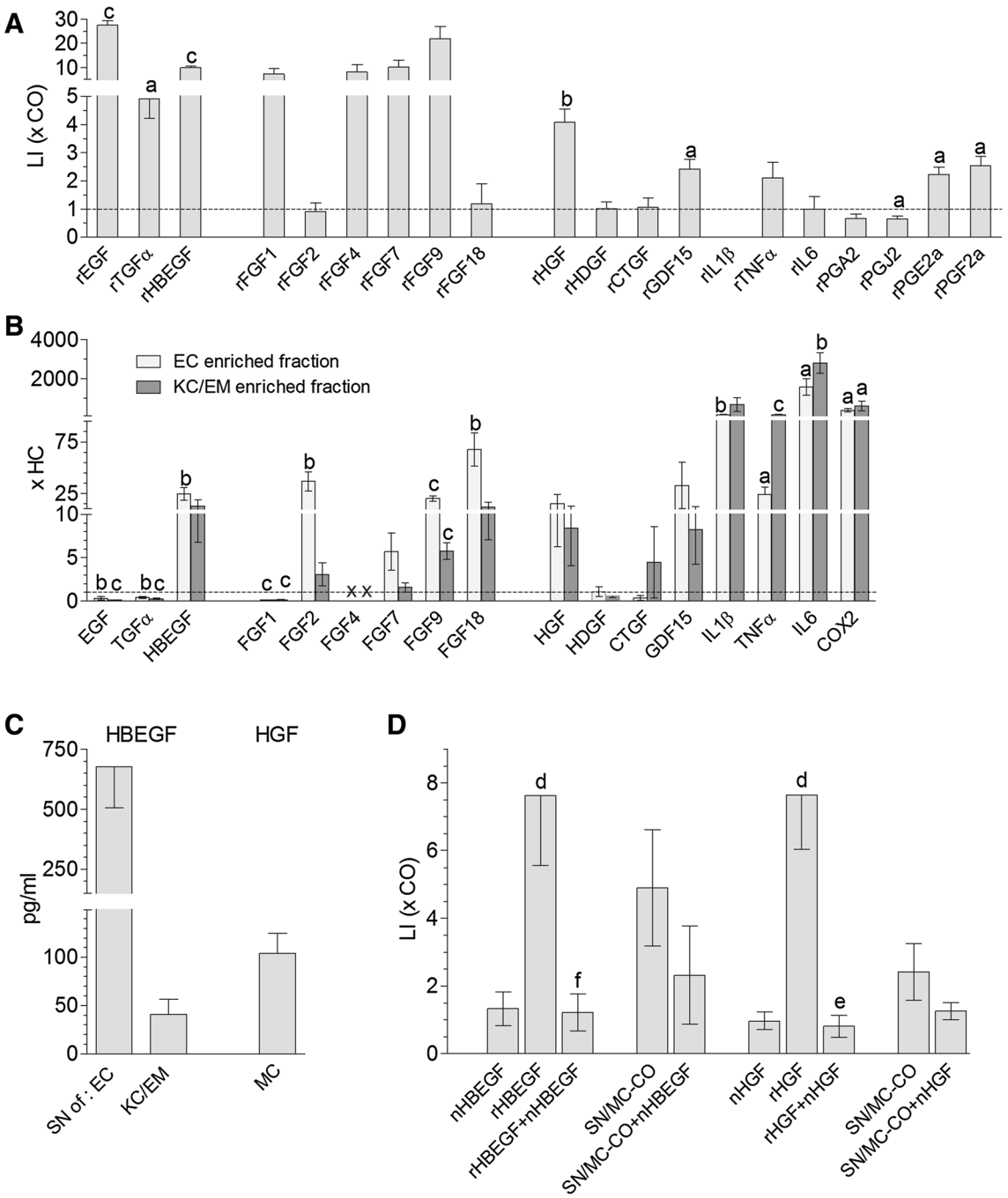

Fig. 2 MC, a rich source of growth factors for HC. **A** Effect of recombinant growth factors on LI of cultured HC. HC, from untreated animals were treated with recombinant proteins applied at 10 ng/ml (rEGF, rTGFα, rHBEGF, rFGF1, rFGF2, rFGF4, rFGF7, rFGF9, rFGF18, rHGF, rHDGF, rCTGF, rGDF15, rTNFα), 30 ng/ml (rIL6), 5 µg/ml (PGJ2), or 17.5 µg/ml medium (PGA2, PGE2a, PGF2a). Treatments were renewed at 48 h and lasted for 72 h. LI was determined by autoradiography and is expressed as fold solvent con- trol. **B** Expression of growth factors in HC, EC and KC/EM from untreated animals. Transcript levels were determined by RT-qPCR and expressed as fold level in HC; x, transcripts were detected in EC- and KC/EM-enriched fractions but not in HC. **C** Concentration of HBEGF in supernatants of EC and KC/EM and of HGF in MC supernatants. EC, KC/EM and MC, obtained from untreated animals, were cultured; supernatants were collected after 24 h for ELISA. **D** Antibodies, neutralizing HBEGF or HGF, impair effect of SN/MC-CO. Medium and supernatants were pre-incubated with nHBEGF or nHGF at 37 °C for 60 min before addition to HC cultures; rHBEGF or rHGF served as positive control. LI was determined by autoradi- ography. **A**–**D** Data are mean ± SEM of ≥ 3 rats. Statistics by one sample *t* test in **A** for different treatment agents vs. control medium and in **B** for the expression level of genes of interest in EC- or KC/ EM-enriched fractions vs. HC: a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$; in **D** for rHBEGF or rHGF vs. CO: d) $p < 0.05$. Statistics by unpaired *t* test in **D** for treatment vs. treatment + treatment neutralizing anti- bodies: e) $p < 0.05$, f) $p < 0.01$

expression pattern of both, the parenchyma and mesenchyme, is altered considerably by both prototypical NGC.

Transcriptome data were subjected to bio-informatic analyses to identify critical, cancer-related signal transduction pathways induced by the NGC. In general, many pathways were affected by PB and CPA in both, HC and MC (Table S2; Riegler et al. 2015). In HC, PB was found to be a potent inducer of drug metabolism, as expected, and of stress-induced pathways (MAPK-, NFkB-, TNFα-mediated signaling), while in MC there were profound upregulation of pathways involved in chemokine and cytokine signaling, as shown recently (Riegler et al. 2015). Genes driving the cell cycle or involved in caretaking of DNA stability and repair were induced by CPA in HC and MC (Table S2).

The hepatic mesenchyme secretes several growth factors following treatment with PB or CPA in vivo

We focused on the identification of growth factors for HC and HC_{PREN}, produced and released by MC-PB or MC-CPA. Transcriptome data showed considerable upregulation of HBEGF, GDF15, TNFα, and COX2 in MC-PB (Fig. 4A). This was paralleled by elevated concentrations of HBEGF, GDF15 and TNFα in SN/MC-PB (Fig. 4B). MC-CPA showed pronounced elevations of HGF, CTGF, GDF15, and COX2 transcripts (Fig. 4A; for data validation see also Fig. S1). In SN/MC-CPA HGF occurred at concentrations of up to 300 ng/ml medium (Fig. 4B). For CTGF no rat-specific ELISA was available. PGE2 could not be detected in SN/MC-PB or SN/MC-CPA by the ELISA used (detection limit 31 pg/ml, data not shown).

Mesenchymal growth factors may mediate the anti-apoptotic and growth-inducing effects of NGC in hepatocytes and cancer prestages

We addressed the question whether the growth factor concentrations, detected in SN/MC-PB or SN/MC-CPA, are sufficiently high to explain the supernatant effects. We tested these factors in recombinant form with regard to their potency to induce DNA replication in HC and HC_{PREN} (Fig. 5). In SN/MC-PB GDF15 occurred at ~ 40 pg/ml, TNFα at ~ 70 pg/ml and HBEGF at ~ 350 pg/ml. At these concentrations rTNFα as well as rGDF15 were without any effect on DNA replication, while rHBEGF might become effective. In SN/MC-CPA HGF occurred at 0.35 ng/ml, which may elevate replication in HC_{PREN} and to some extent also in HC, as shown by the recombinant form of this factor. rCTGF and rHDGF exerted no significant effects on hepatocellular DNA replication.

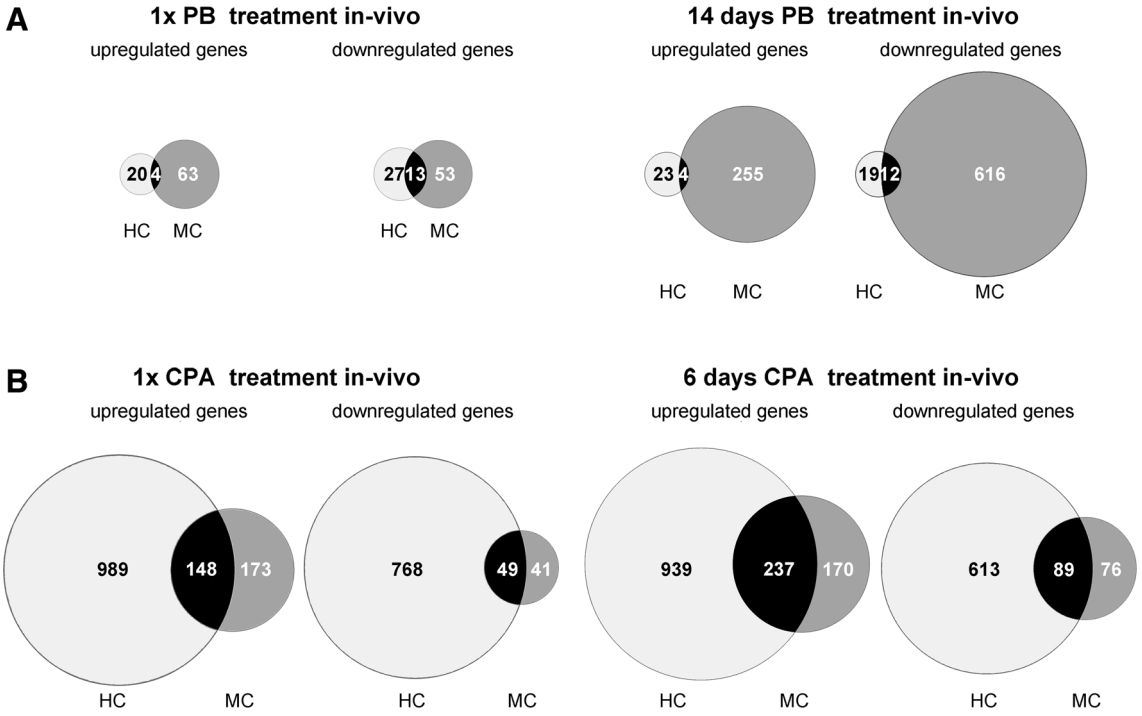

Fig. 3 PB alters the transcriptome profile rather in MC than in HC, while

the opposite applies to CPA. Transcriptome profiles were deter- mined by oligo-array analyses. Venn diagrams illustrate number of genes being up-/downregulated ≥ 2-fold in HC and/or MC after one application of PB or after 14 days of PB treatment in **A** or after one or 6 applications of CPA in **B**. Data are expressed as fold solvent con- trols and give means of ≥ 3 animals per treatment and time point

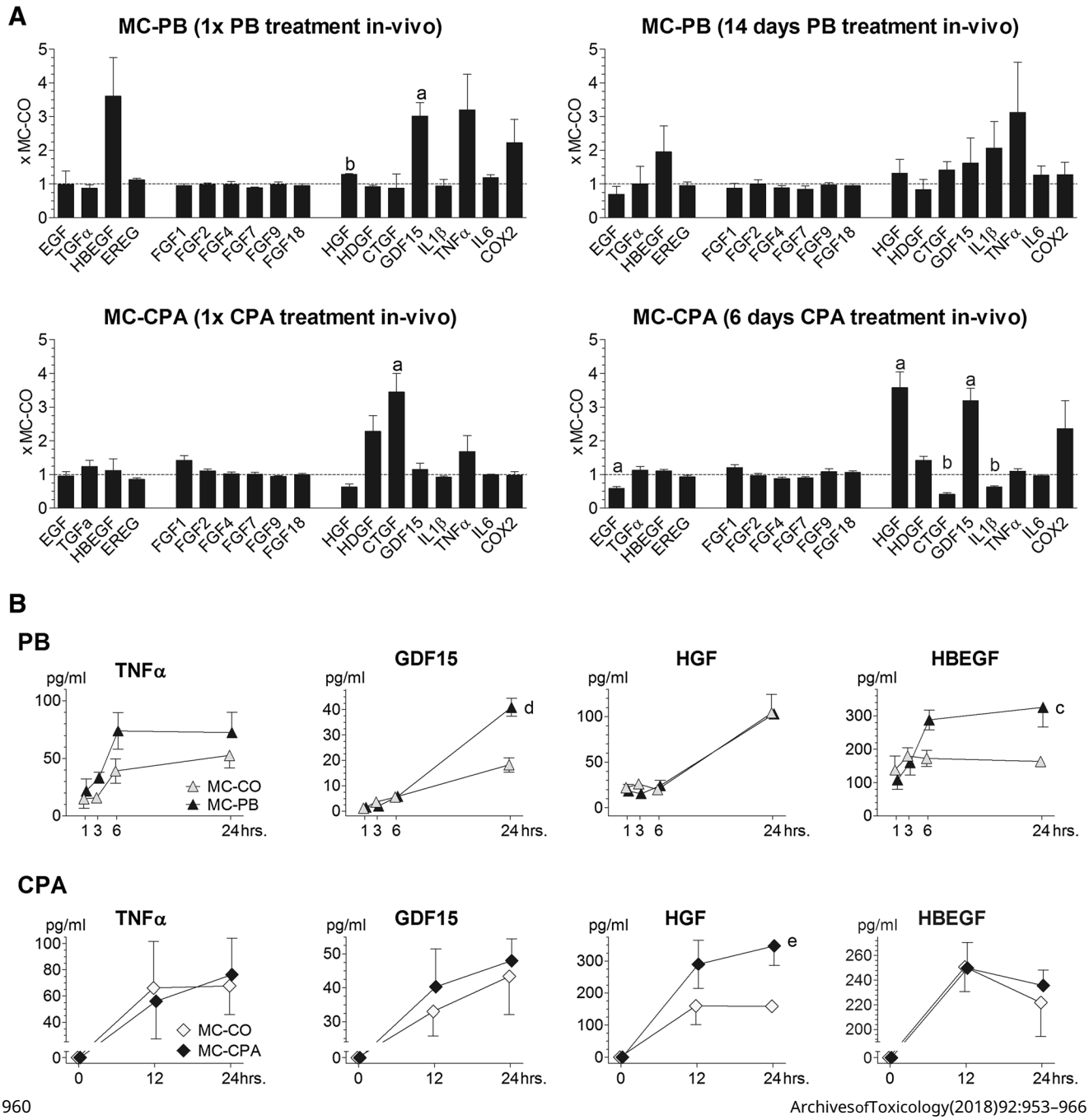

Fig. 4 Effect of PB or CPA in vivo on production and secretion of growth factors by MC in vitro. **A** Oligo-array analyses of growth factors in MC-PB and MC-CPA. No further growth factors were up-/downregulated ≥ 2-fold at any of the two time points than those given here. **B** Secretion of growth factors by MC-PB and MC-CPA. After a single dose of NGC or solvent in vivo, MC were separated and cultured. Aliquots of culture supernatant were collected at time points indicated and factor concentrations were determined by ELISA. **A**, **B** Data are mean ± SEM of ≥ 3 animals per treatment and time point. **A** Statistics by one sample *t* test for MC-CO vs. MC-PB or MC-CPA: a) $p < 0.05$, b) $p < 0.01$. **B** Statistics by unpaired *t* test for MC-CO vs. MC-PB at 24 h: c) $p < 0.05$, d) $p < 0.01$; for MC-CO vs. MC-CPA at 24 h: e) $p < 0.05$

We pre-incubated SN/MC-PB with antibodies blocking HBEGF (nHBEGF), which failed to neutralize the effect of SN/MC-PB on DNA synthesis of HC (data not shown). Antibodies blocking rat GDF15 were not available. Thus, GDF15, an unidentified factor or a combination of some

factors may be responsible for the weak effects of SN/ MC-PB on HC replication (Fig. 1C).

Recently, we described that MC-derived TNFα medi- ates the pronounced anti-apoptotic effects of SN/MC-PB in HC (Riegler et al. 2015). Therefore, we checked whether

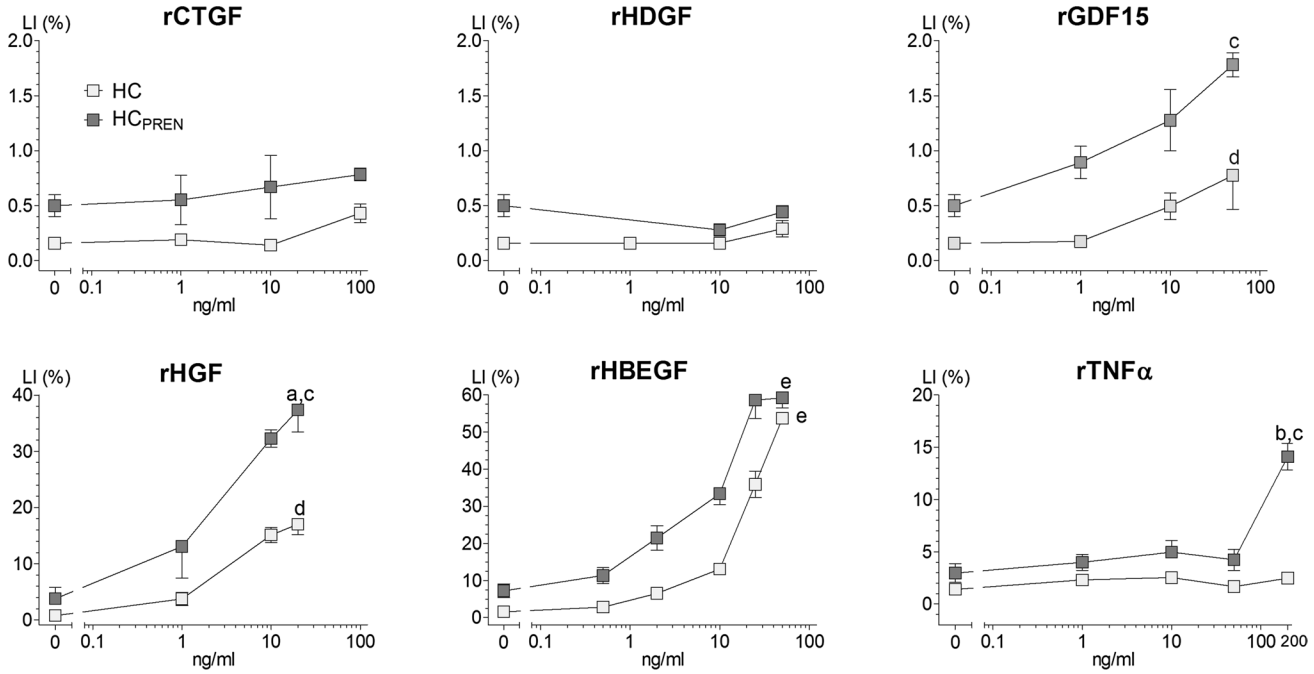

Fig. 5 rGDF15, rHGF and rHBEGF induce DNA replication of HC and HC_{PREN}. HC and HC_{PREN} were isolated from NNM-treated liv- ers for culture. Treatments with recombinant factors started 2 h after seeding of cells, were renewed at 48 h and lasted for 72 h. The LI was determined by autoradiography. Data are mean ± SEM from ≥ 3 rats. Statistics by unpaired *t* test for HC vs. HC_{PREN} at highest concentration: a) $p < 0.05$, b) $p < 0.01$. Statistics by Kruskal–Wallis test: c) $p < 0.05$, d) $p < 0.01$, e) $p < 0.001$

HBEGF or GDF15 may act also as survival factors for HC. Both factors, applied as recombinant form, exerted marginal effects on basal apoptotic activity. To induce a distinct apoptotic response, HC were treated with rTGFß1, which dramatically increased the incidence of apoptosis. Co-application of rHBEGF or rGDF15 antagonized the pro-apoptotic effects of TGFß1 (Fig. 6A). Interestingly, the apoptosis-inducing potency of rTGFß1 was also reduced dramatically by co-application of SN/MC-CO or SN/MC-PB. Obviously, the mesenchymal supernatants contain potent survival factors for HC. Pre-incubation of SN/MC-CO with nHBEGF had no effect on the apoptotic activity of HC. In contrast, pre-incubating SN/MC-PB with nHBEGF significantly elevated the apoptotic activity (Fig. 6B). This strongly suggests that

the elevated HBEGF levels in SN/MC-PB inhibit apoptosis of HC. Due to lack of nGDF15-antibodies direct evidence

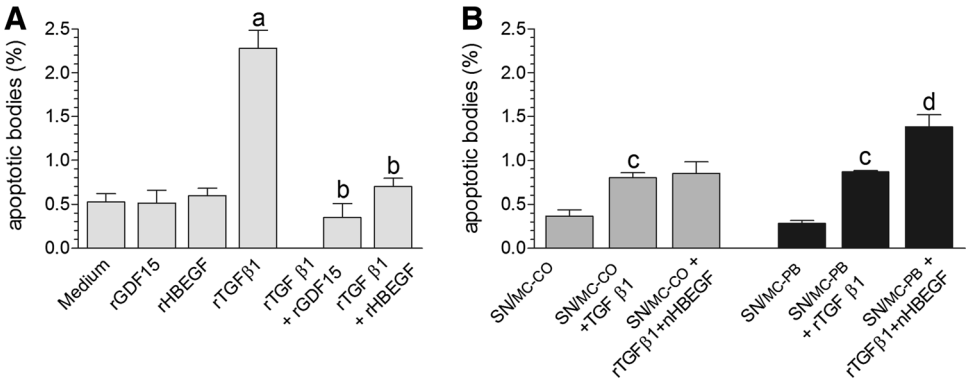

Fig. 6 SN/MC-PB exerts anti-apoptotic effects in HC probably via GDF15 and HBEGF. **A** HC were kept in medium and were treated with rGDF15 (10 ng/ml), rHBEGF (10 ng/ml), and/or rTGFß1 (1 ng/ ml). **B** HC were kept in SN/MC-CO or SN/MC-PB (with/without pre-incubation with nHBEGF; see also Fig. 2) and were treated with rTGFß1 (1 ng/ml). **A**, **B** 24 h after start of treatment HC were harvested to determine apoptoses. At least 2000 HC per experiment and treatment group were screened. Statistics by unpaired *t* test in **A** for TGFß1 vs. medium: a) $p < 0.01$; for TGFß1 vs. TGFß1 + treatment with recombinant factor: b) $p < 0{,}01$; in **B** for SN/MC-CO or SN/ MC-PB vs. SN/MC-CO + TGFß1 or SN/MC-PB + TGFß1, respec- tively: c) $p < 0.01$; for treatment vs. treatment + nHBEGF: d) $p < 0.05$

for an anti-apoptotic effect of this factor in SN/MC-PB could not be obtained.

In contrast to SN/MC-PB, SN/MC-CPA strongly enhanced DNA replication of HC and HC_{PREN}. Pre-incubation of SN/MC-CPA with antibodies neutralizing HGF (nHGF) largely abolished the stimulatory effect of SN/MC-CPA (Fig. 7). This implies that HGF may be the most important growth factor in SN/MC-CPA for normal HC and in particular for early cancer prestages and may thus be one of the key factors in CPA-driven hepatocarcinogenesis.

Discussion

Here, we show for the first time that two prototypical NGC increase the production and release of survival and growth factors from MC, which may promote outgrowth of first stages of carcinogenesis in rat liver. Such effects are considered causally involved in the tumor promoting and eventually carcinogenic action of these compounds, as discussed in the following.

The hepatic mesenchyme as source of growth factors for hepatocytes

In the liver and other organs the stroma is known to provide crucial signaling for control of tissue homeostasis. Accordingly, any deviation in signaling may cause not only

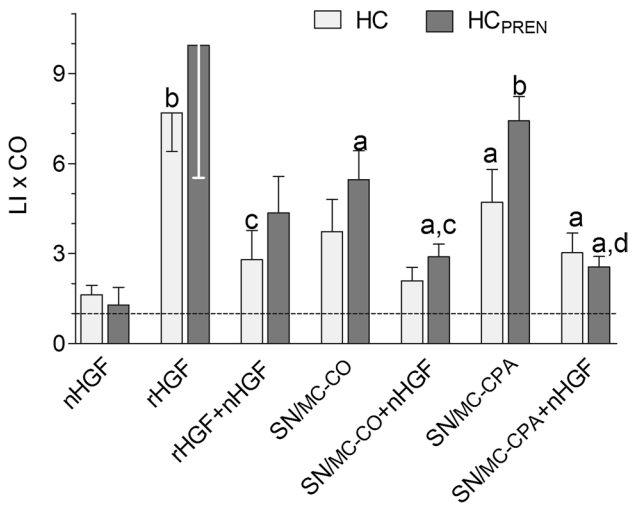

Fig. 7 SN/MC-CPA induces replication of HC and HC_{PREN} via HGF. Medium, SN/MC-CO or SN/MC-CPA were pre-incubated with nHGF before addition to HC cultures (see also Fig. 2). rHGF served as positive control. Cells were harvested 48 h after start of treatment; LI was determined by autoradiography. Data are expressed as fold medium control (CO) and are mean ± SEM from independent experi- ments on ≥ 3 rats. Statistics by one sample *t* test for treatment vs. CO: a) $p < 0.05$, b) $p < 0.01$; statistics by unpaired *t* test for treatment vs. treatment + nHGF: c) $p < 0.05$, d) $p < 0.01$

destabilization of tissue homeostasis but also promotion of premalignant cells towards malignancy (Bissell and Hines 2011).

We tested the effect of the soluble factors, released by hepatic stroma cells surrounding unaltered HC as well as first stages of hepatocarcinogenesis. The overall outcome was slight induction of replication of HC and HC_{PREN} by the supernatant of MC, isolated from untreated livers. This may be due to the fact that we used MC from 6–8 weeks old animals which were still growing. In addition, the disruption of the extracellular matrix may also create experimental conditions favoring cell replication.

Considering the mesenchymal expression profile of growth factors and the ELISA data of the secretomes, MC produce and release a complex mixture of TNFα, HBEGF, HGF, GDF15, and probably other factors, not identified so far. TNFα is secreted mainly by KC to bind to TNFR1/2, which transiently activates NFkB and increases the proliferative response of HC to growth factors in vivo (Michalopoulos 2014). This priming effect of TNFα was also evident in cultured HC, e.g., peroxisome proliferators stimulate DNA synthesis of cultured HC only in the presence of TNFα or TNFα-producing KC (Parzefall et al. 2001). In the present study, TNFα was not a growth stimulator of unaltered HC, but at concentrations of > 50 ng/ml medium it induced replication of HC_{PREN}. This indicates an altered response of the very first stages of hepatocarcinogenesis towards paracrine stimuli. We found that mainly EC produce HBEGF, which binds to erbb1 and erbb4 for signaling. HBEGF is one of the key factors in liver growth and promotes the transition of HC from the G1 into the S-phase (Michalopoulos 2014). HGF transcripts are produced by both, EC and KC, at more or less equal amounts. When released, HGF acts as a multifunctional cytokine on HC via the hepatocyte growth fac- tor receptor to regulate hepatocellular growth, motility and morphogenesis (Michalopoulos 2014). GDF15, a member of the transforming growth factor-β family, is also known as macrophage inhibitory cytokine-1. A specific receptor for GDF15 has not been identified so far. Recently, it was shown that GDF15 enhances the phosphorylation of Erk and Akt, components of growth and survival pathways (Urakawa et al. 2015). Here, we show for the first time that this factor supports survival and replication of HC and HC_{PREN}.

Considering the presence of ~ 0.05 ng of TNFα, ~ 0.1ng of HGF and ~ 0.2 ng of HBEGF per ml SN/MC-CO, rHBEGF and rHGF required five–tenfold higher concentrations to elevate hepatocellular DNA replication in purified HC, being depleted from TNFα-producing KC. It appears possible that the efficacy of the natural factors in SN/ MC -CO was increased by the presence of TNFα, known to prime HC to enter the cell cycle. Also IL6 is occurring in the MC supernatant at ~ 70 pg/ml and is also a priming fac- tor for HC (Michalopoulos 2014; Riegler et al. 2015). This

may explain, that neutralization of only 0.2 ng of HBEGF or 0.1 ng of HGF in SN/MC-CO by blocking antibodies abolished greatly the replication-inducing effect of the supernatant. These observations provide strong evidence that HBEGF and HGF are the main mesenchymal factors driving hepatocellular replication.

PB-treatment in vivo stimulates mesenchymal liver cells to release growth/survival factors for hepatocytes and early cancer prestages

In the present study, DNA replication of HC and HC_{PREN} was suppressed when PB was applied directly to cultures but was stimulated by SN/MC-PB. We considered the possibility of a direct growth-suppressing PB effect due to blockade of Erbb1 activity, as was described recently in cultured primary murine HC (Mutoh et al. 2013). In this system, 2.5 mM PB binds directly to Erbb1 to block phosphorylation and signalling of the receptor and to prevent phosphorylation also of the receptor for activated C kinase 1 (RACK1). De-phosphorylated RACK1 interacts with constitutive androstan receptor (CAR) and protein phosphatase 2A to elicit de-phosphorylation and subsequent translocation of CAR to the nucleus for transcription of PB-target genes (Mutoh et al. 2013). However, in HC of the currently used rat model, 1 mM PB did affect neither rEGF-induced Erbb1-phosphorylation nor rEGF-induced DNA replication (Fig. S3). The reason underlying the apparent discrepancy between murine and rat HC is presently unclear.

In the hepatic mesenchyme PB induced profound alterations of the transcriptome profiles, including growth regulatory genes, such as GDF15. The ~ twofold elevated transcript levels of GDF15 in MC-PB was reflected by a ~ twofold enhanced secretion of this cytokine. HBEGF transcripts were elevated ~ 3-fold in MC-PB resulting in ~ twofold enhanced secretion of this cytokine. HBEGF is a ligand of Erbb1 and Erbb4. PB did not affect DNA replication, if induced by HBEGF (Fig. S3). This indicates that the activity not only of Erbb1 but also of Erbb4 was not affected by PB in HC. HBEGF tended to stimulate growth of HC at ~ 1 ng/ml medium and of HC_{PREN} already at 0.5 ng/ml, a concentration coming close to levels measured in the supernatant of PB-treated MC. However, nHBEGF failed to block the effects of SN/MC-PB on replication of HC and HC_{PREN} indicating a function of HBEGF in PB-driven hepatocarcinogenesis being different from induction of DNA replication.

As reported previously, PB slightly elevated DNA replication but suppressed distinctly the elimination of cells by apoptosis in unaltered tissue and preneoplastic lesions of rat liver in vivo (Schulte-Hermann et al. 1990). This led to liver hypertrophy/hyperplasia and to outgrowth of tumors. Withdrawal of PB increased dramatically the apoptotic activity in the liver, which reversed liver hyperplasia and dramatically

reduced the size of preneoplastic lesions, indicating a dependence of unaltered and preneoplastic HC on this NGC. This PB-elicited shift from death towards replication of preneoplastic cells was found to be crucial for the tumor promoting effect of this compound (Schulte-Hermann et al. 1990). We described recently that PB-treated MC secrete elevated levels of TNFα, which antagonizes apoptosis in HC via activation of NFkB and down-stream survival pathways (Riegler et al. 2015). Therefore, we checked whether HBEGF and GDF15 exert anti-apoptotic activity as well. In fact, rGDF15 and rHBEGF completely blocked rTGFß1-induced HC apoptosis. Interestingly, the pro-apoptotic effect of rTGFß1 remained unaffected when pre-incubating SN/MC-CO with nHBEGF but was elevated significantly when pre-incubating SN/MC-PB with nHBEGF. These observations provide indirect but compelling evidence that the elevated HBEGF levels in SN/MC-PB may mediate the pro-survival effects of PB in HC (Fig. 8).

The direct hepatomitogen CPA recruits mesenchymal HGF for paracrine growth stimulation of hepatocytes and cancer prestages

In rats CPA acts as a strong hepatomitogen inducing pronounced hyperplasia of the liver (Kasper 2001). This considerable intrahepatic growth pressure is considered to be essential for the outgrowth of preneoplasia to malignancy. In cultured HC and HC_{PREN} CPA exerted a direct growth inducing effect but also a considerable indirect one, mediated by factors released from CPA-treated MC. Similar to PB, CPA elicited multiple alterations in the hepatic mesenchyme, which is reflected by elevated transcript levels of growth factors, like CTGF, HDGF and HGF, and enhanced secretion of some of these factors. CTGF and HDGF appear not to interfere with hepatocellular growth regulation, since in recombinant forms they neither induced replication of HC or HC_{PREN} (Fig. 5) nor affected the apoptotic activity of HC (data not shown). SN/MC-CPA contained about 0.35 ng HGF/ml; this concentration induced DNA replication of HC_{PREN}, as confirmed by the HGF-blocking antibodies abrogating completely the SN/MC-CPA effect. Array data showed that the expression of HGF remained elevated in the hepatic mesenchyme also after 6 days of CPA treatment. Taken together, these findings indicate that HGF may be one of the key growth factors in CPA-driven rat hepatocarcinogenesis (Fig. 8).

We described recently that MC express glycine receptors, which may mediate the PB effects (Riegler et al. 2015). In analogy, we raised the question which receptors in the hepatic mesenchyme might transmit the CPA effects. CPA inhibits competitively androgen and glucocorticoid receptors, exerts progestational and mineralocorticoid effects, acts as pregnane-X-receptor (PXR) agonist, and interferes

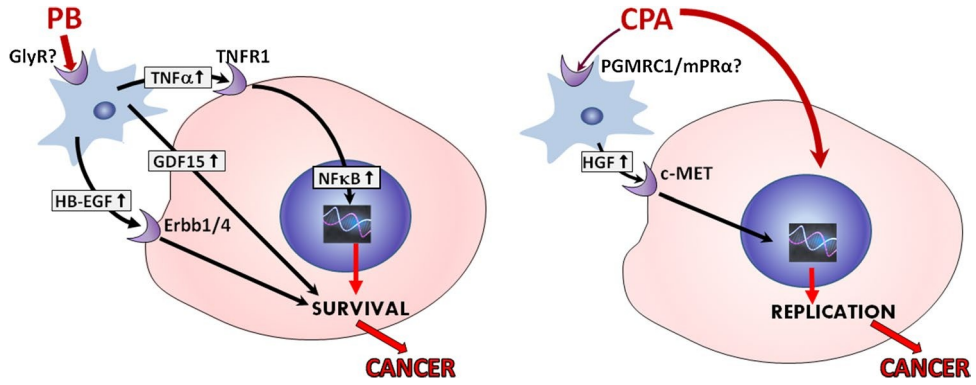

Fig. 8 Hypothesis on PB/CPA-induced alterations in epithelial–mesenchymal interactions supporting tumor promotion in rat liver. Left panel, after treatment of MC by PB, TNFα is released and binds to TNFR1 exposed on HC. This leads to activation of NFκB and nuclear translocation of this transcription factor, activating pro-survival pathways (Riegler et al. 2015). PB also may induce the release of HBEGF and GDF15 from MC. Subsequently HC are protected from pro-apoptotic stimuli. As a consequence, enhanced survival of HC_{PREN} may support indirectly growth of preneoplasia and enhances the probability for development of cancer. Right panel, in contrast with PB, CPA elicits more direct effects on DNA replication of HC_{PREN}. In addition, CPA may also act via PGMRC1 and/or mPRα on MC, which produce and release HGF to support the growth-inducing and tumor promot- ing effect of CPA in cancer prestages

also with CAR (Honer et al. 2003; Kasper 2001; Schuetz et al. 1998). On HC the hepatomitogenic effect appears to be largely mediated by PXR and CAR (Shizu et al. 2013). However, in EC or KC we could not detect significant levels of PXR or CAR (Riegler et al. 2015). The same holds true for the androgen or mineralocorticoid receptors (Fig. S4). The glucocorticoid receptor showed the highest expression level in liver cell-types, when compared to the other receptors investigated. Since CPA antagonizes the effects of the glucocorticoid receptor, the relatively high expression level appears not to be significant for the CPA-mediated effects in MC, as seen in this study. Considering that CPA acts mainly as progestin, it was surprising not to detect any signal for progesterone receptor transcripts, neither in HC nor in any MC type.

The recent discovery of membrane-located progesterone receptors (mPR) and progesterone-receptor-membrane-component 1 (PGRMC1) in mammalians suggests that there are alternatives to mediate progesterone effects particularly in tissues lacking the classical progesterone receptor. mPR belong to the progestin-adipoQ-receptor-family and are coupled to an inhibitory G-protein. Progesterone is the most important ligand, indicating regulatory functions in reproductive tissues (Tokumoto et al. 2016). mPR appear to be involved in the inhibition of apoptosis via MAP-kinase and Akt (Dressing et al. 2012). PGRMC1, a member of the membrane-associated progesterone receptor family, is highly expressed in the liver, and shows high affinity for progesterone, but also testosterone and glucocorticoids are possible ligands (Cahill et al. 2016). PGRMC1 interacts with Erbb1 and binds and activates also cytochrome P450 proteins, facilitating cancer proliferation and chemoresistance (Kabe et al. 2016). In the present study, we found transcripts of mPRa

and PGMRC1 in HC and all MC types studied, at levels being comparable to those in uterus or ovar (Fig. S4). This might indicate that the progestin CPA acts on all liver cell types via the membrane-anchored progesterone receptors.

PB and CPA may also interfere with human MC— putative implications for riskassessment

Much effort has been spent to assess putative health risks for humans being continuously treated with barbiturates or CPA. When exposed to barbiturates an altered functional status of macrophages/monocytes became evident, similar to observations in rats (Park and Brody 1971; Ploppa et al. 2008; Rossano et al. 1992). The elevated secretion of TNFα by PB-treated rat KC suppressed apoptosis of HC and acted as survival factor (Riegler et al. 2015). In human liver, however, enhanced intrahepatic TNFα secretion may lead rather to the development of drug-induced liver injury, as observed occasionally during treatment with anti-epileptic barbiturates (Shapiro et al. 1980). It remains to be elucidated whether barbiturates can enhance the production of HBEGF and GDF15 in liver mesenchyme not only of rats but of humans as well. Species comparisons with regard to the functional reactivity of the hepatic mesenchyme towards PB or other barbiturates would greatly support the estimation of health risks by this class of compounds.

In humans CPA is widely applied for contraception and treatment of androgen-related diseases, such as acne, hirsutism, prostate cancer, or pubertas praecox (Azziz 2007; Bastide et al. 2013). Conflicting data exist with regard to the carcinogenic potency. Most studies do not report an increased incidence of hepatic tumors under therapeutic use of CPA in adults (Kasper 2001). However, in 1552 children

and adolescents, suffering from pubertas praecox or other diseases and receiving CPA for 2.7 years on average, there was a tendency towards an elevated occurrence of malignant hepatoma (Watanabe et al. 1997). With regard to the mode of carcinogenic action in rats, CPA is sex-specifically activated to pro-mutagenic adducts in the liver of females only. In a long-term rat carcinogenicity study very high, hepatotoxic doses of CPA elevated the incidence of tumors in the liver with a somewhat higher incidence in females than in males (Schuppler and Günzel 1979). At lower dose levels no tumorigenic effect became evident in both sexes (Kasper 2001). Schulte-Hermann et al. (1981) showed that in the intact liver CPA induced proliferation dramatically in HC_{PREN} and only slightly in HC indicating that HC_{PREN} are more susceptible to the growth stimulus of CPA than their unaltered counterparts. Consequently, rodent liver tumor formation was considered to depend largely on the mitogenic effects of the compound and a predominantly non-genotoxic and non-linear mode of action has been anticipated. It has not been excluded so far that CPA may act as tumor promoter also in human livers. In previous and the present study CPA was found to enhance the proliferation of rat HC and HC_{PREN} in vitro, as observed in vivo. However, cultured human primary HC were non-responsive to direct mitogenic stimulation by CPA (Parzefall et al. 1991). It is presently unclear whether these cultures were depleted from MC and whether CPA requires the presence of MC to elicit a growth reaction in human HC. Similar to PB, data on the functional reactivity of human MC towards CPA would greatly help to better understand the health risk of exposed humans.

To conclude, homeostasis of liver tissue is dependent on the continuous integration of intra- and extracellular signals controlling growth, survival, and death of the cells. Any disturbances may trigger the outgrowth of cancer. While factors released from MC after PB-treatment appear to interfere mainly with cell survival pathways, CPA-treated MC secrete HGF to stimulate cell replication. Both effects may contribute to the outgrowth of liver tumors (Fig. 8). Thus, our data suggest that the carcinogenic activity of NGC results not solely from effects on the parenchyma as frequently anticipated. However, direct effects on the hepatic mesenchyme may be of high significance in NGC-driven carcinogenesis. This new concept requires testing with other NGC in other organs and species and may improve greatly strategies in risk assessment of NGC.

Acknowledgements Open access funding provided by Medical University of Vienna. The technical assistance of Elisabeth Haslinger, Helga Koudelka and Birgit Mir-Karner is gratefully acknowledged. Venn diagrams were drawn with the program "Venn Diagram Plotter" kindly provided by PNNL and the OMICS.PNL.GOV website. This project ("MARCAR") received funding under European Union Innovative Medicine Initiative (IMI JU) under grant agreement no. 115001 and by the Herzfelder´sche Familienstiftung.

2.2.2.1.1 References

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2.3 Interlude

The second publication also was another part of MARCAR project. The laboratory work of both publications have been done parallel during 2010-2016. In this part, we tried to go into detail of PB induced changes in rat HCC and their potential relevance for human. In this publication, the importance of inflammatory response on the cancer promotion was studied and described with more detail. Animal treatment with PB was done by Theresa Riegler but Marzieh Nejabat does the most of Rat perfusions. Cell separation processes has been done mostly by Theresa Riegler. The analysis of Immunohistochemistry of sections (menechymal cells and hepatocytes) done by Marzieh Nejabat and Istvan Paraszti. The both PhD students of the MARCAR projects (Nejabat & Riegler) worked together on the analysis of the results of gene analysis data

2.4 PDF of second paper

Proinflammatory mesenchymal effects of the non-genotoxic hepatocarcinogen phenobarbital: a novel mechanism of antiapoptosis and tumor promotion

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Abstract

Many environmental pollutants and drugs, including steroid hormones, hypolipidemics and antiepileptics, are non- genotoxic carcinogens (NGC) in rodent liver. The mechanism of action and the risk for human health are still insufficiently known. Here, we study the effects of phenobarbital (PB), a widely used model NGC, on hepatic epithelial–mesenchymal crosstalk and the impact on hepatic apoptosis. Mesenchymal cells (MC) and hepatocytes (HC) were isolated from control and PB-treated rat livers. PB induced extensive changes in gene expression in MC and much less in HC as shown by transcriptomics with oligoarrays. In MC only, transcript levels of numerous proinflammatory cytokines were elevated.

Correspondingly, ELISA on the supernatant of MC from PB-treated rats revealed enhanced release of various cytokines. In cultured HC, this supernatant caused (i) nuclear translocation and activation of nuclear factor-κB (shown by immunoblots of nuclear extracts and reporter gene assays), (ii) elevated expression of proinflammatory genes and (iii) protection from the proapoptotic action of transforming growth factor beta 1 (TGFß1). PB treatment *in vivo* or *in vitro* elevated the production and release of tumor necrosis factor alpha from MC, which was identified as mainly responsible for the inhibition of apoptosis in HC. In conclusion, our findings reveal profound proinflammatory effects of PB on hepatic mesenchyme and mesenchymal–epithelial interactions. The resulting release of cytokines acts antiapoptotic in HC, an effect crucial for tumor promotion and carcinogenesis by NGC.

Introduction

Chemical carcinogens are categorized as genotoxic or non-genotoxic, according to their pathogenic mechanism. While genotoxicity is a key event in tumor formation by agents of the first category, the mode of action of non-genotoxic car- cinogens (NGC) is still unclear (1). Many endogeneous or syn- thetic hormones act as NGC and account for cancer in breast, prostate or other hormone-dependent organs of various mam- malian species (2). Other NGC produce mainly liver tumors in long-term rodent bioassays, as observed for antiepileptics, such as PB, hypolipidemics, antidiabetics and many non-drug agents. Considering the daily intake of these NGC by millions of humans, thorough knowledge of the mode of action is of utmost importance to estimate the health risks (1,2).

Most NGC are ligands/activators of nuclear receptors, such as hormone receptors or the constitutive androstane receptor (CAR). Following activation receptors mediate adaptive increases in specific enzymes, organelles and/or cell number in target tissues. Adaptive cell multiplication is a self-limited process and

Received: May 11, 2015; **Revised:** August 25, 2015; **Accepted:** September 8, 2015

Abbreviations

BAY	BAY11-7082
CAR	constitutive androstane receptor
CO	untreated control rats or cells
EC	endothelial cells
EM	exsudative monocyte
ELISA	enzyme-linked immunosorbent assay
GABA	γ-aminobutyric acid
HC	hepatocytes
IL1ß	interleukin-1 beta
KC	Kupffer cells
MC	mesenchymal liver cells
MC-CO	mesenchymal cell obtained from an untreated animal
MC-PB	MC isolated from PB-treated animal
NFκB	nuclear factor-κB
NGC	non-genotoxic carcinogen
NOS2	nitric oxide synthase 2
PB	phenobarbital
SN	supernatant
$SN/_{MC-CO}$	supernatant of MC, isolated from untreated animals
$SN/_{MC-PB}$	supernatant of MC, isolated from PB-treated animal
TGFß1	transforming growth factor beta 1
TNFα	tumor necrosis factor alpha
TNFR1	TNF receptor 1
TRAF6	TNFR associated factor 6
USP2	ubiquitin carboxyl-terminal hydrolase 2

per se not carcinogenic (3,4). However, cells carrying mutations in critical (growth regulatory) genes may show an 'over-response' towards NGC, i.e. selectively increased proliferation based on a continuous shift from cell death towards cell replication (5). This strong growth pressure may trigger the outgrowth of mutated cells to malignancy.

For our mechanistic investigations, we chose the CAR activator and barbiturate phenobarbital (PB), an antiepileptic drug for humans, which has been applied and studied as liver tumor promoter in rodents since more than four decades. In experimental mouse models, the key events in PB-induced liver tumor formation are considered to comprise activation of CAR, resulting in altered gene expression and increased cell proliferation, which induces growth of altered hepatic foci and ultimately the development of liver tumors (3,4). In mice, PB strongly promotes HC carrying mutations of the Ctnnb1 gene, encoding a constitutively activated version of ß-catenin (6). Dong *et al.* (7) have shown that activation of ß-catenin alone induces senescence and growth arrest. This is overcome if combined with CAR activation, resulting in uncontrolled proliferation and tumor formation.

In rats, the significance of CAR for PB-driven hepatocarcinogenesis has remained unclear due to lack of transgenic or knock-out models. While PB strongly induces DNA replica- tion in murine liver, it is much less effective in rats—similar to human HC in primary culture or chimeric 'humanized' mice (8,9). Unlike in mice, in rats PB suppresses apoptosis in nor- mal liver and more potently in preneoplastic lesions. Thereby it accelerates the outgrowth of malignancy, a fundamental mechanism of tumor promotion by this NGC in this rodent spe- cies, as shown in previous studies and reproduced also under the present experimental conditions (Supplementary Figure 1, available at *Carcinogenesis* Online) (3,5,8). Furthermore, in rats most PB-generated liver tumors fail to exhibit mutations of the ß-catenin gene as observed in mice (unpublished observation).

These findings imply that other/additional mechanisms may play a role in tumor promotion by PB in rat liver.

Conventional notion regards the action of PB and other NGC as essentially confined to parenchymal liver cells. Accordingly, the potential role of the hepatic mesenchyme has not been investigated in detail. However, MC play a key role in carcinogenesis by genotoxic agents via eliciting proinflammatory states. For example, exposure to genotoxic carcinogens, like dietary aflatoxin-B1 or ethanol, exerts considerable cytotoxicity leading to necrosis of HC and inflammation, followed by regenerative growth and/or replacement of the dying cells by scarring (10). These processes involve an altered epithelial–mesenchymal dialogue wherein Kupffer cells (KC) initiate the inflammatory response by the release of cytokines, like interleukin 1ß (IL1ß) and tumor necrosis factor α (TNFα) (11). TNFα recruits further immune cells to the liver. Moreover, it induces hepatocellular apoptosis via tumor necrosis factor receptor 1 (TNFR1), an event primarily antagonized by activation of the transcription factor nuclear factor-κB (NFκB) (12–15). This balance between proapoptotic stimuli and activated NFκB plays a key role in hepatocarcinogenesis driven by inflammation, e.g. $Mdr2^{-/-}$ mice develop hepatic inflammation and hepatocellular carcinoma; the development of hepatocellular carcinoma, however, can be prevented by expression of an NFκB repressor transgene inducing proapoptotic pathways in HC (16). KC, activated by proinflammatory signals, also release superoxide, which appears to contribute to hepatocarcinogenesis. In previous work, p47-phox knockout mice, lacking superoxide formation by KC, were less sensitive than wild-type animals to the carcinogenic effect of cytotoxic doses of diethylnitrosamine (17).

As to NGC, a few pioneering studies have shown that these agents act not only on HC but also on hepatic MC, resulting in enhanced secretion of growth factors, proinflammatory cytokines and reactive oxygen species (11,18–20). Moreover, some of these growth factors have been found to act selectively on preneoplastic HC, serving as endogenous tumor promoters (21,22). The present study aims at a better understanding of the role of the hepatic mesenchyme for NGC-driven hepatocarcinogenesis. In humans, PB and other barbiturates are known to interfere with the innate immune system by altering the function of leukocytes/monocytes/macrophages (23,24). Here, we show for the first time that PB induces immunological alterations in the mesenchyme of rat liver, which includes elevated secretion of proinflammatory cytokines from MC. In HC, this effect results in enhanced translocation of NFκB to the nucleus and a considerably reduced susceptibility to proapoptotic stimuli. In conclusion, the hepatic mesenchyme appears to be the actual source of the antiapoptotic signal of PB, which plays a crucial role for tumor promotion and carcinogenicity by this NGC (5).

Materials and methods

Animals and treatment

Male Han–Wistar rats, 8 weeks old, were obtained from Charles River (FRG). They were kept under standardized SPF conditions. Animals were treated with 50 mg PB (5-ethyl-5-phenylbarbituric acid sodium salt, Sigma–Aldrich) per 1000 g body weight by a single gavage or via drinking water for a period of 7 or 14 days. Controls received tap water only. All experiments were approved by the 'Committee of Animal Protection' of the Austrian government and performed according to Austrian regulations.

Separation of liver cells and primary cultures

Livers of untreated or PB-treated rats were perfused with collagenase (Worthington, Lakewood, NJ). The cell suspension obtained was used to separate MC from HC by low-speed centrifugation in percoll gradients. MC were further separated by selective cell adherence into an endothelial cell (EC)-enriched fraction and a fraction consisting mainly of exsudative

cd68+/ED1+ exsudative monocytes/macrophages (EM) and liver-resident cd163+/ED2 + KC. For details on procedures see Böhm *et al.* (25). The purities of cell fractions were determined to be: 98.4 ± 0.6 for HC, 99.9 ± 0.1 for MC, 80 ± 19.4 for KC/EM and 94.5 ± 2.8 for EC (25). Stock solutions were prepared of PB, IL1ß, TNFα, TGFß1 and BAY11-7082 (BAY) and aliquots were added to the medium to provide the final concentration (Supplementary Table 1, available at *Carcinogenesis* Online). Treatment of cells commenced 2 h after plating (time point 0).

Gene expression analyses

For reverse transcriptase quantitative PCR (RT-qPCR), the extracted mRNA was processed and measured by the ABI-Prism/7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using TaqMan-based assays (Applied Biosystems). For primers and assays see Supplementary Table 1, available at *Carcinogenesis* Online.

For whole genome expression analyses, the extracted RNA was subjected to quality control (2100 Bioanalyzer System, Agilent, St Clara, CA). Complementary RNA targets were prepared and hybridized according to the manufacturer's procedures on high-density oligonucleotide microarrays (Affymetrix Rat 230 2.0 Array, Cleveland, OH). The microarrays were performed at the core facility 'Genomics' at the Medical University Vienna. For further details, see Supplementary Material, available at *Carcinogenesis* Online. For validation of transcriptome data by quantitative RT-qPCR, see Supplementary Figure 2, available at *Carcinogenesis* Online.

Immunodetection

Antisera and enzyme-linked immunosorbent assay (ELISA) kits used see Supplementary Table 1, available at *Carcinogenesis* Online.

Immunoblotting

Nuclei were isolated via 'NE-PER Nuclear & Cytoplasmic Extraction Kit' (ThermoScientific, Rockford, IL) according to the manufacturer's instructions. Proteins, harvested in RIPA buffer [500 mM NaCl, 50 mM Tris (pH 7.4), 0.1% SDS, 1% igepal CA630, 0.5% Na-deoxycholate, 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluorid; all obtained from Sigma–Aldrich), were homogenized by sonication and centrifugation (12 000*g*, 5 min) and loaded at equal amounts (10 µg) onto 10% SDS gels. Bands, obtained by immunostaining ('ECL Plus Western Blotting Detection Kit'; GE Healthcare, St Gilles, GB), were evaluated by densitometry ('Optimax 2010 X-ray film processor' and 'Quantity One 4.2.1'; Bio-Rad, Hercules, CA). Further details see (25).

ELISA

ELISAs were performed according to the manufacturer's instructions. Material not included in the module set: tetramethylbenzidine peroxi- dase solution for color development (Thermo Scientific); microtiter plates (Costar, Corning, NY).

Immunohistochemistry

Sections, obtained from Carnoy- or formalin-fixed liver tissue, were embedded in paraffin and stained, as given in detail elsewhere (25).

Reporter gene assay

Primary HC were seeded at 5×10^5 cells/cm^2; 24 h later cells were cotransfected by 500 ng plasmid DNA, coding for the NFκB-responsive luciferase vector and 25 ng of the renilla luciferase vector applying lipofectamine 2000 (Life Technologies, Carlsbad, CA). Transfections with basic PGL2- and pRL-SV40 vectors (Promega, Madison, WI) served as control. Mesenchymal supernatant was added 24 h after transfection; 24 h later firefly and renilla luciferase were detected with the 'Dual Luciferase Reporter System' (Promega) according to the manufacturer's instruction. Each set of experiments was performed in triplicate and repeated thrice.

Results

PB affects transcriptome profiles: multiple alterations in MC, few in HC

To analyze the role of the liver mesenchyme in NGC-driven hepatocarcinogenesis, we treated male rats with PB once by gav- age or for 2 weeks. Twenty-four hours after gavage or at the end of

the 14th day period of PB application via drinking water, cells were isolated from the liver and separated into HC and MC. Cell fractions from untreated rats served as control. Subsequent analyses by oligoarrays revealed that a single dose of PB altered the expression of 64 genes in HC and 133 genes in MC. Fourteen days' treatment changed mRNA levels of 58 genes in HC and of even 887 genes in MC (Figure 1A; Supplementary Figure 3, available at *Carcinogenesis* Online). Interestingly, at both time points only a few genes were commonly deregulated in both cell fractions. Overall, these data show that both hepatic cell types, but more prominently MC, are able to react extensively and specifically to PB treatment.

PB induces a stress-response in HC via enhanced secretion of TNF α by MC

Transcriptome data were subjected to bioinformatic analy- ses. Unlike in HC, in MC many different proinflammatory cytokines and respective downstream signaling pathways were found to be deregulated by PB, such as NFκB-driven pathways (Supplementary Table 2, available at *Carcinogenesis* Online). Furthermore, there was over-proportional upregulation of chemokines of the CCL- and CXCL-family in MC (Figure 1B), which was not seen in HC (data not shown).

To study the effect of the upregulated proinflammatory cytokines in PB-treated MC, we chose a holistic approach, i.e. we isolated MC from animals, which had been treated with PB for 7 days ('MC-PB'), cultivated the cells, collected their super- natant ('SN/MC-PB') and exposed cultured HC with SN/MC-PB. Supernatants of MC from rats, which had been treated with solvent ('SN/MC-CO'), served as control. For further details, see Supplementary Figure 4, available at *Carcinogenesis* Online.

Exposure of untreated HC to SN/MC-CO induced mRNA levels of proinflammatory genes, i.e. nitric oxide synthase-2 (NOS2) and TNFα expressions were elevated ~300- and ~4-fold, respectively. This effect became even more pronounced when SN/MC-PB was applied, i.e. 1400-fold (NOS2) and 100-fold (TNFα) elevated transcript levels were obtained when compared with HC in basic medium (Figure 2A). This effect was evident also after one PB application and 14 days of PB treatment (Supplementary Figure 5, available at *Carcinogenesis* Online). The dramatic upregulation of proinflammatory genes was confirmed for NOS2 at the protein level (Figure 2C). Probably due to the very complex post-transcriptional/post-translational regulation of this gene, the PB-induced elevation of the NOS2 protein was less pronounced than of the transcript (26).

When determining proinflammatory cytokines in the supernatants, considerable levels of IL1ß and TNFα were evident in SN/MC-CO indicating spontaneous activation of cultured MC, as shown before (27). However, considerably higher concentrations of these cytokines were found in SN/MC-PB than in SN/MC-CO (Figure 2B). To test for a causal role of these two cytokines for the supernatant effects, we added recombinant TNFα or IL1ß to HC cultures, which simulated the effect of SN/MC-PB on HC (Supplementary Figure 6, available at *Carcinogenesis* Online). To clarify which of the two cytokines is mainly responsible, we pre-incubated SN/MC-PB with neutralizing anti-IL1ß or anti-TNFα. Anti-TNFα abolished distinctly the supernatant effects while anti-IL1ß had little impact (Figure 2D). This implies that TNFα is the main factor in SN/MC-PB, causing the stress–response in HC.

PB *in vivo* enhances anti-inflammatory effects of HC on MC

Interestingly, the supernatant of untreated HC reduced the expression of proinflammatory genes in MC. This effect was even more pronounced with supernatant of HC from PB-treated rats (Supplementary Figure 7, available at *Carcinogenesis* Online). This

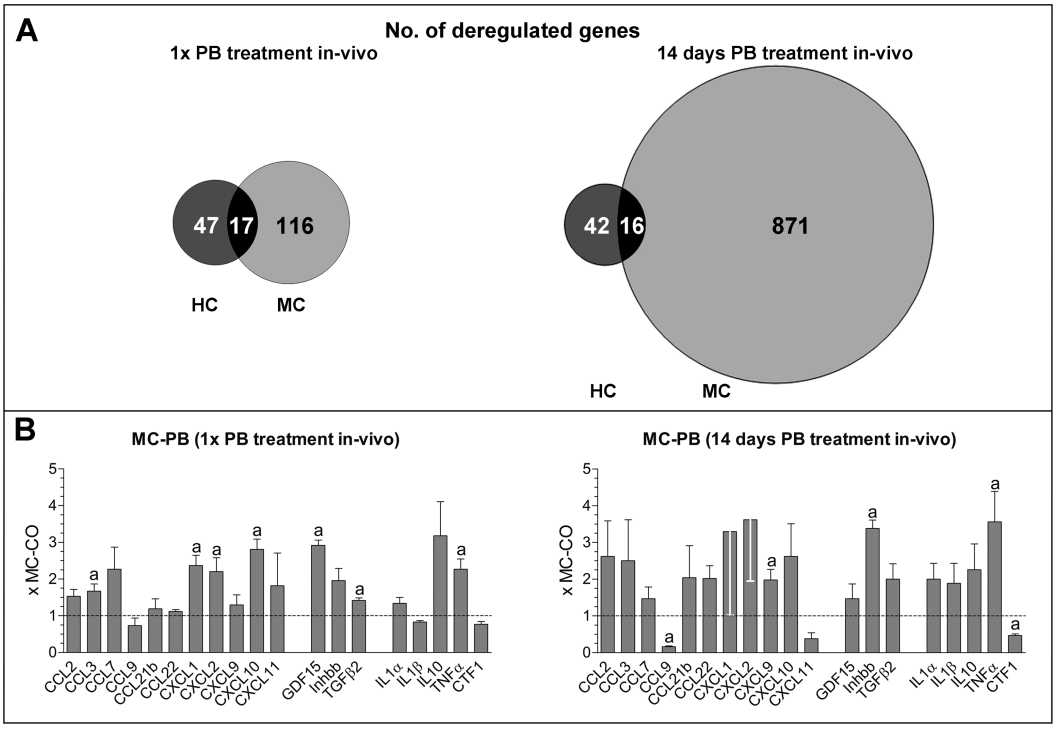

Figure 1. PB alters the transcriptome profile in MC rather than in HC. Transcriptomics was performed by oligoarray analyses. (**A**) Venn diagrams illustrate number of genes being deregulated ≥2-fold in MC and HC after one PB application or 14 days of PB treatment. (**B**) All chemokines of the C-, CC-, CX3C-, CXC-, TNF-, TGFß and interleukin (IL) superfamilies are shown, which were deregulated in MC at any time point investigated. Data are expressed as fold MC of untreated controls (× MC-CO) and as means ± SEM of ≥3 animals per treatment and time point. Statistics by one-sample *t*-test: MC-CO versus MC-PB: (a) $P \leq 0.05$.

suggests that PB-treated HC may counteract the proinflamma- tory action of the PB-activated mesenchyme. The outcome of the transcriptome analysis suggests that in the intact liver the effect of MC is predominating as indicated by upregulation of proinflammatory cytokines in MC and activation of stress–response pathways in HC (Supplementary Table 2, available at *Carcinogenesis* Online).

PB acts directly on MC to induce proinflammatory genes

Due to complex epithelial–mesenchymal interactions in the intact liver, it remained unclear whether PB acts directly on MC to induce proinflammatory genes. For clarification, MC were isolated from untreated rats and were separated into EC- and KC/EM-enriched fractions, both of which were kept in culture and treated with PB. As shown in Figure 3, PB *in vitro* elevated IL1ß and TNFα concentrations in the supernatant of KC/EM, but not in EC supernatant. This indicates that PB is able to directly acti- vate hepatic KC/EM; these cells appear to be the main source of TNFα in the PB-treated mesenchyme.

MC express hardly PB-responsive nuclear receptors (CAR, PXR) but considerable levels of glycine receptor subunits

The direct effects of PB on isolated and cultured KC/EM raised the question as to the underlying mechanism (Figure 3). We could not detect significant transcript levels of the PB-responsive nuclear receptors CAR or PXR in EC or KC/EM (Supplementary Table 3, available at *Carcinogenesis* Online). Then we tested for expression of receptors known to interact with PB in neuronal/non-hepatic tissues such as γ-aminobutyric acid (GABA) $GABA_A$-receptors, AMPA/kainate receptors, glutamine receptors 3 and 6 and glycine receptor (28–33). We found transcripts only ofthe glycine receptor subunits

with levels being considerably higher in MC than in HC, as described previously for KC (32). The interaction of PB with hepatic glycine receptors and the possible consequences are discussed below.

Supernatant of PB-treated MC activates NF κB in HC to exert antiapoptotic activity

Considering the bioinformatic analyses and the hepatocellular upregulation of several NFκB-target genes, such as NOS2, COX2, IL1ß, IL6 and TNFα by SN/MC-PB, we checked whether this supernatant is able to induce the translocation of NFκB to HC nuclei. In fact, NFκB protein was increased about 2-fold in nuclei of HC exposed to SN/MC-CO and 3-fold elevated with SN/MC-PB (Figure 4A and B). This was reduced by BAY, which blocks irreversibly and selectively the cytokine-induced phosphorylation of NFκB-inhibitor alpha (IkBα), necessary to unmask the nuclear localization signals of NFκB (34). A somewhat elevated occurrence of NFκB-positive HC nuclei was also evident in tissue sections obtained from PB-treated liver (Figure 4C). Furthermore, SN/MC-PB was able to enhance NFκB activity in HC, as shown by a reporter-gene assay (Figure 4D).

It is well established that activation of NFκB serves as a primary mechanism in protecting HC from proapoptotic stimuli. We therefore checked whether the SN/MC-PB-induced activation of NFκB is involved in the antiapoptotic activity of PB in HC. Under the present experimental conditions 0.7 ± 0.3% of HC in primary culture underwent spontaneous apoptosis 24 h after seeding, which agrees with published data (Figure 5) (35). SN/ MC-CO, SN/MC-PB and recombinant IL1ß and TNFα had only marginal effects on basal apoptotic activity. In order to induce a distinct apoptotic response, HC were treated with TGFß1, which elevated the occurrence of apoptosis 3.3-fold. Coapplication of IL1ß further enhanced the TGFß1 effect, while SN/MC-CO, TNFα and—most pronounced—SN/MC-PB decreased the rate of apoptosis. Thus, the elevated level of TNFα in SN/MC-PB will

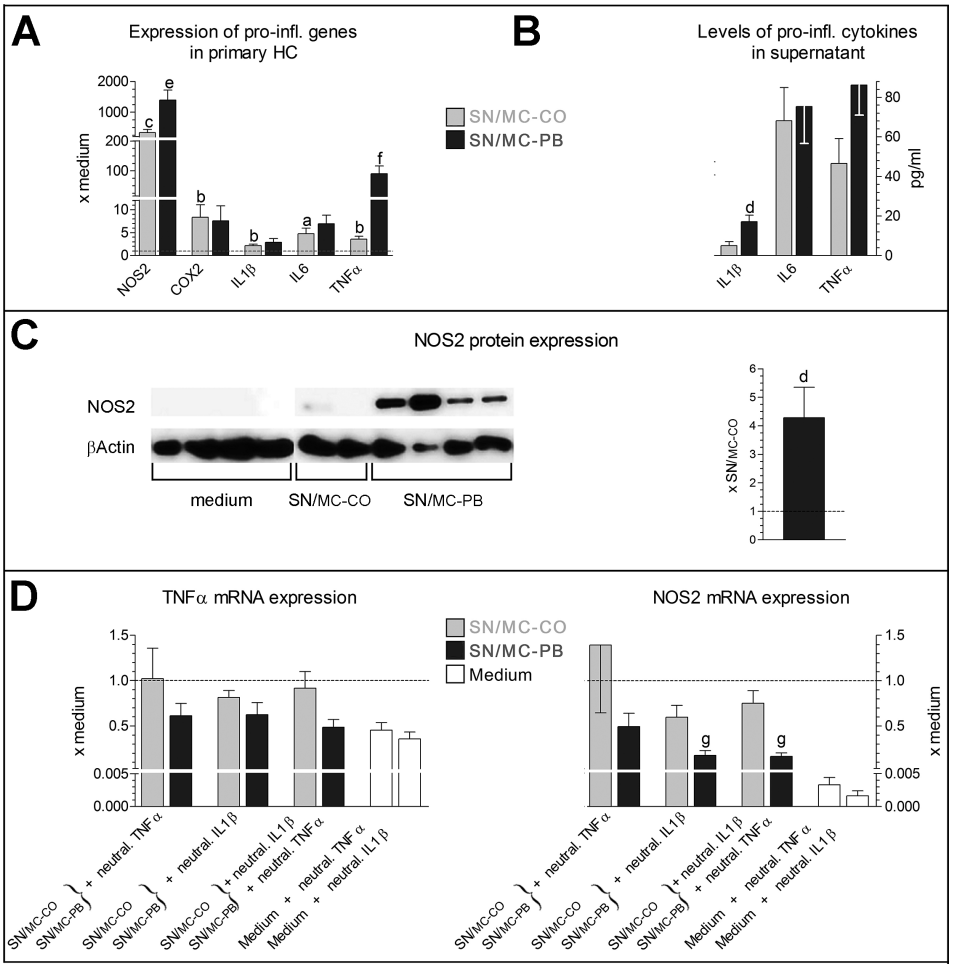

Figure 2. Supernatants of untreated (SN/MC-CO) and PB-treated MC (SN/MC-PB) induce proinflammatory genes in HC—TNFα identified as major proinflammatory cytokine. Rats were left untreated or treated with PB for 7 days to generate SN/MC-CO or SN/MC-PB, which were applied to cultured HC obtained from control rats. (**A**) mRNA was assayed by RT-qPCR. (**B**) IL1ß, IL6 and TNFα concentrations were determined by ELISA. (**C**) HC proteins were separated by SDS page, immunoblotted for detection of NOS2, evaluated by densitometry and expressed as fold SN/MC-CO. (**D**) Medium and supernatants were pre-incubated with blocking antibodies at 37°C for 60 min before addition to HC cultures; 24 h later HC were harvested to determine the mRNA of TNFα and NOS2 by RT-qPCR. (**A–D**) Data are means ± SEM of ≥3 rats. Statistics by Wilcoxon's *t*-test: medium versus SN/MC-CO: (a) $P \leq 0.05$, (b) $P \leq 0.01$, (c) $P \leq 0.001$; SN/MC-CO versus SN/MC-PB: (d) $P \leq 0.05$, (e) $P \leq 0.01$, (f) $P \leq 0.001$; SN/MC-PB versus SN/MC-PB + neutralizing antibodies: (g) $P \leq 0.05$.

support the survival of HC and thereby contribute to the tumor promoting effect of PB.

Discussion

The present work shows for the first time that the NGC PB (i) induces profound alterations in the transcriptome profiles of MC and much less in HC, (ii) increases the production and release of proinflammatory cytokines from MC, (iii) causes a proinflammatory reaction and nuclear translocation/activation of NFκB in HC and (iv) thereby protects HC from proapoptotic stimuli. These alterations appear to be causally involved in the carcinogenic action of this compound as outlined in the following.

We have shown in previous and the present study that the NGC PB is able to induce multiple alterations in the hepatic mesenchyme, which is reflected not only by altered transcriptome profiles but also by deregulated patterns of intracellular as well as secreted proteins (36). PB treatment elevated transcript lev- els of chemokines of the CC-, CXC-type, of the TNF, TGFß and interleukin superfamily. CCl2, CCL7 and CXCL10 specifically

attract monocytes and/or regulate macrophage function and were found to be elevated at both time points of investigation. To understand the impact of the altered intrahepatic chemokine patterns, we determined the occurrence of KC and EM and found no alteration between untreated and PB-treated livers (data not shown). Next, we studied the effect of PB on hepatic inflammation induced by a necrogenic dose of the genotoxic nitrosamine N-nitrosomorpholine. N-nitrosomorpholine alonereduced the frequency of KC but elevated dramatically the occurrence of EM (Supplementary Figure 8, available at *Carcinogenesis* Online). Application of PB appeared to delay both, the recruit- ment of EM to the liver and the repopulation of this organ by KC. Interestingly, also barbiturates have been documented repeatedly to impair the innate host defense in mammalians. In rats, long-term treatment with PB reduced lymphoprolifera- tive responses and anesthesia by thiopentone impaired phago- cytosis of peritoneal macrophages (37,38). Patients sedated by barbiturates often suffer from life-threatening bacterial infec- tions in the post-operative period (23,24). Mechanistic stud- ies revealed that barbiturates inhibit chemotaxis/recruitment

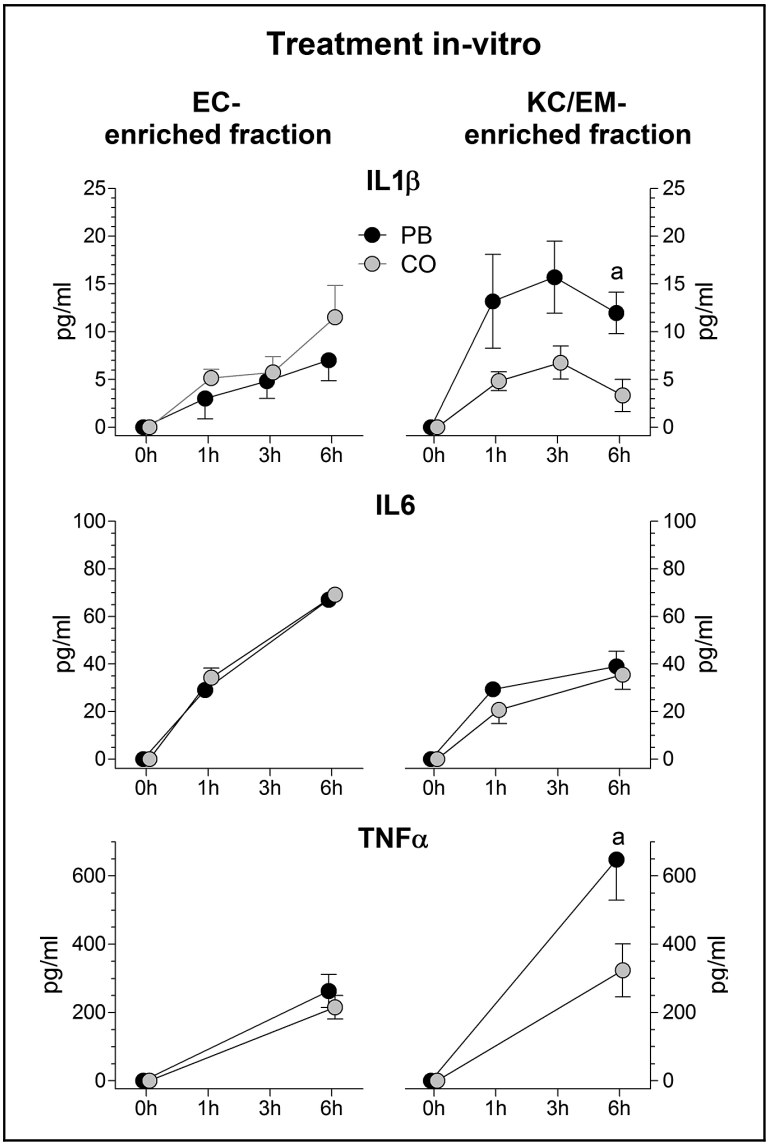

Figure 3. PB *in vitro* induces a proinflammatory state in EC and KC/EM. MC-CO were further separated into EC- and KC/EM-enriched fractions, cultured and treated with 1 mM PB for 6 h. Aliquots of supernatant were collected at the time points indicated and concentrations of IL1ß, IL6 and TNFα in the supernatant were determined by ELISA. Data show means ± SEM of ≥3 rats per treatment. Statistics by two-way ANOVA for time response: CO versus PB: (a) $P \leq 0.05$.

and phagocytosis of human monocytes and/or macrophages (23, 24,39). The upregulated CCL- and CXCL-mRNAs, as observed in the present study in MC-PB, might be a consequence of the body's attempt to counter-regulates the impaired recruitment of KC/EM.

There were also direct effects of PB on isolated and cultured KC/EM, raising questions on the underlying mechanism (Figure 3). As we could not detect significant levels of the PB-responsive nuclear receptors CAR or PXR in EC or KC/EM, we tested for expression of receptors known to bind PB in neuronal/non- hepatic tissues. PB prolongs/potentiates the action of GABA on $GABA_A$ receptors and at higher concentrations was shown to directly activate this receptor (28). Barbiturates also block AMPA/kainate receptors and impair the glutamine receptors 3 and 6 (28–31). However, in HC, EC and KC/EM, we could not find significant transcript levels of components of these recep- tors (data not shown). However, KC/EM express several glycine receptor subtypes, as described previously and confirmed in the

present study (32). Froh *et al.* (32) showed that cell membranes of KC became hyperpolarized by glycine due to a chloride influx. This hyperpolarization prevented lipopolysaccharide-induced activation of the cells, thereby minimizing production of TNFα and various other cytokines and eicosanoids, such as prostaglandins. PB, however, was found to interfere with the response of isolated human α1-glycine receptors to glycine (33). When glycine impairs the proinflammatory function of the cells and PB inhibits the glycine effect, the final outcome might be enhanced production of cytokines under PB treatment, as seen under our present experimental conditions. This also agrees with reports on enhanced production of prostaglandins in PB-treated rat KC (20). Furthermore, when tested at blood concentrations reached during anesthetic administration, the barbiturate pentothal caused a 4–5-fold increased production of TNFα from human monocytes (40). Taken together, these data from literature agree with our finding that despite of a transiently impaired recruiting/repopulation of KC/EM in the inflamed liver by PB, PB-treated

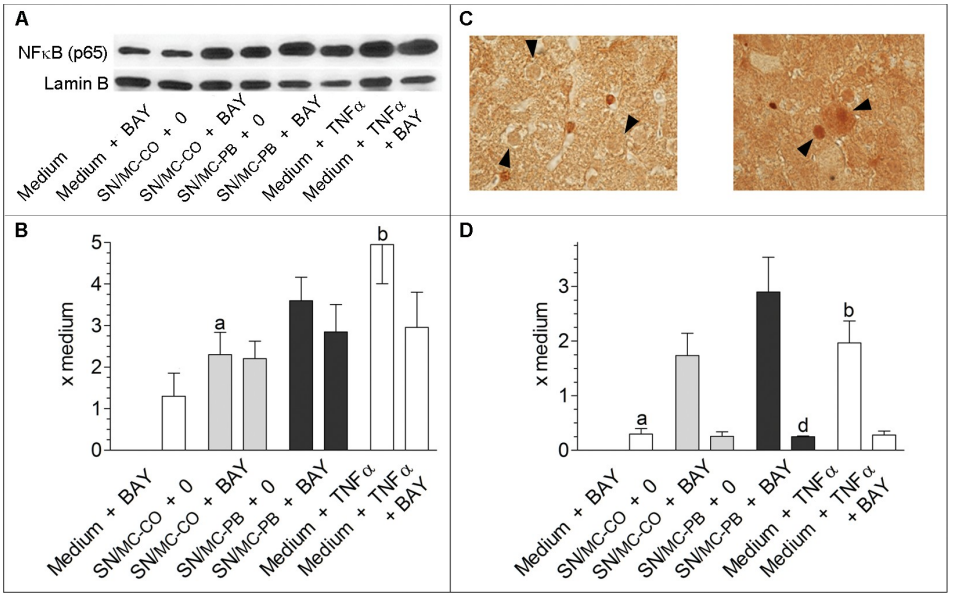

Figure 4. Supernatant of MC from PB-treated rats enhances translocation of NFκB to HC nuclei. (**A**, **B**, **D**) rats were left untreated or were treated with PB for 7 days to generate SN/MC-CO or SN/MC-PB, which were applied to cultured HC, obtained from control rats. Treatment with TNFα (10 ng/ml) served as control. BAY (10 μM) was applied to HC 1 h before the addition of supernatants or TNFα. (**A**) Nuclear proteins of HC were separated by SDS page and immunoblotted for detection of NFκB. (**B**) nuclear NFκB protein levels were evaluated by densitometry and expressed as fold medium control. (**C**) immunostains of formalin-fixed and paraffin-embedded tissue sections obtained from control liver (left panel) or livers subjected to PB treatment for 7 days (right panel); magnification: ×150. (**D**) HC from untreated rats were cotransfected by NFκB-responsive luciferase and renilla luciferase vectors. Supernatants were added 24 h after transfection for 24 h. Data show luciferase activity, normalized to renilla activity and expressed as fold medium control. (**B**, **D**) Data are means ± SEM of ≥3 rats. Statistics by Wilcoxon's *t*-test: medium versus BAY, SN/MC-CO or TNFα: (a) $P \leq 0.05$, (b) $P \leq 0.01$; SN/MC-PB treatment versus SN/MC-PB treatment + BAY: (d) $P \leq 0.05$.

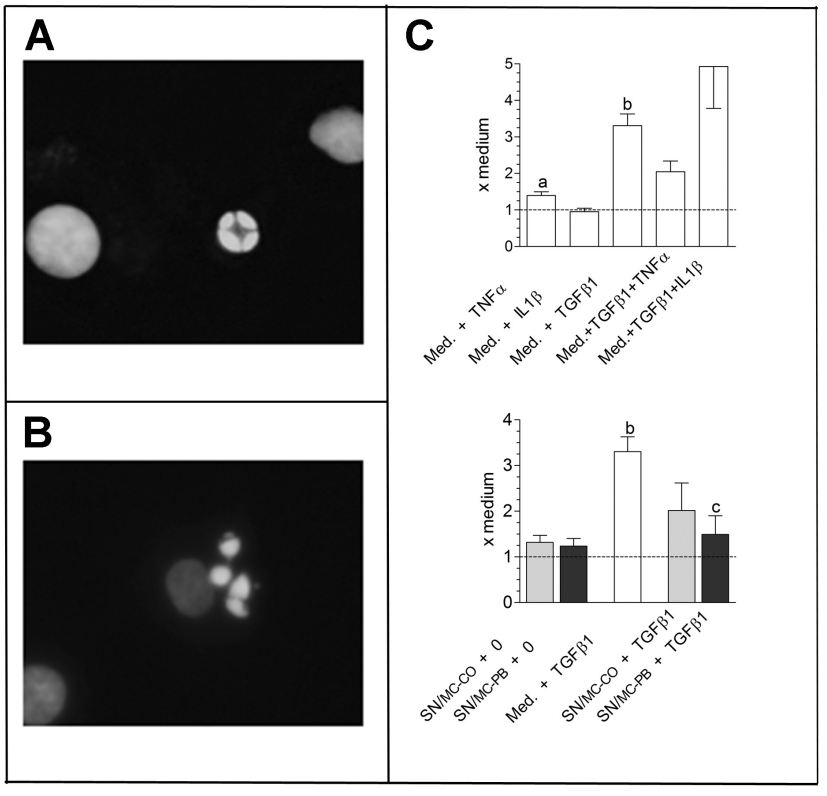

Figure 5. Supernatant of PB-treated MC exerts anti-apoptotic activity in HC. Rats were left untreated or were treated with PB for 7 days to generate SN/MC-CO or SN/ MC-PB, which were applied to cultured HC, obtained from control rats. Parallel cultures were treated with recombinant IL1ß (0.1 ng/ml), TNFα (1 ng/ml) and/or TGFß1 (1 ng/ml). About 24 h later, HC were fixed and stained with Hoechst to count apoptotic bodies: (**A**) shows early stage apoptosis (**B**) an advanced stage. (**C**) At least 2000 HC per experiment and treatment group were screened. Data are expressed as fold medium control and are means ± SEM of ≥3 rats. Abbreviations: Med., medium. Statistics by Wilcoxon's test: medium versus TNFα or TGFß1: (a) $P \leq 0.05$, (b) $P \leq 0.001$; TGFß1 versus TGFß1+SN/MC-PB: (c) $P \leq 0.05$.

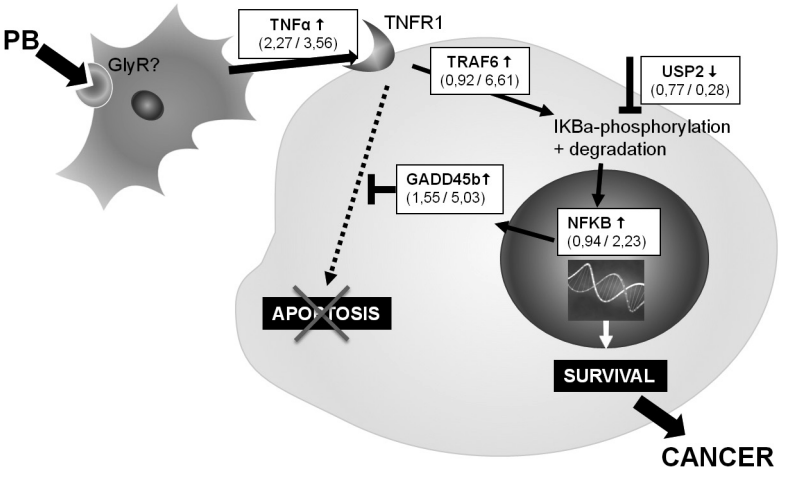

Figure 6. Hypothesis on PB-induced alterations in epithelial–mesenchymal interactions supporting tumor promotion in the liver. Following activation of the mesenchyme by PB via glycine receptor (GlyR), TNFα is released and binds to TNFR1 exposed on HC, with consequent activation of TRAF6 and NFκB. NFκB activation may occur via downmodulation of the deubiquitinating enzyme USP2, which usually prevents the degradation of IkBα to keep NFκB in an inactive state in the cytoplasm. Following nuclear translocation and transcriptional activity of NFκB, GADD45ß may be induced to interfere with JNK-mediated apoptosis. As a consequence, enhanced survival of PB-treated preneoplastic HC supports indirectly growth of preneoplasia and enhances the probability for development of cancer. Symbols: arrows indicate up- or downregulation of genes, as observed by the analyses of oligoarrays; the numbers in brackets give fold deregulation at 24h/14 days of PB treatment when compared with untreated controls. For further details on TRAF6- and NFκB-driven signaling cascades, see Supplementary Table 2, available at *Carcinogenesis* Online.

KC/EM per se release enhanced levels of proinflammatory cytokines. Furthermore, the human relevance of our findings on immunological effects of PB is supported as well.

With regard to the action of PB as NGC in rats, continuous tumor promotion with this compound only marginally elevated DNA replication in the premalignant hepatic lesions but distinctly suppressed the elimination of preneoplastic HC by apoptosis, leading to outgrowth of tumors (Supplementary Figure 1, available at *Carcinogenesis* Online) (3,5). Discontinuation of PB leads to a dramatically elevated apoptotic activity in the liver reversing not only PB-induced hyperplasia but reducing dramatically also the size of the preneoplastic lesions. This PB-induced shift from death towards renewal of preneoplastic cells was found to be the base of the tumor promoting effect of this compound (3,5).

Homeostasis in mammalian tissues is dependent on the continuous integration of cell survival and cell death signals mainly deriving from the extracellular environment. Extensive cross-talk between these antagonistic signaling pathways emanate from TNFR1 stimulation. The proapoptotic downstream signaling includes cJun-activating kinase (JNK), erk and caspase-8 activation. Antiapoptotic signaling events are IκBα phosphorylation/ubiquitination and NFκB translocation into the nucleus (41). The HC-specific deubiquitinating enzyme ubiquitin carboxyl-terminal hydrolase-2 (USP2), which is involved in degrading IkBα, is considered critical, i.e. USP2-knockdown inhibited actinomycin D/TNFα-induced apoptosis of HC via elevated levels of the anti-apoptotic protein c-flip(L/S), while USP2 overexpression exerted the opposite effect. Thus, Haimerl *et al.* (42) suggested that TNFα-induced USP2 downregulation is an effective antiapoptotic mechanism for HC. Papa *et al.* (43) observed that NFκB activation promotes growth arrest and DNA-damage-inducible, beta (GADD45ß) activation, which also blocks the TNFα-induced apoptosis of HC. Analogous alterations were observed in PB-exposed rat HC under our experimental conditions. A counter-regulated and thus truncated TNFα and interferon response was evident, involving USP2 downmodulation, nuclear translocation of NFκB and upregulation of GADD45ß (Figure 6). To conclude, our present work provides strong evidence that mesenchyme-induced

NFκB activation in HC is deeply involved also in non-genotoxic hepatocarcinogenesis, driven by PB.

Much effort has been focused on the assessment of the putative health risks for humans being continuously treated by barbiturates. For better estimation, cross-species compar- ison may be helpful. We analyzed the hepatic mesenchyme in PB-treated mice and failed to see significant proinflamma- tory alterations (Supplementary Figures 9 and 10, available at *Carcinogenesis* Online). This observation might be due to profound functional differences of the innate immune system between rats and mice (44–46). This is also in line with the observation that in mice apoptosis suppression appears to be of minor importance in both, PB-driven hepatocarcinogenesis (outlined above) and regulation of liver mass (8). Accordingly, withdrawal of the NGC WY-14.643 or food led to regression of the murine liver without considerable induction of the hepatocellular apoptotic activity (47). However, in rats and humans, the effects of barbiturates on the innate immune system appear to be similar, i.e. a delayed recruitment of monocytes/macrophages and an increased production of pro-inflammatory cytokines by these cells were observed in both species. The TNFα-mediated antiapoptotic activity in HC can easily switch towards proapoptotic effects of this cytokine. Accordingly, death ligands like TNFα or FasL are often mechanistically involved in the development of drug-induced liver injury. In fact, during long-term PB treatment a mixed pattern of hepatocellular and/or cholestatic damage is seen in a small fraction (<1%) of the patients (48). Liver biopsies from these patients often show granulomatous infiltrations but enhanced apoptosis/necrosis of HC has also been observed (49). Further research in human-relevant systems is required to clarify whether PB-treated human hepatic mesenchyme produces elevated levels of TNFα which might lead to induction of apoptosis and drug-induced liver injury or apoptosis suppression and tumor promotion.

To conclude, our findings imply that the carcinogenic activity of NGC may not, or not always, result primarily or exclu- sively from effects on the parenchyma as generally assumed. Rather, direct effects on the hepatic mesenchyme seem to play

important roles in NGC carcinogenesis. This new concept needs to be tested with other NGC as well as with other organs and species. Eventually, new insight generated along these lines will improve concepts of risk assessment of NGC.

Supplementary material

Supplementary Figures 1–11 and Tables 1–3 can be found at http://carcin.oxfordjournals.org/

Funding

European Union Innovative Medicine Initiative (IMI JU) (Nr 115001).

Acknowledgements

The technical assistance of Helga Koudelka, Birgit Mir-Karner and Suchitra Sharma is gratefully acknowledged. Venn diagrams were drawn with the program 'Venn Diagram Plotter' kindly provided by PNNL and the OMICS.PNL.GOV website.
Conflict of Interest Statement: None declared.

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3 CHAPTER THREE: DISCUSSION

3.1 General discussion

HCC is a common cause of death from malignancies in humans. Therefore, improving our knowledge about the risk factors involved is of particular importance. Genotoxic carcinogens, such as AFB1 or ethanol, are well studied and the underlying mechanisms by which they contribute to the development of HCC are somewhat clear; but but it is still uncertain how NGCs, which do not show genotoxic activity, lead to HCC in long-term animal experiments. Approximately 40% of tested NGCs are hepato-carcinogenic [144]. Thus, this means that a clear screening assay system for NGCs, which detects the potential non genotoxic carcinogenic effects of chemicals and/or drugs, would be helpful for the prevention of HCC and, more important, in the improvement of drug safety strategies.

Recent studies show that the majority of NGCs exert their carcinogenic effect by affecting liver homeostasis with the purpose of creating an imbalance between cell proliferation and cell death. This dysregulation gives the mutated/initiated cells more chance to enhance cell replication and progress to pre-neoplastic cells [16, 145].

Many of the NGCs—such as CPA and PB—act on hepatocytes via nuclear receptors (CAR, PXR, PPAR, etc.)[146]. It has been observed that, in the rodent liver, these receptors mediate adaptive increases in specific enzyme groups (e.g., lipid or drug metabolism) and /or organelles (like SER or peroxisomes) and liver growth via hypertrophia or hyperplasia. Some of these enzyme inductions, by virtue of NGC receptors, have also been observed in humans, but do not lead to liver enlargement [147]. However, the key question of how activated enzyme cascades lead to proliferation in the liver of rodents, but not in humans, is still unclear.

A further possible mode of action for NGCs that has not yet been well studied is the effect of NGCs on the mesenchyme and the significance of mesenchymal-parenchymal interactions in the process of hepato-carcinogenesis. MCs cannot transfer to malignant cells, but, as discussed previously, recent evidence suggests that mesenchymal cells may be targeted by NGCs and may have a contributory role in the process of NGC-driven rodent hepatocarcinogenesis by selective stimulation of pre-neoplastic HCs via production and release of growth factors [148, 149].

The present work is based on the concept that liver tumor formation by NGCs is not a mere epithelial disease. We, therefore have focused on the role of MC in

responses of pre-neoplastic lesions toward the action of NGCs.

We have chosen PB and CPA as two known tumor-promoting drugs in the rodent liver. It has been shown before that these drugs interact with PXR and CAR receptors, which, after stimulation, lead to induction of CYP2B, CYP3A, and P450 ADDIN EN.CITE [120, 150, 151]. In our study, we could observe and prove that, after treatment with some NGCs, such as PB and CPA, the mesenchyme of the liver is significantly altered. There are recent published studies that have also shown the activation of the mesenchyme after treatment with PB [144, 152]. Furthermore, these activations lead to the release of factors that promote and /or inhibit the outgrowth of premalignant hepatocytes. These factors could be produced by HC and /or mesenchymal cells. This bidirectional signaling between hepatocytes and mesenchymal liver cells (endothelial, kupffer cells, and stellate cells) seems to be essential for regulating homeostasis in the liver and also for the balance between growth stimulatory and inhibitory factors. The present studies attempt to elucidate the influence of receptor-mediated hepatocyte activation and the activated mesenchyme in NGC-induced carcinogenesis.

3.2 Possible underlying mechanism of action for PB

In an initial step, we looked at purified hepatic mesenchyme without any treatment to observe which factors produced from MCs are responsible for the induction of proliferation in normal and pre-neoplastic HCs. According to our mesenchymal cell gene expression profile and experiments at the protein level, MCs can produce and secrete different growth factors, such as HGF, HBEGF, GDF 15, and TNF-α, which induces a slight proliferative response in normal HCs, as well as pre-neoplastic HCs [14]. After treatment of MCs —*in vivo* and *in vitro*—with PB, these growth factors show a significant upregulation in the oligo array.

It seems that the gene induction that is induced by PB treatment has a compound-specific pattern in both cell preparations (HCs & MCs). We observed these alterations not only in *in vivo* treatment, but also in cultured purified cells, which showed that purified MCs are also able to react toward NGCs even though the mRNA of CAR and PPARα were barely detectable in EC and KC. Only PXR is transcribed at a low level in ECs.

Subsequently, we detected glycine receptor subunits on MCs as possible receptors for PB. When compared to the treatment with PB *in vivo*, the application of PB in purified cells *in vitro* affected different genes; therefore, we focused on *in vivo* experiments with PB, as it seemed to be more reliable for the detection of underlying mechanisms of action of PB on MC/HC ADDIN EN.CITE [14, 115, 153]. Klepeisz et al. also found that *in vivo* PB treatment has a more profound effect and is even largely different on HC and MCs compared to the *in vitro* treatment[144].

Our data showed that PB treatment severely affects the mesenchymal transcriptome even more than hepatocytes (120 genes versus 1200 genes). So, we concluded that it was essential to study the role of MCs in NGC-driven hepatocarcinogenesis in more detail ADDIN EN.CITE [14, 153]. Recently performed proteome profiling assays and other applied techniques have already identified and confirmed some of the effects of PB on HCs that we had observed in our study, such as the induction of cytochrome P450, the proto oncogene RAF, and GST enzymes ADDIN EN.CITE [154-157].

Further, we studied the secretome that is produced by PB-treated MCs/HCs. We found that several proteins appeared only in the secretome of PB-treated cells (not in controls), demonstrating again that PB can induce a compound-specific reaction profile not only at the gene expression level, but also at the protein level. In HCs, PB was confirmed to be a potent inducer of drug-metabolism and also stress induced-pathways, such as MAPK and TNF-α signaling. Replication of the same results on rodent HCs in our genomic analysis, as well as in the proteome analysis, confirmed that our method is valid.

Formerly, in 1998, it was shown that PB treatment induces TNF-activation in the liver of mice[158, 159]. We could show that PB induces pro-inflammatory alterations in the mesenchyme and somehow counter-regulations in HCs: MCs isolated from PB-treated rats showed a pronounced elevation in the mRNA of many chemo/cytokines related to pro-inflammatory responses, as in the CXCL and CCl family. In isolated HCs, we observed counter-regulation of pro apoptotic genes like TGF-□□:σ, as well as anti-apoptotic pathways, like the activation of the AKT pathway and MIC-1, or downregulation of the USP2 pathway representative of the jnk/Caspase pathway. Haimerl et al. in 2009 also showed that pretreatment of mouse HCs with TNF-□□:σ caused a rapid downregulation of USP2, which is a

cytoprotective mechanism in the liver [160]. These counter reactions can be considered a protective mechanism of the liver to cope with the stress actions induced by MCs. Such counter reactions can lead to survival of pre-neoplastic or mutated lesions in the liver and it can be considered to increase the probability of HCC development in the long-term.

3.3 Possible mechanisms underlying the action of CPA

CPA may be the most studied anti-androgen drug from a steroid family, but most studies have mainly focused on the genotoxic, and even fewer studies on the non-genotoxic effects on HCs. The interactions between the mesenchyme and the parenchyma and the role of this cross-talk in cancer promotion has not been well studied [161].In this study, we tried to clarify the role of MCs in cancer promotion induced by CPA.

We could show that several factors produced by MCs can control cell replication and cell growth in the HCs. Some of them had already been identified, including TNF-α [162], and others, like HBEGF, HGF, and GDF15 had not been discussed before. Interestingly, in our study, TNF-α at a concentration that is produced by normal control MCs, had no significant proliferatory effects on normal HCs. However, at the same concentration [14], TNF-α can induce proliferation in pre-neoplastic/initiated HCs, confirming the known fact that initiated cells are more prone to over-react to cell stimulations.

As mentioned previously, there were significantly more affected genes and possible activated cascades in rat HCs treated *in vivo* with CPA than in MCs, contrary to the results obtained with PB [14]. This, again, suggests that CPA has a compound-specific pattern, different from PB. Genes involved in the cell cycle, stability, and repair of DNA were induced in HCs treated with CPA. In addition, both NGCs seem to induce pathways in HCs that are important for coping with oxidative stress, such as the glutathione-metabolism pathway and the NRF2-mediated stress response, etc. In MCs, CPA treatment profoundly altered the arachidonic acid metabolic pathways and also pathways that cope with oxidative stress. Amino acid pathways that were induced after CPA treatment may also occur due to their role in the

regulation of immunological functions [163].

HCs isolated from CPA-exposed liver cells also showed an increase in DNA synthesis not only in normal HC, but even more in pre-neoplastic HCs. The same results, albeit stronger and more pronounced, were observed when purified normal and pre-neoplastic HCs were directly treated with CPA, confirming the fact that despite PB, CPA acts more potently directly on HCs, likely via known nuclear receptors [14].

In the next step, we focused on the role of MCs in the process of tumour promotion. We observed that CPA caused deregulation of TNF-α, interferon, and NFk B pathways. TNF-α is also important in apoptotic pathways and aging seems to demonstrate counter-regulations against pro-apoptotic stimulus. We could also detect an intense hepato-mitogen induction that explains the hyperplasia that was induced by CPA, as shown in our previous studies. It seems that this hyperplasia plays an important role in the progression of initiated HCs to neoplastic lesions. The activation of CTGF, HGF, and HDGF in CPA-treated mesenchyme reflects the upregulation of growth cascades and further secretion of growth factors. Since CPA is known to act mainly as a progestin and we did not find any activated signal for the progesterone receptor's transcript, we conclude that there should be alternatives by which to mediate the effects of progesterone. In our genome analysis, there is some evidence to support this theory that should be studied further.

3.4 Human relevance of the findings

The present work focuses on the role of MC in hepato-carcinogenesis induced by NGCs and attempts to identify growth factors and chemo/cytokines from both MC and HC that may drive the excessive growth response of pre-neoplastic lesions toward the action of NGCs.

The proliferative response of cultured *in vitro* rat hepatocytes, and/or the inhibition of apoptosis followed by PB treatment in *in vivo*, has, thus far, not been observed in comparable cultures in humans [125, 164]. Recently developed humanised PXR/CAR mouse models displayed the induction of P450s and the induction of HC-hypertrophy, like rodent HCs, but did not show hepatocyte proliferation [165]. In transgenic models that express only human CAR in the liver (hCAR mice),

treatment with PB for one week resulted in relative increases in liver weight and cell proliferation, but, again, no neoplastic changes or even increased mortality was observed. There was also no significant difference in the genome profile after the injection or dietary intake of PB either in an hCAR mouse model or in controls [129, 166].

In another study, long-term treatment with PB induced a highly similar hepatic transcriptional program in wild-type and humanised CAR/PXR mice [115]. This transcriptional response included the upregulation of some cell cycle genes, as well as proliferative markers like Ki67. It has been well demonstrated that PB treatment can induce replicative DNA synthesis (RDS) in hCAR/hPXR mice, but how relevant this system is for humans is yet to be clarified. There are many discrepancies between the results from hCAR/hPXR mice and human cultured HCs or from chimeric mice with human livers (Braeuning et al., 2014; Luisier et al., 2014 Corinne Haines 2018).

After chronic treatment with PB and similar drugs from the same group in humans, immunosuppression, in the form of a decreased number of immune cells in the blood was observed. But, in the context of MCs receptors and the interaction of MCs with HCs, unfortunately, there is no humanised receptor study. Nevertheless, in a recently study published in 2018, an RDS response was observed in HCs of chimeric mice with a humanised liver after *in vitro* treatment with HGF and EGF, which is in agreement with our findings in rat HCs under treatment with HGF produced by MCs (Corinne Haines 2018).

Further research in human-relevant systems, particularly a dose-response evaluation, is required to clarify whether the PB-treated human hepatic mesenchyme produces elevated levels of TNF-α, which might lead to the induction of apoptosis and DILI, or apoptosis suppression and tumour promotion.

As mentioned previously, CPA has widespread use in human therapy, mainly in women's androgen-related diseases, breast cancer, and prostate carcinoma. Previous data indicated that CPA is a purely non-genotoxic carcinogen compound, which enhances the proliferation of hepatocytes in the initiated cells more than normal hepatocytes [167, 168]. Human liver cells were found to be non-responsive to mitogenic stimulation by CPA, suggesting that this tumour-promoting activity is a rodent-specific mechanism of action [127]; but, there has been conclusive new

evidence that shows that CPA forms DNA adducts and induces DNA repair in primary hepatocytes from female rats and humans of both genders [137, 169]. In another study on human liver slices of male and female donors, a dose-dependent formation of main adducts was observed [170]. Mesenchymal cells in primary human cultures have not yet been studied. In contrast, epidemiological studies in human subjects do not support an increase in the incidence of hepatic tumours under the therapeutic use of CPA [171], although a few case reports presented reversible hepatotoxicity induced by CPA after treatment of prostate carcinoma [172].

It seems that our presented model, with the combination of genome analysis and proteome profiling with treatment of purified normal and pre-neoplastic HCs with the secretome (supernatant) of MCs—especially after *in vivo* treatment—can be considered a good model with regard to biological relevance and the clarification of the mode of action of NGCs. Omic data should be evaluated in the context of molecular interactions—MCs and HCs—to achieve more realistic results. To be sure, more optimization and more techniques may be used; for example, a liquid chromatography of the secretome may be useful to obtain an overview of the whole secreted protein and its quantification in order to know which protein has the potential to be used as a marker.

Another possible method, which may be a candidate to be used for prediction of genotoxic and non-genotoxic carcinogens and even their mode of action, is the machine-learning system. This system has been widely used in recent years in oncologic disease to predict cancer behaviour, therapy response, and also the affected genes [173, 174]. Machine-learning systems are based on the mathematical combination of different parameters with regard to the epidemiological features. A machine-learning system that could combine multiple -omic data with the structure of chemicals and epidemiological data and more may also be useful for the estimation of important markers or even to enhance the power of already used markers.

Unfortunately, studies that have focused on extrapolating rodent genotoxic/non-genotoxic data to humans are not convincing. As such, additional research would be necessary to interpret the possible mode of action of CPA and the role of the mesenchyme in human hepato-carcinogenesis.

4 CHAPTER FOUR: MATERIALS AND METHODS

4.1 Materials

Substances:

Substance	Supplier	Product code
(−)-Tetramisole hydrochloride	Fluka, St. Gallen, Switzerland	87963
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA	M7154
5-Bromo-4-chloro-3-indolyl phosphate p -toluidine salt	Sigma-Aldrich, St. Louis, MO, USA	B8503
Ammonium persulfate	Sigma-Aldrich, St. Louis, MO, USA	A3678
Bromophenol blue sodium salt	Sigma-Aldrich, St. Louis, MO, USA	B8026
BSA	Sigma-Aldrich, St. Louis, MO, USA	A8022
$CaCl_2$	Fluka, St. Gallen, Switzerland	21097
Chloroform	Merck, Darmstadt, Germany	1.02445.2500
Collagenase Type 2	Worthington, Lakewood, NJ, USA	CLS-2
Cy™2-conjugated streptavidin	Jackson ImmunoResearch, West Grove, PA, USA	016-220-084
Dexamethasone	Sigma-Aldrich, St. Louis, MO, USA	D4902
D-glucose monohydrate	Merck, Darmstadt, Germany	1.08346.1000
Diethyl pyrocarbonate	Sigma-Aldrich, St. Louis, MO, USA	D5758
EDTA	Sigma-Aldrich, St. Louis, MO, USA	E5134
Ethanol	Merck, Darmstadt, Germany	1.00983.2500
Formaldehyde 37%	Merck, Darmstadt, Germany	1.04003.2500
Gentamycin sulfate	Serva, Heidelberg, Germany	22185
Glucagon	Serva, Heidelberg, Germany	51775
Glycerol	Fluka, St.Gallen, Switzerland	49770
Glycine	Riedel-de Häen, Seelze, Germany	33226
HEPES	Sigma-Aldrich, St. Louis, MO, USA	H4034
Hoechst 33258	Sigma-Aldrich, St. Louis, MO, USA	861405
IGEPAL® CA-630 viscous liquid	Sigma-Aldrich St. Louis, MO, USA	I3021
Insulin	Sigma-Aldrich	I5500

	St. Louis, MO, USA	
Kaiser's glycerol gelatine	Merck, Darmstadt, Germany	1092420100
KCl	Merck, Darmstadt, Germany	4936
KH_2PO4	Merck, Darmstadt, Germany	4783
L-ascorbic acid	Sigma-Aldrich, St. Louis, MO, USA	A4544
Methanol	Merck, Darmstadt, Germany	1.06009.2511
$MgCl_2•6H_2O$	Fluka, St. Gallen, Switzerland	63068
$MgSO_4$	Sigma-Aldrich, St. Louis, MO, USA	M7506
$MgSO_4•7H_2O$	Merck, Darmstadt, Germany	5886
N,N,N',N'-Tetramethylethylenediamine	Sigma-Aldrich, St. Louis, MO, USA	T8133
N,N-Dimethylformamide	Merck, Darmstadt, Germany	822275
$Na_2HPO_4•2H_2O$	Merck, Darmstadt, Germany	1.06580.1000
NaCl	Sigma-Aldrich, St. Louis, MO, USA	S7653
$NaH_2PO_4•H_2O$	Merck, Darmstadt, Germany	1.06346.0500
Na-Heparin	Serva, Heidelberg, Germany	24590
NaOH	Merck, Darmstadt, Germany	1.06469.1000
Nitro blue tetrazolium chloride	Serva, Heidelberg, Germany	30550
Penicillin G sodium salt	Sigma-Aldrich, St. Louis, MO, USA	P3032
Polybrene / Hexadimethrine bromide	Sigma-Aldrich, St. Louis, MO, USA	H9268
2-propanol	Merck, Darmstadt, Germany	1.09634.9025
SeaKem® LE agarose	Lonza, Basel, Switzerland	50004
Skim milk powder	Fluka St. Gallen, Switzerland	70116
Sodium deoxycholate	Sigma-Aldrich, St. Louis, MO, USA	D6750
Sodium dodecyl sulfate	Sigma-Aldrich, St. Louis, MO, USA	L4390
Sodium orthovanadate	Sigma-Aldrich, St. Louis, MO, USA	S6508
Sodium pyruvate	Sigma-Aldrich, St. Louis, MO, USA	P2256
Streptomycin sulfate salt	Sigma-Aldrich, St. Louis, MO, USA	S9137
Triiodotyronine	Serva, Heidelberg, Germany	37041
TRIS	Fluka, St. Gallen, Switzerland	93349
Trypan blue	Sigma-Aldrich, St. Louis, MO, USA	T8154
TWEEN® 20	Sigma-Aldrich, St. Louis, MO, USA	P1379

Table 1 List of substances

Reagents, media, and commercial solutions

Reagent/medium/commercial solution	Supplier	Product code
40% acrylamide/bis solution	Bio-Rad, Hercules, CA, USA	161-0148
5x reaction buffer	Fermentas, Vilnius, Lithuania	EP0441
6x loading dye solution	Fermentas, Vilnius, Lithuania	R0611
Amersham ECL prime Western blotting detection reagent	GE-Healthcare, Little Chalfont, UK	RPN2232
Amersham Hybond-P PVDF membrane	GE-Healthcare, Little Chalfont, UK	RPN303F
CL-XPosure film	Thermo Scientific, Waltham, MA, USA	34089
Complete, mini, protease inhibitor cocktail tablets	Roche, Basel, Switzerland	11 836 153 001
DNA primer 40nmol, lyophilized	Eurogentec, Cologne, Germany	BA-DN001-004
dNTP Mix, 10 mM each	Fermentas, Vilnius, Lithuania	E0192
Endoprime Kit	PAA, Pasching, Austria	U050-042
Foetal bovine serum, heat inactivated	PAA, Pasching, Austria	A15-104
GeneRuler™ 50 bp DNA ladder	Fermentas, Vilnius, Lithuania	SM0371
GIBCO® GLUTAMAX	Invitrogen, Carlsbad, CA, USA	35050
GIBCO® Willams medium E	Invitrogen, Carlsbad, CA, USA	22511
GoTaq® green master mix	Promega, Madison, WI, USA	M7113
Minimum essential medium Eagle	Sigma-Aldrich St. Louis, MO, USA	M0268
Negative control Lentifect™ lentiviral particles	Genecopoeia, Rockville, MD, USA	LP-NEG-LV201-0200
PageRuler prestained protein ladder	Thermo Scientific, Waltham, MA, USA	26616
Paper blotting grade GB005 1.2 mm thickness 580 mm x 580 mm	Schleicher & Schuell, Keene, NH, USA	10426994
Percoll	GE-Healthcare, Little Chalfont, UK	17-0891-01
Polybead® microspheres 1.00 µm	Polysciences, Warrington, PA, USA	07310
Polyester mesh, 105 µm pores	VWR, Darmstadt, Germany	510-9509
Protein assay dye reagent concentrate	Bio-Rad, Hercules, CA, USA	500-0006
Random hexamer primer 100 µM	Fermentas, Vilnius, Lithuania	SO142
RevertAid reverse transcriptase (200 U/µL)	Fermentas, Vilnius, Lithuania	EP0441
RiboLock RNase inhibitor (40 U/µL)	Fermentas, Vilnius, Lithuania	EO0381
RPMI-1640 medium	Sigma-Aldrich, St. Louis, MO, USA	R6504

Serva DNA stain clear G	Serva, Heidelberg, Germany	39804
Ssniff® R/M-H extrudate	ssniff, Soest, Germany	V1536-000
Streptavidin/HRP	DakoCytomation, Cambridge, UK	P0397
Peq GOLD TriFast™	peqLab, Erlangen, Germany	30.2020

Table 2: List of reagents, media, and commercial solutions

Plastics:

Plastic	Supplier	Product code
0.2 ml SoftTubes®	Biozym Scientific, Hessisch-Oldendorf, Germany	711004
6 well multiwell plate	BD Falcon, Franklin Lakes, NJ, USA	353224
48-well cell culture plate	BD Falcon, Franklin Lakes, NJ, USA	353078
96-well microplate	BD Falcon, Franklin Lakes, NJ, USA	353075
Micro tube 0.5 ml, PP	Sarstedt, Nümbrecht, Germany	72699
Micro tube 1.5 ml, PP	Sarstedt, Nümbrecht, Germany	72690001
Micro tube 2 ml, PP	Sarstedt, Nümbrecht, Germany	72691
Millex-GP, 0.22 µm, polyethersulfone, 33 mm, gamma sterilized	Millipore, Billerica, MA, USA	SLGP033RB
Nunc* dishes, cell culture/petri, 35 mm dish, with airvent	NUNC, Roskilde, Denmark	153066
Small cell scraper	Corning, Corning, NY, USA	3010
Steritop-GP, 0.22 µm, polyethersulfone, 250 mL 45 mm	Millipore, Billerica, MA, USA	SCGPT02RE
Tube, 15 ml, PP, screw cap	Greiner Bio-One, Kremsmünster, Austria	188271
Tube, 50 ml, PP, screw cap	Greiner Bio-One, Kremsmünster, Austria	227261

Table 3 List of plastics

Antibodies:

Antibody	Supplier	Product code
Anti-rat hepatic sinusoidal endothelial cells (SE-1) mouse IgG MoAb	Immuno-Biological Laboratories, Fujioka, Japan	10078
Monoclonal antibody mouse anti rat OX -62	AbD Serotec, Kidlington, UK	MCA1029G
Monoclonal anti-β-actin antibody produced in mouse	Sigma-Aldrich, St. Louis, MO, USA	A5441

Monoclonal mouse anti-human desmin clone D33	DakoCytomation, Cambridge, UK	M0769
Mouse anti-rat CD68 monoclonal antibody	AbD Serotec, Kidlington, UK	MCA341GA
Polyclonal goat anti-mouse immunoglobulins/HRP	DakoCytomation, Cambridge, UK	P0447
Polyclonal goat anti-rabbit immunoglobulins/AP	DakoCytomation, Cambridge, UK	D0487
Polyclonal goat anti-rabbit immunoglobulins/biotinylated	DakoCytomation, Cambridge, UK	E0423
Polyclonal goat anti-rabbit immunoglobulins/HRP	DakoCytomation, Cambridge, UK	P0448
Polyclonal rabbit anti-human albumin	DakoCytomation, Cambridge, UK	A0001
Polyclonal rabbit anti-mouse immunoglobulins/biotinylated	DakoCytomation, Cambridge, UK	E0464
Rabbit polyclonal anti-CD3 antibody	Abcam, Cambridge, UK	ab5690
Rabbit polyclonal anti-CD45 antibody	Abcam, Cambridge, UK	ab10558
Rabbit polyclonal CD19 antibody	Abbiotec, San Diego, CA, USA	250585
Table 4 List of antibodies		

Devices:

Device	Supplier	Product Code
Assistent® Zählkammer Neubauer improved	Hecht, Sondheim, Germany	442/2
BD FACSCalibur	Becton Dickinson, San Jose, CA, USA	-
C1000™ Thermal Cycler	Bio-Rad, Hercules, CA, USA	184-1000
Centrifuge 3-18K	Sigma-Aldrich, St. Louis, MO, USA	-
Centrifuge 5415 R	Eppendorf, Hamburg, Germany	5426 000.018
Confocal microscope LSM 710	Zeiss, Jena, Germany	-
Fluorescence microscope Eclipse Ti	Nikon, Tokyo, Japan	-
Heraeus megafuge 40R	Thermo Scientific, Waltham, MA, USA	-
Mini PROTEAN Tetra cell system	Bio-Rad, Hercules, CA, USA	-
NanoDrop® ND-1000	peqLab, Erlangen, Germany	91-ND-1000
Optimax 2010 X-ray film processor	Protec, Oberstenfeld, Germany	-
Orion 3 Star pH-Meter + electrode	Thermo Scientific, Waltham, MA, USA	1112001
Sub-Cell® GT cell system	Bio-Rad, Hercules, CA, USA	-
Tecan Reader Inifinite M200Pro	Tecan, Männedorf, Switzerland	-
Thermomixer comfort	Eppendorf,	5355 000.011

	Hamburg, Germany	
Ultrasonic homogenizer	Bandelin Electronics, Berlin, Germany	UW 2070
Ultrasonic homogenizer power supply	Bandelin Electronics, Berlin, Germany	HD 2070

Table 5: List of devices

Buffers & Solutions:

50 bp DNA ladder-solution	
Gene Ruler 50 bp DNA ladder (Fermentas)	40 µl
6x DNA loading dye	32 µl
DEPC treated water	128 µl
store at 4°C	

10 % APS	
APS	10 g
distilled water	100 ml
store at room temperature	

AP-staining-solution	
NBT-stock	3.5 µl
BCIP-stock	4.5 µl
AP-1-solution	ad 1 ml
always prepare fresh	

AP-stop-solution	
TRIS	6.05 g
NaCl	2.92 g
EDTA	18.61 g
distilled water	500 ml
adjust pH=9.5, store at 4°C	

AP-1-solution	
TRIS	6.05 g
NaCl	2.92 g
$MgCl_2 \cdot 6H_2O$	1.02 g
Tween 20	500 µl
Levamisol-stock	1 ml
distilled water	ad 500 ml
adjust pH=9.5, store at 4°C, add Levamisol just before using	

100x ascorbate-solution	
ascorbic acid	265 mg
distilled water	100 ml
sterile filter, store at -20°C, actually used aliquot at 4°C	

BCIP stock	
BCIP	50 mg

N,N-dimethylformamide	1 ml

store at -20°C

blocking solution	
skim milk powder	0.45 g
PBSt	15 ml

always prepare fresh

Bradford-solution	
Protein Assay Dye Reagent Concentrate (BioRad)	400 µl
distilled water	1600 µl

always prepare fresh

BSA-buffer	
BSA-buffer stock solution	20 ml
Gentamycin-solution	500 µl
BSA	2.5 g
distilled water	ad 500 ml

adjust pH=7.4, sterile filter, store at 4°C

BSA-buffer stock solution	
NaCl	103.75 g
KCl	6.25 g
HEPES	28.7 g
1M NaOH	75 ml
distilled water	ad 500 ml

adjust pH=7.5, sterile filter, store at -20°C

BSA-solution	
BSA	296 mg
distilled water	237 ml

always prepare fresh

BSA-stock	
BSA	10 mg
distilled water	10 ml

store at -20°C

BSA/TBS (2.5% BSA)	
TBS	100 ml
BSA	2.5 g

store at 4°C

100x $CaCl_2$-solution	
$CaCl_2$	2.94 g
distilled water	100 ml

sterile filter, store at -20°C, actually used aliquot at 4°C

Collagenase buffer	
distilled water	87 ml
salt buffer	10 ml
Pen-Strep-solution	1 ml
H2-Mix	1 ml
Pyruvate-solution	0.2 ml
$CaCl_2$-solution	1 ml
Collagenase	40 mg

adjust to pH=7.5-7.6, sterile filter, always prepare fresh

Culture medium	
Williams-Medium E	96 ml
Glutamax	1 ml
HEPES	1 ml
Gentamycin-solution	1 ml
H2-mix	1 ml
Ascorbate-solution	1 ml

store at 4°C

DEPC treated water	
DEPC	1 ml
distilled water	ad 1000 ml

stir over night, autoclave, store at room temperature

0.5 M EDTA pH= 8.0	
EDTA	93.5 g
distilled water	500 ml

adjust pH=8.0 store at room temperature

1000x genatmycin-solution	
Gentamycin	1 g
0.9% NaCl	100 ml

sterile filter, store at -20°C, actually used aliquot at 4°C

Glucagon-solution	
glucagon	7 mg
0.1 M HCl	100 µl
0.9% NaCl with 15 mg BSA / 10 ml	9.9 ml

store at -20°C

Insulin-solution	
insulin	0.92 mg
0.1 M HCl	100 µl

store at -20°C

10x HBSS-buffer	
NaCl	80 g

KCl	4 g
$MgSO_4 \cdot 7H_2O$	2 g
KH_2PO_4	0.6 g
glucose	10 g
$NaH_2PO_4 \cdot H_2O$	0.6 g
distilled water	1000 ml

sterile filter; store at 4°C

Heparin-2-solution	
heparin-Na	12 mg
0.9% NaCl	50 ml

sterile filter, store at -20°C, actually used aliquot at 4°C

100x Heparin-3-solution	
Heparin-Na	62 mg
0.9% NaCl	50 ml

sterile filter, store at -20°C, actually used aliquot at 4°C

100x HEPES-solution	
HEPES	23.8 g
4 M NaOH	6-8 ml
distilled water	ad 50 ml

adjust to pH=7.4, sterile filter, store at -20°C, actually used aliquot at 4°C

Hoechst-solution (8 µg / ml)	
Hoechst 33258	2 mg
distilled water	250 ml

store at 4°C in the dark

100x H2-Mix	
Insulin-solution	100 µl
Glucagon-solution	84 µl
BSA-solution	237 ml
T3-solution	2.4 ml
Dexmethasone-solution	240 µl

sterile filter, store at -20°C, actually used aliquot at 4°C

10x Laemmli running buffer	
TRIS	30 g
glycine	144 g
SDS	10 g
distilled water	1000 ml

store at 4°C

6x Laemmli sample buffer	
3 M TRIS pH=6.8	1 ml
glycerol	6 ml
SDS	1 g

bromophenol Blue sodium salt	2.5 mg
2-mercaptoethanol	700 µl
distilled water	ad 10 ml
store at -20°C	

Levamisol-stock	
(−)-tetramisole hydrochloride	2.41 g
distilled water	10 ml
store at -20°C	

Lille's buffered formalin	
formaldehyde 37%	200 ml
$NaH_2PO_4 \cdot H_2O$	8 g
$Na_2HPO_4 \cdot 2H_2O$	16.3 g
distilled water	2000 ml
store at 4°C	

NBT-stock	
NBT	75 mg
N,N-dimethylformamide	700 µl
distilled water	ad 1000 µl
store at -20°C	

10x PBS	
$Na_2HPO4 \cdot 2H_2O$	14.4 g
$NaH_2PO_4 \cdot H_2O$	26.2 g
distilled water	1000 ml
adjust pH=7.4, autoclave, store at room temperature	

PBSt	
10x PBS	100 ml
Tween 20	500 µl
distilled water	Ad 1000 ml
store at room temperature	

PBS/EDTA	
EDTA	744 mg
1x PBS	100 ml
store at 4°C	

PBS/1.5 % FA	
formaldehyde 37%	4.05 ml
1x PBS	ad 100 ml
store at 4°C	

PBS/1.5 % FCS	
FCS	1.5 ml
1x PBS	ad 100 ml
store at 4°C	

100x Pen-Strep-solution	
penicillin G-Na	300 mg
streptomycin-SO_4	500 mg
0.9% NaCl	50 ml
sterile filter, store at -20°C, actually used aliquot at 4°C	

25% Percoll-solution	
SPS	4.75 ml
1x PBS	14.25 ml
prepare one day before perfusion, store at 4°C	

50% Percoll-solution	
SPS	22.5 ml
1x PBS	22.5 ml
prepare one day before perfusion, store at 4°C	

Perfusion buffer	
distilled water	217 ml
salt buffer	25 ml
Pen-Strep-solution	2.5 ml
H2-Mix	2.5 ml
heparin-3-solution	2.5 ml
pyruvate-solution	0.5 ml
adjust to pH=7.4, sterile filter, always prepare fresh	

Plating Medium	
culture medium	90 ml
FCS	10 ml
store at 4°C	

100x Pyruvate-solution	
Na-pyruvate	1.1 g
0.9% NaCl	20 ml
sterile filter, store at -20°C, actually used aliquot at 4°C	

RIPA-buffer	
1 M NaCl	50 ml
0.2 M TRIS pH=7.4	25 ml
10% SDS	1 ml
Igepal CA 630	1 ml
sodium deoxycholate	0.5 g
Complete, Mini, Protease Inhibitor Cocktail Tablets (Roche)	10 pcs
distilled water	ad 100 ml
store at -20°C	

RPMI-medium	
RPMI 1640	400 ml
gentamycin-solution	400 µl
store at 4°C	

R10-medium	
RPMI 1640	400 ml
FCS	40 ml
gentamycin-solution	444.44 µl
store at 4°C	

10x Salt Buffer	
NaCl	34 g
KCl	2 g
HEPES	5 g
glucose	5 g
4 M NaOH	1ml
distilled water	ad 500 ml
store at 4°C	

10% SDS	
SDS	10 g
distilled water	100 ml
store at room temperature	

SIP-solution	
Percoll	45 ml
HBSS-buffer	5 ml
always prepare fresh	

Stock Percoll solution (SPS)	
Percoll	31.5 ml
10x PBS	3.5 ml
always prepare fresh	

Streptavidin-Cy2-solution (2.4 µg / ml)	
Streptavidin-Cy2-stock	2.86 µl
distilled water	ad 1000 µl
always prepare fresh	

Streptavidin-Cy2-stock (1700 mg / ml)	
Streptavidin-Cy2	1.1 mg
glycerol	0.65 ml
distilled water	ad 1.3 ml
store at -20°C in the dark	

5x TBE	
TRIS	54 g
0.5 M EDTA pH= 8.0	20 ml
boric acid	27.5 g
distilled water	1000 ml
autoclave, store at room temperature	

TBS	
TRIS	6.06 g
NaCl	17.53 g
distilled water	1000 ml
adjust pH=7.6, store at 4°C	

2.5x transfer buffer	
TRIS	15 g
glycine	72 g
methanol	250 g
distilled water	ad 2000 ml
store at 4°C	

T3-solution	
triiodothyronine	1.35 mg
0.1 M HCl	100 µl
0.9% NaCl with 15 mg BSA / 10 ml	19.8 ml
store at -20°C	

Wash Medium	
MEM-medium	391.6 ml
Glutamax	4 ml
HEPES-solution	4 ml
gentamycin-solution	0.4 ml
store at 4°C	

Table 6: List of Solutions

4.2 Methods

4.2.1 Perfusion of rat livers

Perfusions were carried out by Marzieh Nejabat, M.D., except for the preparation of the required solutions.

Rats weighing 200-300 g were anesthetized with chloroform and disinfected with 70% EtOH. Heparin-2-solution (1 ml / kg body weight) was injected into the leg vein. Collagenase and perfusion buffer were heated to 37°C and pumped into a canula through a water-jacketed heating coil to ensure the correct temperature. The pump had to be started before the beginning of the perfusion to avoid the inflow of air bubbles into the liver. All instruments were disinfected with 70% EtOH and the stomach of the rat was opened with scissors. The intestines were pushed gently aside to expose the liver and a disinfected thread was loosely knotted around the portal vein to make it easier to fix the canula afterward. The liver portal vein was cut, and the canula was inserted into the portal vein and fixed with the thread. After cutting the vena cava inferior, the liver was perfused with the perfusion buffer at 14 ml / min until the entire amount of blood in it was washed out, which usually required 8-10 min. Then, the aspiration tube of the pump was put into the collagenase buffer and the liver was perfused for 5-10 min at 12- 13 ml / min, depending on the size of the liver, until it was swollen and soft. The ligaments were cut and the liver was ready for further use.

4.2.1.1 Rat collagen-solution

The preparation of the solution was carried out by Birgit Mir-Karner.

Rat tails were broken into 1-2 cm long pieces with two pliers and collagen threads were drawn. The remaining tissue pieces were cut off and the threads were dried for 24-48 h at room temperature on a filter paper. The dry threads were cut into 0.5 1 cm long pieces and 1.66 g were sterilized in a Petri dish under UV-light for two hours. The threads were stirred in 500 ml distilled water and 2 ml acetic acid for three days at 4°C. The solution was split into Falcon tubes and centrifuged for 60

min at 43000g and 4°C. The resulting solution was used as a coating for isolated primary cells.

4.2.1.2 Isolation of hepatocytes

The cell isolation process was carried out by Marzieh Nejabat and Teresa Riegler.

The day before perfusion, collagen-coated plates were prepared. Rat collagen solution was diluted 1:10 with sterile distilled water and a 100 µl / cm² area of the plate were applied. The collagen solution in the plates was dried overnight in the work hood.

The liver was put into a beaker with 20 ml cold wash medium, the liver capsule was cut, and cells were shaken out. The resulting suspension was filtered through a 100-µm mesh. This procedure was repeated until a 100 ml filtered cell suspension was obtained. Two Falcon tubes were filled with 50 ml of the suspension, and the following operations were carried out with both Falcon tubes. Cells were centrifuged for 5 min at 78 g and 4°C. The resulting pellet consisted of HC, and the supernatant contained the mesenchymal cells. For the HC isolation, the supernatant was removed and the pellet was resuspended in 20 ml cold wash medium and centrifuged for 5 min at 20 g and 4°C. The pellet was resuspended in 25 ml cold wash medium and 24 ml SIP-solution was added. After centrifugation for 10 min at 55 g and 4°C, the supernatant was discarded and cells were resuspended in 40 ml wash medium, followed by centrifugation for 2 min at 55 g and 4°C. The resulting pellet was resuspended in 25 ml wash medium and the suspension from one tube was transferred into the other. Cells were precipitated by centrifugation for 5 min at 20 g and 4°C and were resuspended in 20 ml plating medium. The cell density was determined using a Neubauer counting chamber, and dead cells were identified by staining with trypan blue. The amount of necessary cells was plated on collagen-coated dishes at a density of approximately 30000 cells / cm². After two hours, the HC were washed once with 37°C warm wash medium and cultivated at 37°C and 5% CO2 in warm culture medium.

4.2.2 Isolation of mesenchymal cells

The resulting supernatant from section 3.1.4 after the first centrifugation was transferred into two Falcon tubes and centrifuged for 10 min at 1200 g and 4°C. At this time, 20 ml of cold 50% Percoll-solution was carefully pipetted under 19 ml cold 25% Percoll-solution in Falcon tubes. The pellet consisting of mesenchymal cells was resuspended in 5 ml cold BSA-buffer per Falcon tube and slowly layered on top of the Percoll gradient. The Falcon tubes with the gradient and the cell suspension on top was centrifuged for 30 min at 1200 g and 4°C. The acceleration and deceleration for this centrifugation step were adjusted to the smallest possible value. After the centrifugation, two rings and a pellet were observed. The upper ring consisted mainly of dead cells and was removed, and the lower ring located between the 10 ml and 30 ml label was collected, each in a new F alcon tube. The tubes were filled with cold BSA-buffer up to 50 ml and centrifuged for 10 min at 1200 g and 4°C. At this time, 100 µl / cm² 1:10 diluted rat collagen was pipetted on the culture dishes. The two pellets were resuspended in 5 - 10 ml R10-medium and were pooled. The cell density was determined using a Neubauer counting chamber, and dead cells were identified by staining with trypan blue. This suspension was used to plate mesenchymal cells or for further purification of KC and EC. After removal of the collagen solution, the amount of necessary cells was plated on the collagen-coated dishes at a density of approximately 300000 cells / cm². After two hours, the mesenchymal cells were washed vigorously, once with 37°C warm 1x PBS, and cultivated at 37°C and 5% CO2 in warm RPMI-medium.

4.2.2.1 Selective adherence

The cell suspension obtained in section 3.1.5 was plated on uncoated dishes at a density of approximately 600,000 cells / cm². KC was affixed to uncoated polystyrene dishes, and, after 35 min, the EC-containing supernatant was aspirated, collected in a Falcon tube, and replaced with 37°C warm R10-medium. The suspension was centrifuged for 5 min at 220 g; meanwhile, 100 µl / cm² of 1:10 diluted rat collagen solution was pipetted on the culture dishes. The pellet consisting of EC was resuspended in endothelial cell medium, the cell density was determined using a Neubauer counting chamber, and dead cells were identified by staining with trypan blue. After removal of the collagen solution, the amount of necessary cells was plated

on the collagen-coated dishes at a density of approximately 300,000 cells / cm². After two hours in culture, both cell types were washed vigorously once with 37°C warm 1x PBS, and further cultivated at 37°C and 5% CO2 in warm RPMI-medium

4.2.3 Determination of cell fraction composition

4.2.3.1 Immunostaining and microscopy

Cells were cultured in 35 mm dishes for 5 h, then the medium was removed and cells were washed once with 1x PBS. Lillie's buffered formalin (150 µl / cm²) was added to the dishes and for 90 min the fixation was carried out at 4°C. KCs showed phagocytic activity. Accordingly, the addition of 250 million 1 µm beads per three million KCs two hours before the fixation served as a marker for the cell type. The surplus of the beads was washed away by washing five times with 1x PBS before the addition of formalin. The formalin was removed and the dishes were washed three times with distilled water. Covered with distilled water, the dishes could be stored for months at 4°C, when the water was changed regularly.

The water covering the fixed cells in the dishes was removed by aspiration and 2 ml TBS per 35 mm dish were added. After 10 min, the TBS was replaced by 2 ml BSA/TBS and applied for at least 30 min. Meanwhile, the primary antibody was diluted in BSA/TBS to the desired concentration, and, after aspiration of the TBS/BSA, 1 ml was pipetted in every dish. The antibody was allowed to bind over night at 4°C while shaking. After washing six times with TBS, 2 ml BSA/TBS were applied for at least 30 min. At this time, the secondary antibody was diluted in BSA/TBS to the desired concentration, and, after aspiration of the TBS/BSA, 1 ml of the solution was added to every dish. After 1 h, the solution was removed and the dishes were washed six times with TBS. For biotinylated secondary antibodies, 1 ml Streptavidin-Cy2-solution was applied for 1 h hour followed by washing six times with distilled water. AP-conjugated antibodies were covered by 2 ml AP-1-solution for 10 minutes. Then, an AP-staining-solution was applied for 5-7 min, depending on the microscopically observed staining process. The reaction was stopped by the application of 2 ml AP-stop solution for 10 min and the dishes were layered with distilled water for 5 min. Independently of the treatment procedure after the application of the secondary antibody, 1 ml Hoechst-solution was applied per dish for 5 min, followed by 5 min with distilled water. The

water was removed by aspiration and the dishes were dried at room temperature. The bottom of the dish was punched out and mounted in Kaiser's glycerol gelatine with a round glass cover-slide. Omitting the first antibody served as a control.

Stained cells were observed in a Zeiss LSM 710 with various contrast methods, including brightfield, phase contrast, and differential interference contrast, depending on the optimal visualization of the desired details. The nuclear staining and Cy-2-coupled antibodies were visualized by fluorescence microscopy. Hoechst emitted blue wavelengths, and Cy-2 showed green light emission. For quantification, at least 1000 Hoechst-positive cells per dish were counted.

4.2.4 PCR

The PCR was done by Marzieh Nejabat and Teresa Riegler.

4.2.4.1 RNA isolation

START HERE. The RNA isolation was carried out in the hood. Hepatocytes, mesenchymal cells, or endothelial and Kupffer cells were cultured for 24 h in six-well plates. The medium was removed by aspiration and 600 µl for HC or 300 µl for the other cell fractions of peq GOLD TriFast (peqLab) was portioned onto three wells. Cells were vigorously scratched off the well bottom with a Small Cell Scraper (Corning). The resulting solution from three wells was collected in a 1.5 ml micro tube. One-fifth of the amount of used volume TriFast chloroform was added, and the tube was turned upside down 15 times and incubated for 10 min at room temperature. After centrifugation at 12000 g for 10 min at 4°C above the red organic phase, a clear aqueous phase could be seen. This phase was transferred into a new micro tube and 1/2 of the amount of the used volume of TriFast 2-propanol was added and mixed well by pipetting. The RNA was precipitated overnight at -20°C. After centrifugation at 12000 g for 10 min at 4°C, the supernatant was poured away and the amount of the used volume of TriFast ethanol was added and gently shaken. After centrifugation at 14000 g for 8 min at 4°C, the alcohol was completely removed with a pipette. The remaining traces of ethanol were allowed to evaporate for approximately 10 min. The nearly dried pellet was solved in 50 µl DEPC-treated water in the case of HC, and the pellet from all other cell fractions was dissolved in

30 µl DEPC-treated water. The RNA concentration was measured using a NanoDrop ND 1000 photometer with DEPC-treated water as a blank.

4.2.4.2 Reverse transcription of RNA

Sample preparation was carried out on ice. The following master mix was prepared:

Mastermix for reverse transcription

	Per sample
5x buffer for M-MuLV RT (Fermentas)	5 µl
dNTP mix (Fermentas)	1.56 µl
RNAse inhibitor (Fermentas)	0.625 µl
DEPC-treated water	1.19 µl
M-MuLV reverse transcriptase (Fermentas)	1 µl
always prepare fresh, add RT just before using	

Table 7 Mastermix for reverse transcription

An RNA solution containing 2 µg of RNA was filled with DEPC-treated water to 15 µl in a 0.2 ml PCR tube. A random hexamer primer (Fermentas, at 0.625 µl) was added and the samples were incubated 70°C for 2 min and put back on ice immediately, at which point 9.375 µl of the Mastermix were added. After vortexing and spinning down of the samples, the tubes were incubated in the thermocycler according to the following program:

42°C	1 h
94°C	5 min
4°C	forever

Table 8: Thermocycler program for reverse transcription

The cDNA samples were filled with 75 µl DEPC-treated water, vortexed, spun down, and stored at -20°C.

Sample preparation was carried out on ice. The following Mastermix was prepared:

Mastermix for PCR	per sample
Primer forward 10 µM	1 µl
Primer reverse 10 µM	1 µl
DEPC-treated water	9.5 µl
GoTaq Green Master Mix (Promega)	12.5 µl
always prepare fresh	

Table 9 Mastermix for PCR

The following primer pairs (Eurogentec) were used:

primer	sequence	size	cycles
rCD32b_for	5' ATG TGC TCT CAC GGA CTT TG 3'	235 bp	34
rCD32b_rev	5' TAG TTG GCT TGG GCT TGA TG 3'		
rCD68_for	5' TAC GGA CAG CTT ACC TTT GG 3'	435 bp	34
rCD68_rev	5' AGA GTG GAC TGG AGC AAA TG 3'		
rDesmin_for	5' GCA CCA ACG ACT CCT TGA TG 3'	289 bp	38
rDesmin_rev	5' CTT TGC TCA GGG CTG GTT TC 3'		
rNTPDase2_for	5' TGC TAC TTT GCG TCC CTA CC 3'	181 bp	38
rNTPDase2_rev	5' GAG ATG CCA CCA CCT TGA AC 3'		
rOX62_for	5' GGT TAT GGT GGT GCT TAC TG 3'	249 bp	40
rOX62_rev	5' TGG ATG ATC CTC TGC TGT AG 3'		
rLYVE-1_for	5' GTC CAA GTG CAA GAC CTT TC 3'	265 bp	36
rLYVE-1_rev	5' GGA CAC CTT TGC CAT TCT TC 3'		
rNKR-P1A_for	5' GCC GAG TGC TTA TTC AAG AG 3'	455 bp	40
rNKR-P1A_rev	5' TCG CAG TCA GGA GTC ATT AC 3'		
rCK19_for	5' AGA CCT GCG TCC CTT ATC CC 3'	477 bp	40
rCK19_rev	5' GGA TCT TGT CGC GCA AGT CC 3'		
rCD45_for	5' TCT TCA GTG GAC CCA TTG TG 3'	147 bp	40
rCD45_rev	5' ATC TCT GTC GCC TTA GGT TG 3'		
rβActin_for	5' ATG TTG CCC TAG ACT TCG AG 3'	175 bp	40
rβActin_rev	5' TCA TGG ATG CCA CAG GAT TC 3'		

Table 10 : Primer pairs for PCR

NTPDase2 is a marker for portal fibroblasts (88), OX62 is specific for dendritic cells (89), LYVE-1 is expressed in lymphatic endothelial cells (90), NKR-P1A is produced

in pit cells (91), CK19 is characteristic for cholangiocytes (92), and CD45 is a general leukocyte marker (93).

A 1-µl cDNA sample or DEPC-treated water as a negative control was pipetted into 0.2 ml PCR tubes and 24 µl of the Mastermix was added. After vortexing and spinning down, the tubes were incubated in the thermocycler according to the following program:

95°C	5 min	x1
94°C	30 sec	repeat 40 times
60°C	30 sec	
72°C	30 sec	
72°C	7 min	x1
4°C	forever	x1

Table 11:Thermocycler program for PCR

The optimal number of cycles was determined for each primer pair with regard to the optimal band intensity for detection and quantification.

If fewer cycles were necessary for samples, they were removed in the 72°C phase and placed on the other thermocycler module, which was preheated to 72°C. After 7 min incubation, the samples were then put on ice. The samples were loaded directly onto the gel or stored at -20°C until subsequent analysis.

To prepare a 1.5% agarose gel, 1.2 g of agarose were heated in 80 ml 1xTBE in the microwave until the solution was completely clear. The gel was poured into the gel tray, and 4 µl of Serva DNA stain Clear G were added. By tilting in both dimensions for a minute, a homogeneous distribution was achieved. The comb was inserted and the gel was allowed to cool for at least 30 min. The gel tray was transferred into the BioRad SubCell GT cell chamber filled with 1x TBE. The comb was removed and 10 µl of the samples were loaded onto the gel, with 5 µl of 50 bp DNA Ladder-solution used as marker. At 125 V, this required about 45 min until the yellow dye

front had nearly reached the end of the gel.

4.2.4.3 Detection and data analysis

The gels were photographed under UV light using a BioRad Gel Doc XR+ System and corresponding Quantity One software. The grey levels of the gel photos were adjusted with a commercial graphics program to eliminate the background signal. The corrected images were opened with Image J software (version 1.46, NIH), the relevant lanes were selected, and the signal intensity along the lanes was plotted. The signal of the amplified fragments was quantified via the area under the corresponding peak

4.3 Analysis of the transcriptome of NGC-treated rats

4.3.1 Long-term treatment with phenobarbital

In brief, male Wistar rats were treated with 250 mg N-nitrosomorpholine / kg body weight at the age of three weeks. After three weeks recovery time, the rats received a daily dose of 50 mg PB / kg per day admixed to the diet. The rats were sacrificed by guillotine under CO2 asphyxiation after 17 months of treatment, and liver and tumor samples were obtained. The animal treatment was carried out by former lab members.

4.3.2 Treatment with cyproterone acetate

In brief, female Wistar rats were treated with either one day or six consecutive days of CPA via gastric gavage at 10 mg/10 ml corn oil/1000 mg body weight.

4.3.2.1 Histological analysis

Aliquots of the samples were fixed and paraffin-embedded. Sections of the lesions were characterised by H/E and GSTp staining. The histological analysis was carried out by former lab members.

4.3.2.2 RNA isolation from snap-frozen aliquots

Snap frozen samples were cryosectioned under RNAse-free conditions. Thirty mg of normal tissue and 15 mg of tumour tissue were used for RNA extraction with an miRNeasy Mini Kit (Quiagen) after homogenization with Precellys Ceramic beads

(peqLab) for 15 sec at 6000 rpm in a homogeniser. The quality of the RNA was determined by analysis with a Bioanalyzer 2100 (Agilent). The isolation was carried out by Teresa Riegler.

4.3.2.3 Array

Extracted RNA was processed, labelled for application, and applied on a rat genome 230 2.0 Array (Affymetrix) by members of the core facility genomics of the Medical University of Vienna.

4.3.2.4 Data analysis

The resulting array data were analysed using a literature research. Bioinformatic analysis (Gene Set Enrichment Analysis (GSEA) in KEGG and GO database) was carried out in the program InCroMAP (Integrated analysis of Cross-platform MicroArray and Pathway data).

Bioinformatic quality control of array data was carried out by Johannes Eichner at the University of Tübingen using arrayQualityMetrics 3.16.0 software.

Data analysis using the program InCroMAP

Files containing gene names and log2 fold expression, normalized by surrounding tissue, were loaded into the program. By selecting a desired level of minimal fold deregulation, a gene pool from the data set was created. For all arrays, ≥ |1.0| log2 was selected, because only >2 fold deregulated genes should be considered. The program calculated p-values for pre-defined gene sets (KEGG pathways, GO terms) for the gene pool. Results are presented in a new table. The number of genes from the gene pool found in the gene set was determined. This result was denoted together with the gene pool size in the “List ratio” column. The “BG ratio” column contains the total number of genes in the gene set and the total number of genes in all gene sets. Using a hypergeometric test, the p-value was calculated from these two values. In the last column, genes from the gene pool that were found in the specific gene set were denoted. Every row corresponded to one gene set.

KEGG gene sets could be visualized, and the resulting picture was based on the KEGG pathway picture. Therefore, circular nodes are small molecules and rectangular-shaped nodes correspond to genes or gene families with several members. Upregulated genes were coloured in red, and downregulation was

indicated by the blue colour. A darker colour indicates stronger deregulation. The log2 value that corresponded to the darkest colour could be defined. Genes that were not found in the gene pool were coloured in grey.

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6 Appendix

Curriculum Vitae

Personal Information

Name: Marzieh Nejabat, MD

Date & place of Birth: 9th Sep 1978, England (UK)-Durham

Nationality: British/Iranian citizenship

Address: Austria: Margaretenstrasse 154/1A, 1050 Vienna

Language: Persian (mother language), English (advanced level), German (advanced level), Arabic

E-Mail: marzieh.nejabat@meduniwien.ac.at,
marzieh.nejabat@gmail.com

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+989124769228

Educational Background

- March 2014-April 2020: Resident in radiology and nuclear medicine at Medical University of Vienna
- May 2013: Registered as a medical doctor with the Medical association of Austria after completing evaluation; medical diploma.
- Jan.2011-now PhD student in "Malignant disease" (oncology), Division of Liver Cancer, Internal Medicine department II, Medical University of Vienna
  Thesis title: Development of Mechanism-Based Markers to Predict Non-Genotoxic Hepatocarcinogens in Early Drug Development
- Jan.2011- 2017: Mastership" in Toxicology" at Medical University of Vienna
- 1998-2006: Diploma study of medicine, School of Medicine, Fatemieh University of Medical Science (medical graduate thesis project: Effect of subcutaneous irrigation in Caesarean sections on post-operative wound infection)
- 1993-1997: High school diploma obtained from Shahed High School, in Qom, Iran

Research experience:

- Project manager and researcher at Department of Radiology and Molecular Imaging at the Medical University of Vienna since 2014-2020
- Assistant researcher and project manager in Gastroenterology and Hepatology research Centre (GEHRC) at Shiraz University of Medical Science in Iran, 2003-2005
- Project manager in ”prevalence of hepatitis B & C in Fars Province, Iran.”
- Project manager in “phenotypes of hepatitis B & C in Iran.”
- Project manager in ”correlation of celiac disease and osteoporosis.”
- Project manager in ”prevalence of upper & lower gastroenterology symptoms in an apparently healthy population of Fars province.”

Clinical work experience

- General Physician (GP) in Boarding Clinic of Zarghan (one village near Shiraz City) 2006-2008
- General Physician in boarding clinic in Qom city between 2007 -2008
- General Physician of dormitory in Naragh Azad University 2009-2010
- Part-time lecturer at Azad University of Iran from 2009-2010

Laboratory experience:

- **Molecular and cell biological:** ELISA, Western blot, PCR (reverse PCR & real-time PCR), RNA isolation, cell culturing with different cell lines (cancer cell lines, blood cell lines, etc.), primary cell culture, viability assays (neutral Red). TACE activity assay, immunohistochemical staining with formalin/Canoy –fixed tissues.
- **Animal lab**: Managing projects with rodents (rat & mouse), such as liver perfusion, intravenous/dermal/Intraperitoneal injection, capable of performing entire process of purification of liver cells of rats and mice into hepatocytes, Kuppfer cells, and endothelial cells.

Computer skills:

- MS Office,
- Graph Pad Prism software
- LUCIA (image analysis software)
- Rew. manager

Honours

- Certificate for 'excellent research of the year' for research output in 2018 at the Clinical Department of Radiology and Nuclear Medicine
- Prize for best short-communication (oral presentation) at the Joint Scientific Symposium of the Austrian Societies of Toxicology (ASTOX), Pharmacy (ÖPhG), Analytic Chemistry (ASAC), and Forensic Medicine (ÖGGM), and the Comprehensive Cancer Centre Vienna (CCC), Vienna, March 21st/22nd, 2013
- Prize for young researcher and presenter at the Seoul International Digestive Disease Symposium, Seoul, Korea, November 2008

Scholarships

- Performance scholarship of the Medical University of Vienna (June 2011 –now)

Membership in Scientific Societies

- Member of the European Society of Hybrid Imaging since 2017
- Member of the Iranian Society of Internal Medicine
- Member of the European Society of Medicine

Publications

1. **Marzieh Nejabat**, Asha Leisser, et al. [11C] acetate PET as a tool for diagnosis of liver steatosis. **J. Abdom Radiol 2018**
2. **Nejabat** M, Riegler T,et al. Mesenchyme-derived factors enhance preneoplastic growth by non-genotoxic carcinogens in rat liver. **Arch. Toxicology** 2018 Feb. 92(2): 953-966
3. Leisser A, **Nejabat** M, et al. Haematopoiesis is prognostic for toxicity and survival of ^{223}Radium treatment in patients with metastatic castration-resistant prostate cancer. **Hell J Nucl Med** . 2017 Sep-Dec;20 suppl:157
4. Leisser A, **Nejabat M**, et al **.** Analysis of haematological parameters as prognostic markers for toxicity and survival Ra ^{223} treatment. **Oncotarget** 2018 Mar 5;9(22)16197-16204.
5. Riegler T, **Nejabat M**, et al. Proinflammatory mesenchymal effects of the non-genotoxic hepatocarcinogen phenobarbital: a novel mechanism of antiapoptosis and tumor promotion. Carcinogenesis. 2015 Dec;36(12):1521-30
6. Parzefall W, Freiler C, Lorenz O, Koudelka H, Riegler T, **Nejabat** M, Kainzbauer E, Grasl-Kraupp B, Schulte-Hermann R. Superoxide deficiency attenuates promotion of

hepatocarcinogenesis by cytotoxicity in NADPH oxidase knockout mice . **Arch Toxicol**. 2014 Sep 3.

7. Food-derived peroxidized fatty acids may trigger hepatic inflammation: a novel hypothesis to explain steatohepatitis. Therese Böhm, Heidi Berger, **Marzieh Nejabat**, Teresa Riegler, Mario Kuttke, Sandra Sagmeister, Monika Bazanella, Klaus Stolze, Anahita Daryabeigi, Nora Bintner, Michael Murkovic, Karl-Heinz Wagner, Rolf Schulte-Hermann, Nataliya Rohr-Udilova, Wolfgang Huber, Bettina Grasl-Kraupp. **Journal of Hepatology available online 9 May** . 2013
8. Khademolhosseini F, Mehrabani D, **Nejabat M**, Beheshti M, Heydari ST, Mirahmadizadeh A, Salehi M, Zare N, Saberi-Firoozi M Irritable bowel syndrome in adults over 35 years in Shiraz, southern Iran: prevalence and associated factors. **J Res Med Sci** . 2011 Feb;16(2):200-6.
9. Saberi-Firouzi M, Omrani GR, **Nejabat M**, Mehrabani D, Khademolhosseini F. Prevalence of celiac disease in Shiraz, southern Iran. **Saudi J Gastroenterol** . 2008 Jul; 14(3):135-8.
10. SaberiFiroozi M , **Nejabat M** .Helicobacter Pylori Treatment regimens in Iran (Review Article) M, **IJMS (Iranian Journal of Medical Science)** 2007Sep;54:43-48

Presentations

1. Haug A, Leisser A, **Nejabat M**, et al. Using pre-existing hematotoxicity as predictor for severe side effects and number of treatment cycles of Xofigo therapy
2. **M. Nejabat**, A. Leisser, G. Karanikas, M. Mayerhöfer, W. Wadsak, M. MitterhauserM. Hacker, A. Haug.[11C]acetat PET for diagnosis of non-alcoholic fatty liver disease. SNNM 2015 USA BaltimoreJune 2015 (oral presentation)
3. **M. Nejabat**, A. Leisser, G. Karanikas, M. Mayerhöfer, M. Hacker, A. Haug. Liver [11C]acetat uptake is increased in patients with non-alcoholic fatty liver disease. DGN 2015 Hannover (Poster)
4. Therese Böhm, Teresa Riegler, Heidi Berger, **MarziehNejabat**, Florian Kellner, Mario Kuttke, Sandra Sagmeister, Klaus Stolze, Rolf Schulte-Hermann, Nataliya Rohr-Udilova, Wolfgang Huber, Bettina Grasl-Kraupp. Peroxidierte Fettsäuren in Der Nahrung: Beteiligt an der Bildung einer Fettleberhepatitis? GOECH/ASTOX Seminar 23-25. April 2014 Vienna (Poster)
5. **Nejabat M**., Riegler, T., Schulte-Hermann R., Huber W. W. and Grasl-Kraupp B. Development of mechanism-based markers to predict non-genotoxic hepatocarcinogens in early drug development. Joint Scientific Symposium of the Austrian Societies of Toxicology (ASTOX), Pharmacy (ÖPhG), Analytic Chemistry (ASAC), and Forensic Medicine (ÖGGM), and the Comprehensive Cancer Centre Vienna (CCC) ,Vienna, March 21st/22nd, 2013 (Oral Presentation)

6. Riegler T., **Nejabat M**., Kellner F., Schulte-Hermann R., Huber W. W. and Grasl-Kraupp B.The mesenchymal – parenchymal interactions are critical for tumour promotion by phenobarbital. Joint Scientific Symposium of the Austrian Societies of Toxicology (ASTOX), Pharmacy (ÖPhG), Analytic Chemistry (ASAC), and Forensic Medicine (ÖGGM), and the Comprehensive Cancer Centre Vienna (CCC) ,Vienna, March 21st/22nd, 2013 (Poster)
7. Kellner F., Riegler T., **Nejabat M.,** Schulte-Hermann R., Huber W. W. and Grasl-Kraupp B.Adaptive Responses of PreneoplasticHepatoctyes: Essential for Tumour Promotion by Non-Genotoxic Hepatocarcinogens?Joint Scientific Symposium of the Austrian Societies of Toxicology (ASTOX), Pharmacy (ÖPhG), Analytic Chemistry (ASAC), and Forensic Medicine (ÖGGM), and the Comprehensive Cancer Centre Vienna (CCC) ,Vienna, March 21st/22nd, 2013 (Poster)
8. M. Saberi-Firoozi, **M. Nejabat**. Experiences with Helicobacter pylori treatment in Iran.Seoul International Digestive Disease Symposium 2008, Seoul, Korea, November 20-22. (Presentation)
9. M. Saberi-Firoozi, **M. Nejabat**, Gh. Omrani, D. Mehrabani, F. Khademolhosseini. "Prevalence of celiac disease in Shiraz, southern Iran". Seoul International Digestive Disease Symposium 2008, Seoul, Korea, November 20-22(Poster)
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11. Mehdi Saberi-Firoozi;MD, **MarziehNejabat;MD**, Maryam Ardebili;MD, Alirezaserati, Leila Kazemi, Mahmood Beheshti, S TaghiHeydari, Saeed Amirizadeh-Maryam Nejabat. Gastroenterohepatology Research Centre, Shiraz University of Medical Sciences, Shiraz I.R.Iran"Comparison of HBV infection in school children with and without involvement inmass HBV vaccination program in Shiraz city and rural areas." 6th Iranian InternationalCongress of Gastroenterology & Hepatology, Dec 5-7, 2006, Tehran, Iran.(Poster)
12. Mehdi Saberi-Firoozi,MD; Maryam Ardebili,MD; Mahmood Beheshti,MD;AlirezaSerati,MD;SaeedAmirizadeh;**MarziehNejabat MD;** Maryam Nejabat" The results ofC13 UBT for Helicobacter Pylori (HP) eradication in different regimens used in dailyclinical practice; Shiraz center experiences ": 6 th Iranian International Congress of Gastroenterology & Hepatology, Dec 5-7,2006, Tehran, Iran(Poster)
13. Mehdi- Saberi-Firoozi, Mahmood – Beheshti, **Marzieh – Nejabat** , Ali Reza Mirahmadi."Reflux Symptoms as the most common GI symptoms in our community". Journal ofGastroenterology and Hepatology 2005 20. 5 th Iranian International Congress ofGastroenterology & Hepatology, Dec 5-7, 2005, Tehran, Iran.(Poster)
14. Saberi-Firoozi ,M; Mosallaei, M ; **Nejabat, M** ; Beheshti, M; Ghaedian , T; Saki,N;Monavrian." Epidemiological survey of inflammatory bowel disease (IBD) and colorectaltumours (CRT) according to the colonoscopy findings between 1994& 2004 in the FarsProvince." 5 th Iranian InternationalCongress of Gastroenterology & Hepatology,

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