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The role of hypoxia and NOTCH signalling on the expression of liver progenitor cell characteristics in primary liver cancer

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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

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LIST OF ABBREVIATIONS

AFP	alfa fetoprotein
ALB	albumin
CDE	choline deficient - ethionine supplemented
CHC	combined hepatocellular- cholangiocarcinoma (also HCC- CC)
CK19	cytokeratin 19 (also KRT19)
Cre	causes recombination / cyclization recombinase
CSC	cancer stem cell
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DEN	diethylnitrosamine/ N-nitrosodiethylamine
DMOG	dimethyloxaloylglycine
EMT	epithelial mesenchymal transition
EPCAM	epithelial cell adhesion molecule
ER	estrogen receptor
FACS	fluorescent activated cell sorting
GFP	green fluorescent protein
GLUT1	glucose transporter 1 (*SLC2A1*)
GSI	gamma secretase inhibitor
HCC	hepatocellular carcinoma
HCC-CC	hepato-cholangiocarcinoma (also CHC)
HES1	hairy and enhancer of split 1
HEY	hes related with YRPW motif
HIF	hypoxia inducible factor
HIFα	hypoxia inducible factor 1 or 2 alpha subunit
HNF1b	hepatic nuclear factor 1 beta
HSC	hepatic stellate cell
iCC	intrahepatic cholangiocarcinoma
iCre	inducible Cre recombinase
JAG1	jagged1
LoxP	Locus of X-over P1
LPC	liver progenitor cell
KRT19	cytokeratin 19 (also CK19)
MACS	magnetic activated cell sorting
MDR1	multi drug resistance protein 1
NICD	Notch intracellular domain
OPN	osteopontin

OpnCre	tamoxifen inducible osteopontin promoted CreERT2
PFK	phosphofructokinase
PHD	prolyl hydroxylase domain
PHD2+/-	prolyl hydroxylase domain 2 haplodeficient
PROM1	prominin1
RosaNicd	Rosa26-LoxP-STOP-LoxP-NICD1- ERAD-GFP
TACE	Trans-arterial chemo-embolization
VEGFA	Vascular endothelial growth factor alpha
WT	wild type
YFP	yellow fluorescent protein

Chapter 1

Introduction

1. LIVER CANCER

1.1. Introduction

With an average 5 year survival of 17,5%, primary liver cancer poses a major health issue(1, 2). Primary liver cancers can be categorised as hepatoblastoma (cancer of fetal liver cells or hepatoblasts), angiosarcoma (cancer of the inner lining of blood vessels or endothelium), intrahepatic cholangiocarcinoma (iCC, tumour of bile duct epithelial cells or cholangiocytes) and hepatocellular carcinoma (HCC, tumour of liver epithelial cells or hepatocytes). The latter two, being the most common in adults, also occur together as the mixed hepato-cholangiocarcinoma (HCC-CC), which could be liver progenitor cell (LPC) derived. HCC, which accounts for 80% of all primary liver cancers, is the fifth most common -and the second most deadly cancer worldwide (1-3).

Cancer generally develops in different stages. In short, during tumour initiation, one or more cells gather enough pro-oncogenic mutations to initiate neoplastic transformation, which allows them to escape normal tissue homeostasis and increase their proliferation capacity (4). During tumour progression, the transformed cell(s) proliferate and form small neoplastic nodules.

The increased proliferation rate allows more mutations to build up, causing tumour heterogeneity. Continuous exposure to different stressors like oxygen and nutrient shortage during further growth leads to a selection process where only the best adapted and most resilient tumour cells survive (4).

Eventually, the tumour can become malignant and cells invade the connective tissue and penetrate blood and lymphatic vessels, which leads to local and distant metastasis (Figure1) (4).

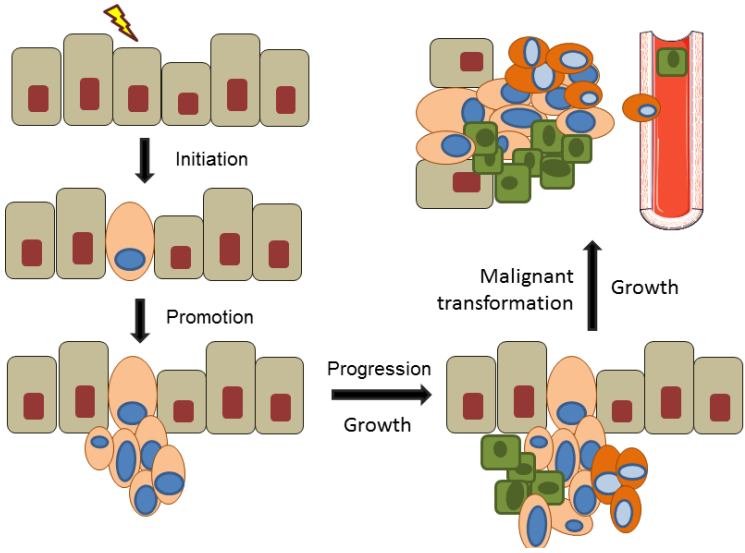

Figure1.Stages of cancer development.

After tumour initiation, the initiated cell starts dividing so a neoplastic nodule is formed. While the tumour grows, cells in the nodule progress by acquiring more mutations, causing tumour heterogeneity, indicated by different shapes and colours in the figure, increasing malignancy. Eventually cells invade the extracellular matrix and can metastasise through blood and lymphatic vessels.

Over 80% of primary liver tumours develop in a background of chronic liver disease, where continuous injury leads to (1) DNA damage, resulting in an accumulation of pro- oncogenic mutations and (2) continuous tissue damage and repair which is associated with excessive proliferation (3, 5). Furthermore, continuous activation of inflammatory and repair pathways help create ideal conditions for tumour growth (3, 6, 7).

1.2. Clinical aspects of hepatocellular carcinoma

The major cause of HCC in Asia and sub- Saharan Africa is hepatitis B virus infection, where carriers may develop HCC with or without development of cirrhosis. AflatoxinB1 exposure and alcohol abuse are also major risk factors. In Western countries, hepatitis C virus-induced cirrhosis is the most common risk factor. Due to the obesity epidemic, non- alcoholic fatty liver disease and non-alcoholic steatohepatitis-induced HCC incidences are on the rise (7, 8).

Ultrasound abdominal scanning is used to screen patients with liver disease, when a suspicious nodule is found this is then further characterised using computed tomography or magnetic resonance imaging. Diagnosis can then be reinforced and/or specified by performing biopsies and blood tests for alpha-fetoprotein (AFP). While AFP measurements are inexpensive, simple and a very helpful tool to measure therapy response or potential recurrence, increased AFP levels are detected in only 40-65% of HCC patients, decreasing its usefulness to detect small/new HCC lesions(8).

Unlike other types of cancers, staging of HCC is not performed through the classical TNM (tumour, lymph Nodes and Metastases) system. Since the underlying liver disease is a major factor in patient survival and success or failure of the treatment, the Child Pugh score, which categorises liver function (Table1), is integrated in the evaluation of the tumour stage (3). Several scoring systems have been proposed, all integrating both liver and tumour characteristics; the Barcelona Clinic Liver Cancer (Figure 2) is most commonly used to determine treatment (7).

Table 1: Child Pugh Score (adjusted from(3))

	Points		
	1	2	3
Encephalopathy	None	Grade 1-2 (Or precipitant-induced)	Grade 3- 4 (Or chronic)
Ascites	None	Mild to moderate (diuretic responsive)	Severe (diuretica refractory)
Bilirubin (mg/dl)	<2	2 - 3	>3
Albumin (g/dl)	>3,5	2,8 – 3,5	<2,8
INR	<1,7	1,7 – 2,3	>2,3
Child Pugh class obtained by adding scores for each parameter			
Class A	5 – 6 points	Least severe liver disease	
Class B	7 – 9 points	Moderately severe liver disease	
Class C	10 – 15 points	Most severe liver disease	

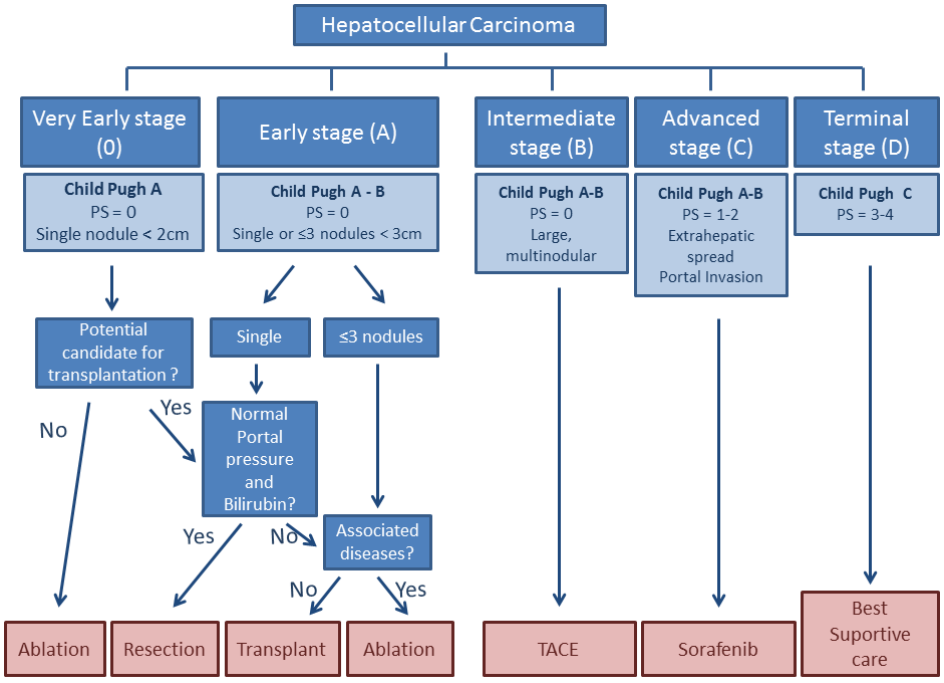

Figure 2: **the Barcelona clinic liver cancer treatment plan.**

Scheme, showing different treatment options depending characteristics of the hepatocellular carcinoma, as well as the severity of the underlying disease (7)

Potentially curative treatment options

For small lesions with minimal underlying liver disease (Child Pugh A), ablation (destruction of tumour cells without physical removal by surgery) or resection (surgically removing nodules) are first-in-line treatment strategies. Five-year survival rates are however still estimated between 40% and 80% due to high recurrence rates (3, 7). For tumours that are within the Milan criteria, defined as a single tumour of ≤5 cm in size or up to three nodules of ≤3 cm in size without vascular invasion and are not eligible for resection, liver transplantation is the best option. Since transplantation cures both hepatocellular carcinoma and the underlying liver disease, the Child-Pugh score is not an issue (3, 7, 9).

Non- curative treatment options

Non-curative treatment options are mostly based on depriving the tumour from its oxygen and nutrient supply, decreasing tumour growth and increasing patient survival time.

This can be applied locally or systemically. Most of the normal liver's blood supply comes from the portal vein; in contrast, hepatic tumours receive over 80% of their blood supply from outgrowths of arterial branches. This provides a unique tool, in which chemotherapeutics or radio-active particles can be administered arterially and/or embolization of the feeding artery can be performed to cause maximal damage (3, 7, 9). The combination of the two strategies is called transarterial chemo–embolization (TACE) or trans-arterial radio-embolization respectively.

The main purpose is to increase the patient's life expectancy but these treatments are also used to suppress tumour growth in patients within the Milan criteria waiting for transplantation and can, in some cases, decrease tumour volume to fit the Milan criteria (3, 7, 9). Even though the non- tumorous, 'healthy' liver is mostly spared, some damage still occurs and the remaining liver function has to be sufficient, excluding Child-Pugh C patients.

Over the years, many systemic treatment options have been tested, with very little success due to high cytotoxicity and small effects on tumour size and characteristics. Up until now, the only approved systemic treatment is the multi-kinase inhibitor Sorafenib (Nexavar©) (3, 7, 9-11). The effects of Sorafenib are not specific, but the most important mode of action related to HCC therapy is blocking the vascular endothelial growth factor and platelet derived growth factor beta receptors on endothelial cells. This results in inhibition of the formation of new blood vessels (angiogenesis) towards the growing tumour, denying tumour cells adequate oxygen and nutrient supply and thus decreasing tumour growth.

Indeed, Sorafenib treatment was shown inefficient in reducing tumour volume, but was capable of reducing the density of tumour vasculature, attributing to the increased survival time (11).

SHARP (sorafenib HCC assessment randomised protocol trial) investigators found a mean overall survival of 10,7 months in the sorafenib treated group compared to 7,9 months in the control population (11) and an Asian- Pacific study observed a mean overall survival of 6,5 compared to 4,2 months (10). Unfortunately, beside this very mildly increased survival time, sorafenib treatment includes significant toxicities (10, 11), and treatment has been shown to induce therapy resistance and increased local invasion and distant metastatic capacities (12, 13).

1.3. Clinical aspects of iCC and mixed phenotype tumours.

iCC is staged using an adjusted TNM staging system (Table 2) and, depending on the stage of the tumour upon diagnosis, average 5 year survival of iCC is 2 – 15%. The only curative treatment is full resection of the tumour, which, unfortunately, is only an option for very few patients and is still accompanied by a five year survival of only 8 – 47 % (5, 6). So far, no benefits have been confirmed for using adjuvant radio- and/or chemotherapy. Furthermore, due to high recurrence rates (up to 90% within 2 years), liver transplantation for cholangiocarcinoma is contraindicated. Palliative treatment is the only option for the majority of patients, with a mean overall survival of 3 to 6 months depending on the possibility of biliary drainage (5, 6, 14).

HCCs with a cholangiocytic phenotype and HCC-CC tumours, clinically present as HCC tumours. However, both tumour phenotypes have to be taken into account for treatment, decreasing treatment options and survival rates compared to single phenotype tumours. Mixed phenotype tumours can occur in different “subtypes” depending on the interaction between the HCC and iCC compartment.

Firstly, HCC and iCC can present at different locations in the same liver. Secondly, tumours can present next to each other, intermingling only at the site of overlap. Lastly, one tumour can carry histological features of both HCC and iCC.

It is unclear which of these tumours originate from hepatocytes/cholangiocytes and alter their phenotype throughout disease progression to adapt to environmental stresses, and which HCC-CC tumours arise from a common liver progenitor cell (LPC), able to differentiate towards both a hepatocytic and a cholangiocytic phenotype (15-17).

Table 2: TNM and AJCC/UICC staging systems for intrahepatic CCA (5)

TNM stage	**Criteria**		
T0	No evidence of primary tumour		
Tis	Carcinoma in situ		
T1	Solitary tumour without vascular invasion		
T2a	Solitary tumour with vascular invasion		
T2b	Multiple tumours with or without vascular invasion		
T3	Tumour perforating the visceral peritoneum or Tumour involving local extrahepatic structures by direct invasion		
T4	Tumour with periductal invasion		
N0	No regional lymph node involvement		
N1	Regional lymph node metastases		
M0	No distant metastases		
M1	Distant metastases		
Stage	**Tumour**	**Node**	**Metastasis**
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
IVa	T4	N0	M0
	Any T	N1	M0
IVb	Any T	Any N	M1

Currently, research is focusing on predicting prognosis and therapy response through identification of specific characteristics, like stem/progenitor cell markers, or activation specific pathways, like hypoxia or Notch signalling, which could be used as markers to predict prognosis and response to therapy(15, 18-21).

Therapeutic options for advanced liver cancer are mostly based on depriving the tumour of its nutrient and oxygen supply, which can lead to activation of the hypoxic adaptive response and result in increased expression of liver progenitor cells, both linked to therapy resistance and poor prognosis (13, 22-28). Further research, investigating the effect of hypoxic conditions on disease progression and the expression ofliver progenitor cell characteristics in HCC is necessary to unravel the interplay between these tumour characteristics.

1.4. Mouse models for Liver Cancer

Carcinogenesis evolves from a multitude of genetic alterations and mutations that eventually lead to the transformation of cells, enabling them to bypass the immune response and cell death and increase their proliferative capacity (4). The different kinds of mouse models that are being used to study hepatocarcinogenesis can be classified into genetic, carcinogen-induced and xenograft models.

Indeed, several mouse models have been established, to mimic the most common genetic alterations (genetic models), allowing the investigation of hepatocarcinogenesis which evolves from multiple “at random” hits (carcinogen-induced), or attempting to study the behaviour of tumour cells *in vivo*, dissociated from normal progression from liver disease to liver cancer (xenograft models) (29).

Genetic models

Inducing a mutation, which is commonly observed in HCC, allows researchers to study its effect and importance in the induction of specific pathways, tumour growth, progression and therapy response.

However, inducing one specific mutation is not entirely representative of human disease, where a plethora of interactions and underlying disease precede tumourigenesis.

Constitutive gene expression (target gene is expressed or deleted in all cells) can be used for non- lethal mutations, that only affect the organ or cell-type of interest. For liver cancer this method has been used to replicate hepatitis B virusand hepatitis C virus–mediated carcinogenesis by inserting constructs coding for viral particles involved in the induction of HCC (30, 31). Tumourigenesis can be activated by overexpressing (proto)oncogenesor reducing the expression of tumour suppressor genes, a short overview of mutations used for HCC induction, are provided in table 3. Since constitutive over/under expression of these genes can be lethal, interfere with embryonic development or induce random tumours in different organs, several cell-specific (conditional) gene expression systems were developed, of which we will describe the Cre-lox system as it was used in this work.

In the Cre-Lox system, expression of the site specific Cre-recombinase (Bacteriophage P1 derived) is controlled by a specific promoter sequence, resulting in tissue/cell specific inversion, deletion or translocation of sequences flanked by constitutively inserted LoxP sequences (32-34).

LoxP sites that are oriented in the same direction result in Cre-mediated excision of the loxP flanked gene/sequence, however, if LoxP sites are oriented in opposite directions, Cre activity results in an inversion of the sequence between the lox-sites, and loxP sites on different chromosomes will induce a Cre-mediated translocation (Figure 3A) (32). The development of these systems has allowed researchers to investigate the effect of (proto) oncogenes and tumour suppressor genes on specific cell populations in the liver. Most commonly used liver specific promoters are albumin (*Alb*, expressed by mature hepatocytes) and *Afp* (targeting all hepatoblast derived cells: hepatocytes, cholangiocytes and LPCs) (33, 34). Sox9, hepatocyte nuclear factor 1 beta (*Hnf1-b*) and Osteopontin (*Opn*) promoters have been used to examine the effect of gene expression in the biliary line (cholangiocytes and LPCs) (34, 41-45).

Table 3: Genetically induced liver tumours (adjusted from (35))

Type	Gene	Liver specific	Tumour type	Time to induction	Ref
(Proto) oncogenes overexpression	TGF alpha	Yes	HCC	9 -15 months	(35, 36)
	c-Myc + TGF alpha	Yes	HCC	8 months	
	Beta Catenin+ H-RAS	No	HCC	6 months	(35)
	PDGF	No	HCC	12 months	
	c- Met	No	HCC	12 months	
	HBV large envelope polypeptide	No	HCC	18 – 21 months	
	HBx	No	HCC	11- 15 months	
	HCV core protein	No	HCC	16 – 19 months	
	NRAS and AKT1	Yes	HCC	6 months	(37)
	Notch1 intracellular domain	Yes	HCC / HCC-CC	8 - 12 months	(38, 39)
	Notch2 intracellular domain	yes	HCC	12 months	(40)
Tumour suppressor Knock outs	PTEN	Yes	HCC	18 months	(35)
	MDR2	No	HCC	16 months	
	TAK2	Yes	HCC	4 – 8 months	
	Nemo	Yes	HCC	12 months	
	CYLD	No	HCC	10 – 12 months	
	TSC1	Yes	HCC	9 – 10 months	
	Mcl1	Yes	HCC	18 months	
	BCL-xL	Yes	HCC	18 months	
	APC	Yes	HCC	8 – 9 months	
	P53	Yes	HCC-CC	5 months	

The Cre- lox system was further improved by fusing the Cre protein with the tamoxifen sensitive domain of the estrogen receptor (ERT or ERT2 systems) resulting in a Cre protein that is cytosol bound until the administration of tamoxifen, allowing migration to the nucleus and lox excision (Figure 3C) (46, 47). This iCre is only activated while tamoxifen is present, so only cells expressing iCre at the time of tamoxifen induction, and their direct progeny, will present with the mutation after tamoxifen is cleared (46, 47).

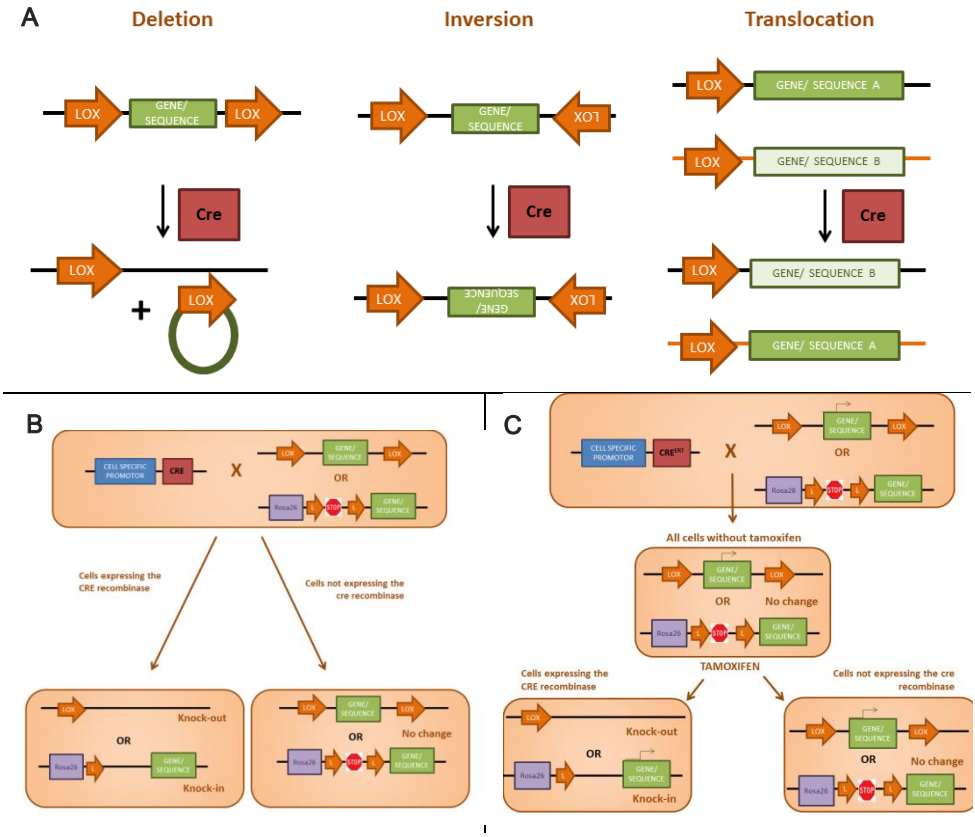

Figure 3: Models for site specific recombinase

A. LoxP sites that are oriented in the same direction result in Cre-mediated excision of the loxP flanked gene/sequence, however, if LoxP sites are oriented in opposite directions, Cre activity results in an inversion of the sequence between the lox-sites, and loxP sites on different chromosomes will induce a Cre-mediated translocation **B.** Cre activity is restricted to cells expressing the Cre protein **C.** iCre- lox system: The fusion of the Cre protein with the tamoxifen sensitive domain of the estrogen receptor (ERT) ensures that Cre activity occurs only in cells expressing a specific promoter sequence after tamoxifen is administered.

For a cell specific knockout, the Lox sequences are placed before and after (a crucial intron of) the gene of interest (also called “floxing”), resulting in gene inactivation upon Cre-mediated Lox excision (Figure 3B,C) (32). The Rosa26 promoter, which has been shown to be ubiquitously expressed in all cell types, followed by a lox flanked stop codon and the gene of interest is used for tissue specific gene induction. Here, the stop codon inhibits expression of the inserted gene in all non-Cre expressing cells (Figure 3B,C)(48).

Chemically induced HCC

Introducing mutagenic toxins to the liver allows researchers to follow the entire process of carcinogenesis, starting with the induction of DNA damage and inflammation, resulting in continuous activation of damage and repair mechanisms, eventually leading to neoplastic transformation and carcinogenesis.

Commonly used carcinogens to induce liver cancer are Aflatoxine, thioacetamine and, most commonly used, diethylnitrosamine (or N-nitrosodiethylamine; DEN) (30, 31, 49).

The downside to using chemicals to induce liver cancer is that due to the unpredictability of the mutations, there is a high variability between animals. However, these variations are a better representation of the human population which is of interest for drug testing studies.

Since we used DEN to induce HCC in our studies, we will shortly discuss this compound. Upon liver uptake, DEN is first hydroxylated into α-hydroxylnitrosamine by the cytochrome P450 enzyme-system, after which the acetaldehyde group is cleaved, and the highly reactive, electrophilic ethyldiazonium ion is formed. Culmination ofDNA damage-induced by ethyldiazonium and reactive oxygen species, formed by the cytochrome P450, results in dose-dependent DNA damage, resulting in mutations and eventually tumour initiation.

DEN mostly induces activation of the proto- oncogene *H-RAS*, which is also seen in human HCC with poor prognosis(50). The effects of DEN are dose, interval, age, sex and strain related (30, 49, 51, 52). All mice strains can develop HCC, however, there appears to be a delay of several months between highly resistant (like C57Bl6) and very susceptible strains (like C3H). In younger mice, the enzymatic competence rises to reach a peak at 7 to 15 days of age, at this time susceptibility to hepatocarcinogenesis is at a maximum for both male and female mice. Next, the metabolic capacity decreases, and does so faster in females than males (52).

In our lab, we have previously validated a protocol in which male sv129 mice are injected with 35mg/kg of DEN at a weekly base, starting from 5 weeks of age. In this model, neoplastic lesions are observed from 15 weeks onwards and mice develop HCC with 100% penetrance from 25 weeks onwards (29). In this model, we can observe HCC lesions in a background of minor fibrosis (metavir score F1 – F2) (29).

Xenograft models

Axenograft is defined as the transplantation of cells or tissues from one species to another. In cancer research, human cancer cell lines are often used to test the response of cancer cells to therapeutic regimens or conditions. To investigate the effects in an *in vivo* situation, these cancer cells can also be injected subcutaneously or orthotopically (in the organ in which the original tumour originated) in immunodeficient mice (30, 31, 49).

Severely compromised immunodeficient or nude athymic mice are mostly used since the lack of T-cell response in these mice ensures that cancer cells will not be cleared by the adaptive immune response. The major advantage of using a xenograft model is that the process of tumour formation is fast(2-6 weeks) compared to orthotopic models. These models are also excellent for preclinical cytotoxicity, pharmacological and pharmacokinetic drug tests (53).

A major downside to using established cancer cells is that each cell line has specific, characteristics, which cannot always be correlated to heterogeneous tumours observed in the clinic (53). Furthermore, interaction between the immune system and the cancer cells is by definition lacking, further decreasing transferability to the clinic (53).

One way around this is the use of a syngeneic model, like Hepa1-6 mouse hepatoma cells originating from C57/Bl6 and transplanting these cells in the liver of C57/Bl6 mice. Since these mice are frequently used to study liver damage, this allows injection of tumour cells in a diseased liver, permitting researchers to investigate the role of the underlying chronic liver disease in tumour growth and progression. Next, cells can be extracted from human tumours, and directly used in xenograft models, to evaluate the response of the tumour to specific treatment regimens (54). Optimisation of this technique can prove to be a valuable clinical tool and will open a window for personalised treatment.

2. LIVER PROGENITOR CELLS

2.1. Introduction

Microscopically, the liver is made up of many hexagonal structures, which are each comprised of a central vein, which will eventually debouch into the vena cava inferior, surrounded by several portal triades, consisting of tracts from the portal vein, hepatic artery and a bile canaliculus (Figure 4A).

The two main epithelial cell populations in the liver are hepatocytes (or liver cells) and cholangiocytes (or bile- duct cells). Hepatocytes are responsible for the major part of liver function, including elimination of toxins and regulation of the carbohydrate and lipid metabolism. Hepatocytes are arranged in cords called hepatic plates (Figure 4B), stretching from a portal triad to a central vein. On one side, at the basal surface, a plate is lined by liver sinusoidal endothelial cells (Figure 4B), guiding blood flow from the portal tract to the central vein, generously allowing diffusion of oxygen, nutrients and toxins along the way. Apically, bile is excreted from hepatocytes, where it flows in the canals of Hering (bile canaliculus), which are lined by neighbouring hepatocytes (Figure 4B), towards the portal triad. At the portal triad the canals of Hering evolve into bile canaliculi, creating a junction between cholangiocytes and hepatocytes (Figure 4B), where LPCs can be found.

In healthy liver, hepatic stellate cells (HSC, liver specific fibroblasts) and Kupffer cells (liver resident macrophages) can be found intrasinusoidally (Figure 4B). During liver injury, this non-parenchymal fraction is enriched by infiltrating portal myofibroblasts and inflammatory macrophages which play an important role in disease progression and resolution by contributing to the inflammatory response and fibrogenesis(55, 56). Moreover, liver macrophages and myofibroblasts were shown to play a major role in the regulation of LPC- cell fate (57).

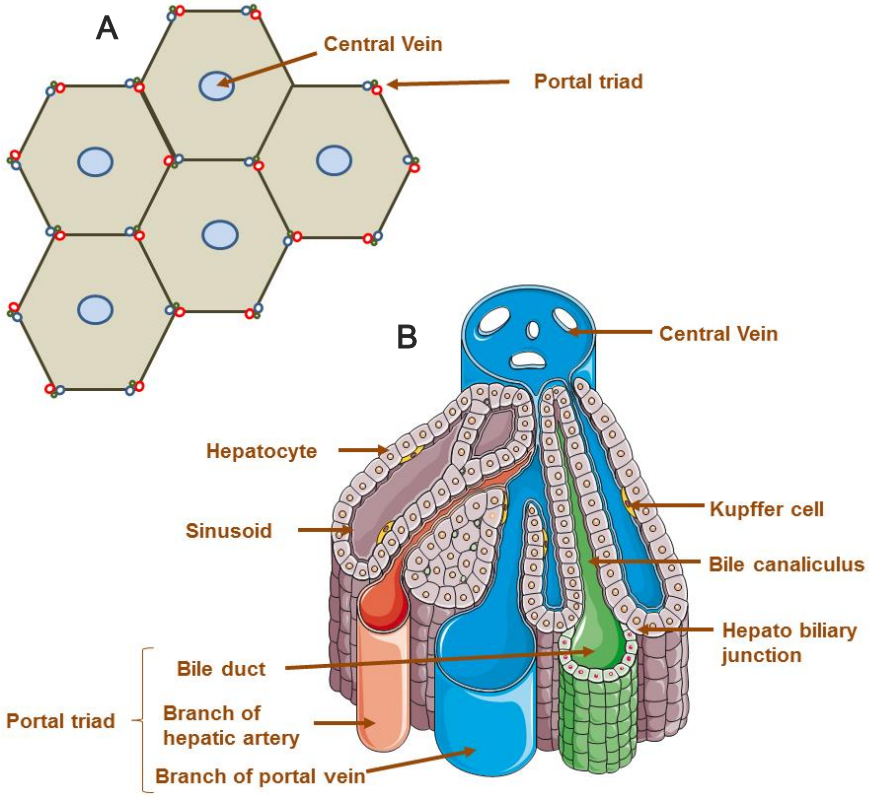

Figure 4: Microscopic structure of the liver

A. Hexagonal structure of liver plates showing the location of the central vein and the portal triade**. B.** Microscopic structure of the liver showing the location of different cell types.

Histologically, hepatocytes are defined as large cells that represent the bulk of the liver mass (Figure 5). Bile ducts are lined by cubical cholangiocytes that are easily distinguished from endothelial cells on haematoxylin-eosin (H&E) stained sections (Figure 5A). Biliary structures, including bile ducts and LPCs, can further be distinguished from other cell types in the liver by performing a cytokeratin 19 (KRT19) staining (Figure 5B).

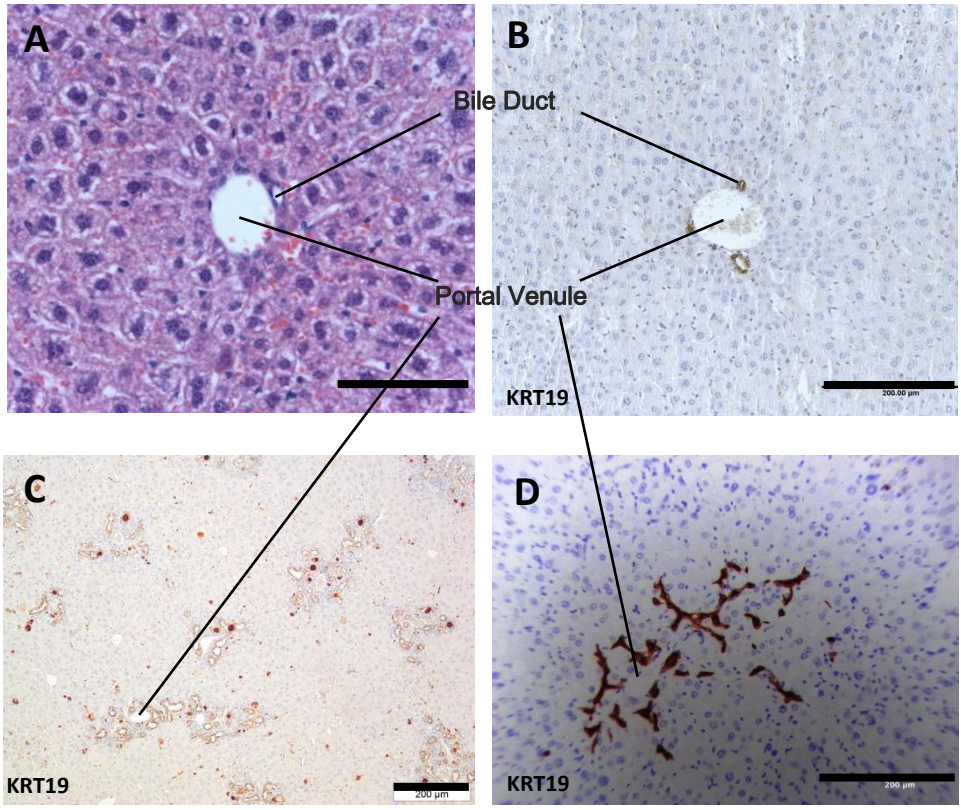

Figure 5: Histology of the Liver

A. H&E and **B.** cytokeratin 19 (KRT19) stained sections of normal liver, showing biliary structures (including LPCs) in brown.**C**. KRT19 stained sections of mouse liver after receiving the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet for biliary damage-induced LPC response. **D.** the choline deficient ethionine supplemented (CDE) diet for hepatocellular damage-induced LPC response. Scalebars: 20µm for A,B,D and 200µm for C

2.2. Regulation of LPC differentiation

Due to the immense self- replicative capacity of hepatocytes and cholangiocytes, it is believed that LPCs have little to no function in normal physiological conditions. However, upon severe acute or chronic injury, when hepatocytes and cholangiocytes can no longer restore liver function by self-replication alone, LPCs are activated. LPCs are bipotential stem cells that can differentiate towards hepatocytes and cholangiocytes and are most commonly believed to reside in the junction between both cell types in the canals of Hering(22, 44, 59).

While interest in the role of LPCs in liver injury, disease and carcinogenesis has been massively expanding in the last decade, the exact role for LPCs in liver pathology has not yet been determined(60). Upon activation, LPCs proliferate and migrate to the site of injury: dispersed throughout the liver lobules to the site(s) of hepatocellular damage (Figure 5C) or around the portal triad in case of biliary damage (Figure 5D) (42, 44, 59).

The Notch signalling pathway plays a pivotal role in the regulation of the cell fate of LPCs, which hasthoroughlybeen described by Boulter et al. (57). Cholangiocyte damage attracts portal myofibroblasts carrying jagged1 (JAG1) ligands, which activate the NOTCH signalling pathway in LPCs, resulting in biliary differentiation(57). However, during hepatocyte damage, macrophage-produced WNT, induced by phagocytosis of hepatocytic debris, inhibits cholangiocellular differentiation and drives LPCs towards the hepatocellular lineage (Figure 6)(57).It is howeverhypothesised that when these mechanisms fail to adequately repair cell damage, a ductular reaction, consisting of cholangiocytes, inflammatory cells, stellate cells and LPCs, is formed (pathological repair), which contributes to fibrogenesis and further liver damage(57, 61).Further investigating the exact triggers for hepatocyte debris-mediated, macrophage-derived Wnt signalling and cholangiocyte damage-induced NOTCH ligand expression could allow the identification of new therapeutic targets to inhibit or ameliorate the response in pathological conditions.

2.3. Liver progenitor cell characteristics in liver cancer

LPCs are a heterogenous cell population characterised by the expression of stem cell characteristics, cholangiocyte markers and early hepatocyte features, each to a variable extent (22, 44, 59). This heterogenousity severely complicates LPC research, different groups use different markers for characterisation and isolation studies, making it unclear whether results can be extrapolated or if we are even investigating the abilities of the same cell population (22, 44, 59, 62).

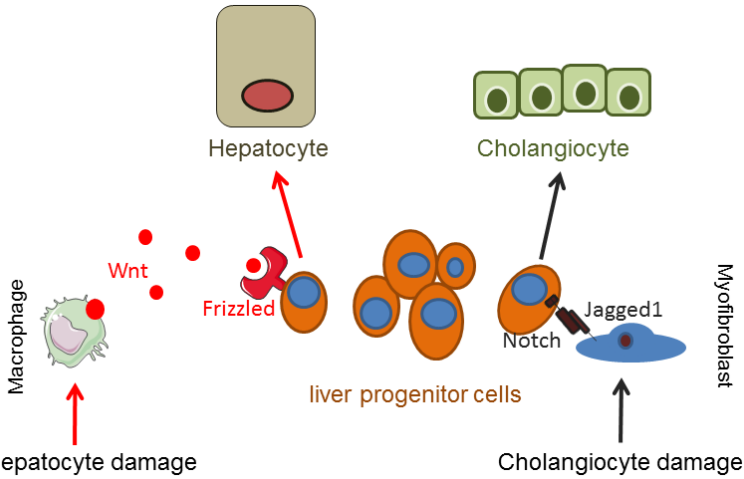

Figure 6: Notch is a crucial factor in LPC cell fate decisions

In biliary regeneration, the interaction of LPC with myofibroblasts attracted by biliary injury results in jagged 1 mediated activation of the Notch signalling pathway in LPCs, leading to biliary specification During hepatocellular regeneration macrophage derived Wnt signalling activates the Wnt- pathway through interaction with Frizzled receptors in LPCs which results in differentiation towards a hepatocellular phenotype

Cell populations exhibiting LPC characteristics have been called “ductular hepatocytes”, “atypical ductal cells”, “intermediate hepatobiliary cells”, “hepatic progenitor/stem cells” and “oval cells”, and the same general set of characteristics are expressed by so- called side population cells (which are isolated from other tumour cells by their ability to efflux Hoechst 33342, caused by the expression of multi drug resistance proteins) and to define the “cancer stem cell” (CSC) (22, 44, 59).

Throughout this work, we will define LPC as single KRT19 positive cells (in contrast to KRT19+ cholangiocytes that are part of a canalicular structure) residing around the portal tract and “cells with (increased) expression of LPC characteristics” for cells of which the cellular ontogeny is unclear, i.e. in liver cancer. Several studies have demonstrated that cells with LPC characteristics are part of the tumour niche in primary liver tumours (19, 63, 64) and increased expression of LPC markers like KRT19, prominin1 (PROM1), epithelial cell adhesion molecule (EPCAM) and AFP have been shown to be related to poor prognosis in HCC (19-21, 65-68).

The predisposition of primary liver tumours to develop in a background of chronic liver disease in which there is an increased proliferation of progenitor cells (27, 69), increases the likelihood of progenitor cells accumulating and stabilizing enough mutations to obtain a cancerous phenotype. It may thus be possible for LPCs to transform into (hepatic) cancer stem cells and grow into primary liver tumours (70, 71). It has been shown that HCC-CCs can be progenitor cell derived, however, there is a broad range of tumours that carry both phenotypes that are not necessarily LPC derived (17). In HCCs with cholangiocellular or LPC features for example, these characteristics can be gathered over time, during tumour progression. Researchers have demonstrated that HCC-cells and hepatocytes can, in certain conditions, de- or transdifferentiate towards a more cholangiocyte or LPC- like phenotype, characterised by expression of biliary markers like KRT19, EPCAM and SOX9(72-74). This suggests that HCC-CC tumours could also arise through de- or transdifferentiation of HCC-cells. In the mouse models used throughout this thesis, HCC with increased LPC or cholangiocellular characteristics will be defined by coexistence of (pre)neoplastic hepatocellular lesions and biliary neoplastic lesions in the same liver and/or increased expression of biliary and LPC markers like KRT19, EPCAM, SOX9 and PROM1.

Many different factors are believed to be involved in the regulation of the expression of LPC characteristics in HCC, some of which have been extensively described in our review entitled “the roles of transforming growth factor – beta, WNT, NOTCH and hypoxia on liver progenitor cells in hepatocellular carcinoma” (22). For the purposes of this thesis, we will only focus on hypoxic and Notch signalling in the next section.

3. HYPOXIA AND NOTCH SIGNALLING

3.1. Molecular factors in the hypoxic response

When the oxygenation of cells or tissues is insufficient, they become hypoxic. Hypoxia can result from reduced oxygen tension in the blood flow or from an insufficient blood supply to affected cells. Cellular adaptation to hypoxic conditions is mainly regulated by the hypoxia inducible factor (HIF) pathway, which is mostly regulated by prolyl hydroxylase domain (PHD) proteins that serve as oxygen sensors: in normoxic conditions, they use available O2 to hydroxylate the continuously produced hypoxia inducible factors 1 and 2 alpha (HIFα), resulting in ubiquitination and HIFα – degradation (Figure 7) (75-78). When the oxygen supply is low, PHDs can no longer hydroxylate HIFα, resulting in stabilisation and migration to the nucleus. Here, HIFαdimerises with the hypoxia inducible factor 1 beta subunit, and binds DNA, where the complex acts as a transcriptional regulator. Altered gene expression then changes the cell's metabolic state by, firstly ensuring survival, proliferation and adequate energy production in anaerobe conditions (like glucose transporter 1, GLUT1; phospho-fructokinase, PFK) and secondly, several pro–angiogenic (like VEGFA) and erythropoiesis stimulating cytokines (like Erythropoietin) increase oxygen supply (27, 75, 79, 80). This reaction to hypoxic conditions is called the 'hypoxic adaptive response' (Figure 6) (75-77, 79).

There are three main mammalian PHD homologs: PHD1, PHD2 and PHD3 and while their regulation and function is mostly similar, some discrepancies do exist. While PHD1 and 3 are believed to hydroxylate HIF2α more efficiently, PHD2 is more abundant and has a strong preference for the hydroxylation of the hypoxia inducible factor HIF1α subunit over HIF2α(81).

This explains why PHD2 deletion cannot be overcome, while other PHDs can compensate for the loss of PHD1 or 3.

Moreover, expression of *PHD2* and *3*is upregulated upon increased HIF signaling, a negative feedback loop that ensures rapid HIFα degradation upon reoxygenation(81). PHDs can also hydroxylate other substrates, like the IκB kinase (IκK), inactivating its activity(81, 82). In hypoxic conditions, unhydroxylated IκK is able to phosphorlylate the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha resulting in transcriptional activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), inducingpro-survival, anti-apoptotic and pro-inflammatory signaling. Moreover reports have shown that NF-κB signaling is essential for activation of the HIF signaling pathway (82, 83).

In physiological conditions, this system prevents massive cell death during organogenesis, wound healing and decreased oxygen intake. However, the same mechanism is also activated in pathological conditions coinciding with reduced oxygen tension, like chronic liver disease and cancer (25, 27, 28, 79, 80, 84). In this case promoting cell survival and inducing angiogenesis contributes to disease progression.

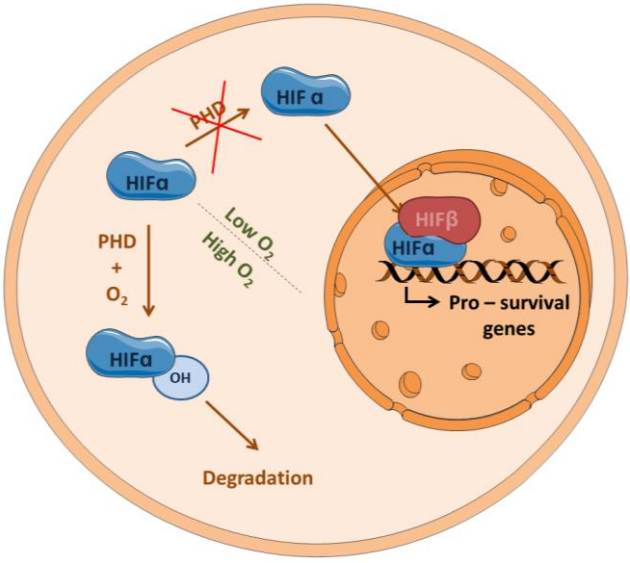

Figure 7. General schematic of the activation of the hypoxic adaptive response.

In normoxic conditions (high O_2) the prolyl hydroxylase domains (PHD) can hydroxylate the hypoxia inducible factor alpha (HIFα) resulting in its degradation. In hypoxic conditions (low O_2) PHD can no longer utilize oxygen for HIFα hydroxylation, causing stabilisation and migration of the HIFα protein to the nucleus. Here the HIF complex acts as a trans-activator for pro- survival genes.

3.2. Molecular factors in NOTCH signalling

The Notch pathway is important in stem cell self-renewal, and plays a special role in the control of many binary cell fate choices in embryonic and adult cells (85). Notch signalling is also involved in several fundamental cell regulatory processes such as proliferation, apoptosis and epithelial to mesenchymal transition (EMT)(85). There are 4 NOTCH receptors and 2 types of ligands described in mammals: the NOTCH 1, NOTCH 2, NOTCH 3 and NOTCH 4 receptors, and the jagged and Delta ligands. Ligand binding to the N- terminal extracellular domain of the receptor triggers cleavage of the C- terminal NOTCH intracellular domain (NICD) (Figure 8) (34, 85-89). NICD cleavage is a two-step process, the second step, mediated by the presenilin-gamma-secretasecomplex, which is composed of 5 subunits: presenilin 1 and 2, nicastrin, presenilin enhancer 2, and anterior pharynx-defective 1(85, 89). Upon its release into the cytoplasm NICD migrates to the nucleus, binds toCSL (CBF1/Su(H)/Lag-1), and recruits co-activators, such as mastermind–like, to induce NOTCH-dependent gene transcription (Figure 8).

The two major targets are the Hairy and HES-related repressor protein families of transcription factors (HES and HEY) and the MYC transcription factor (85, 89). The list of target genes and role in different physiological and pathological events are both cell type and receptor specific (89-91).

3.3. Hypoxia in primary liver cancer

Chronic liver disease is characterised by inflammation which, amongst others, causes an increased metabolic rate, oxygen need and NF-kB activation, leading to activation of the hypoxic adaptive response(83). Moreover, the accompanying fibrogenesis results in increasing rigidity of the organ, which causes portal hypertension and reduced blood flow to the liver. Together this indicates that, in tumours arising in a background of chronic liver disease, the hypoxic adaptive response can already be activated at tumour initiation.

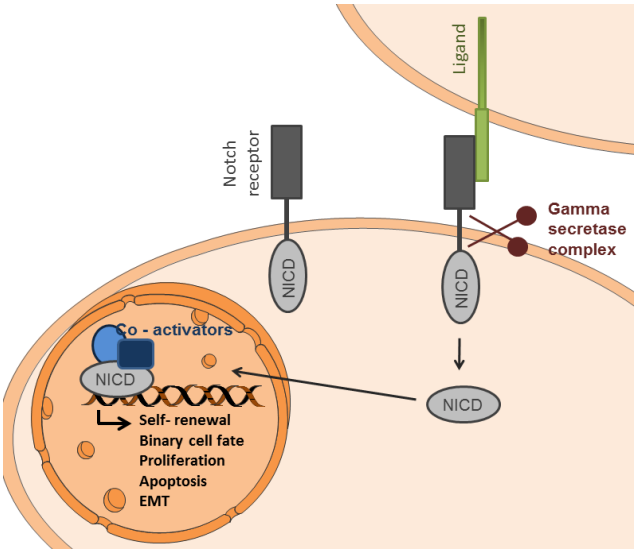

Figure 8: Mechanism of NOTCH pathway activation

Ligand binding induces gamma secretase-mediated cleavage of the NOTCH intracellular domain (NICD). NICD then migrates to the nucleus were it transactivates genes involved in several cell-regulatory processes.

During progression, oxygen deprivation is further accomplished by inadequate blood supply to growing tumours (29, 92). In addition, treatment strategies for advanced HCC, like TACE and Sorafenib are based on depriving the tumour from its oxygen supply to reduce tumour growth, however, this could also result in activation of the hypoxic adaptive response, which stimulates survival and pushes the environment to increase oxygen delivery, which can severely aggravate tumour growth and survival and certainly influence progression (79).

Increased expression/stabilisation of HIFα has independently been correlated with poor prognosis (25, 79, 93, 94) but has also been shown to induce therapy resistance (12, 23, 26, 95), metastasis (13, 28, 96) and expression of stem/progenitor cell characteristics (24, 97, 98) in several cancers, including HCC. Furthermore, in tumours recurring in HCC patients that underwent TACE treatment followed by transplantation, the more aggressive HCC-CC phenotype was observed in recurring tumours (99, 100). This phenotypic switch was accompanied by increased expression of LPC characteristics, which was also observed after tumour resection (24, 101).

While little is known concerning the role of PHD1 and 3 in HCC, previous studies in our lab have shown that in DEN-mediated HCC induction in PHD2 haplodeficient mice also results in the development of a mixed HCC-CC phenotype with increased expression of LPC characteristics (27).

A HepG2 xenograft study has also shown an increased amount of side population cells after laminin treatment (16), showing the potential of HCC- cells to dedifferentiate, and an *in vitro* study has shown that this de- or transdifferentiation can also be induced by exposure of HCC cells to reduced oxygen tension (74).

In addition, it was also shown that hepatocyte transdifferentiation is reversible *in vitro*(102, 103). Together, these data show that increased hypoxic signalling can induce de- or transdifferentiation of HCC cells, but also that the original phenotype could be restored. A better understanding of the mechanisms involved in the increased expression of LPC characteristics by HCC cells, could lead to the discovery of new therapeutic targets that could inhibit the observed hypoxia-induced phenotypic changes.

3.4. NOTCH signalling in primary liver tumours

Aberrant NOTCH signalling is well described in many different kinds of cancer, such as breast, lung, colorectal, pancreatic and hepatic cancer (24, 63) and has been described as both oncogenic and tumour suppressive, depending on tissue type and circumstances (63-65). The role of NOTCH signalling is now also being extensively studied in hepatocellular carcinoma(34).

Liver specific overexpression of NICD1 (using both Afp:Cre and Alb:Cre mice) has been shown to induce liver tumours with biliary features (38, 39), which were also observed in livers with hepatocyte- specific NICD2 overexpression (104).

NOTCH2 overexpression in hepatocytes was shown to rapidly induce hepatocyte dedifferentiation (104), which indicates a role for NOTCH signalling in the dedifferentiation of hepatocytes.

The gamma secretase complex is not only an essential factor in NOTCH cleavage, it is involved in the intramembranous cleavage of several proteins like E-Cadherin, N-Cadherin, CD44 and the amyloid precursor protein (105), which is important in the pathogenesis of Alzheimer. This resulted in the development of many compounds to inhibit this complex, gamma secretase inhibitors (GSIs), to decrease amyloid plaque formation in the progression of Alzheimer's disease (105). However, due to the overlapping function, these GSIs have also been shown effective in inhibiting NOTCH signalling pathways (106, 107). Because of the importance of NOTCH signalling during development, in proliferation and cell fate, it is not surprising that NOTCH inhibitors, like GSIs could be therapeutic in neoplastic malignancies (107, 108).

Since increased NOTCH signalling induces HCC with the same LPC-like phenotype as observed after hypoxic stimuli, and activation of both pathways has been shown during hepatocarcinogenesis, it is likely that the NOTCH pathway and hypoxic signalling pathways are interconnected in the establishment of the tumour's phenotype in HCC. Indeed, hypoxia has also been shown to induce or maintain a dedifferentiated phenotype in different neuronal and myofibroblast-derived cell lines, coinciding with increased expression of NOTCH ligands and downstream targets (109, 110),administration of a GSI was able to inhibit these effects (109).Moreover, using GSI's in hypoxic conditions, the NOTCH signalling pathway has been shown crucial for hypoxia-induced EMT, cell motility and invasiveness (111, 112). Interestingly, activation of the HIF signalling pathway was also shown to increase the expression of NOTCH ligands and downstream targets (109, 111, 112) and HIFα has also been shown to induce increased gamma secretase activity(113, 114) and/or to bind NICD, augmenting it's stability.

GSI's could prove interesting tools to investigate the crosstalk between the HIF and NOTCH pathways in hepatocellular carcinoma. However, GSI's inhibit the four NOTCH receptors, and while NICD1 and NICD2 have been shown to be functionally equivalent (115), specific NOTCH receptor inhibition had distinct outcomes in hepatocarcinogenesis (90).

Interestingly, NOTCH1 inhibition was shown to have a beneficial effect on HCC but drastically increased the iCC burden, while NOTCH2 inhibition positively affected HCC load (90). The latter indicates that, while sharing hepatocyte-related effects, both receptors possibly have distinct effects on the biliary compartment. Using a HNF4:Cre mouse, researchers demonstrated that NOTCH2 overexpression in the biliary compartment induces severe ductular reactions (104). *In vitro*, NOTCH2 and 4 have been shown essential for LPC proliferation, while NOTCH3 was shown to induce hepatocytic differentiation, and no clear NOTCH1 mediated effects were described (91). The discrepancies between NOTCH1 and NOTCH2 inhibition in HCC could be caused by cell specific effects of inhibiting different receptors. It would therefore be important to investigate the effect of NOTCH1 upregulation in the biliary compartment.

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5. The roles of transforming growth factor beta, Wnt, Notch and Hypoxia on liver progenitor cells in hepatocellular carcinoma (Review)

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5.1. Abstract

Primary liver tumours have a high incidence and mortality. The most important forms are hepatocellular carcinoma and intrahepatic cholangiocarcinoma, both can occur together in the mixed phenotype hepatocellular-cholangiocarcinoma. Liver progenitor cells (LPCs) are bipotential stem cells activated in case of severe liver damage and are capable of forming both cholangiocytes and hepatocytes. Possibly, alterations in Wnt, Transforming growth factor-β, Notch and hypoxia pathways in these LPCs can cause them to give rise to cancer stem cells, capable of driving tumourigenesis. In this review we summarize and discuss current knowledge on the role of these pathways in LPC activation and differentiation during hepatocarcinogenesis.

5.2. Introduction

Liver cancer is one of the most frequently diagnosed cancers worldwide. Despite efforts made, these tumours are often detected in an advanced stage, making liver cancer the 3rd most deadly cancer worldwide (1). The most important types of primary liver cancer are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC). HCC often develops in a background of chronic liver disease caused by chronic alcohol abuse, viral hepatitis or non-alcoholic steatohepatitis, while less is known on potential risk factors for ICC. Both primary tumours can be found together in combined hepatocellular–cholangiocarcinoma (CHC), which is characterised by a worse prognosis than HCC or ICC (2, 3).

There are several curative therapeutic options for primary liver tumours including resection, transplantation and radiofrequency ablation.

However, more often than not, these tumours are detected in late stages. At this point, existing therapies like anti-angiogenic compounds such as sorafenib and transarterial chemoembolisation (TACE) (4), mainly aim to slow down tumour growth and increase survival.

Unfortunately, these treatment strategies still hold various serious adverse effects and therapy resistance, relapse and metastasis remain a real threat (4-6). Importantly, anti-angiogenic treatment also sometimes causes increased local invasion and metastasis, worsening tumour progression (5). Finally, a phenotypic switch from HCC to CHC has been reported after both TACE and increased hypoxia inducible factor alpha (HIFα) stabilisation in a mouse model for HCC (6, 7).

Cancer stem cells (CSC) are cancer cells that possess stem cell characteristics such as the ability to differentiate to all cell types found in a particular cancer sample and are associated with relapse and metastasis (8, 9). Recently, interest has grown in the existence of liver CSC with a liver progenitor cell (LPC) gene signature.LPCs are triggered during severe acute or chronic liver injury, during which proliferation of mature hepatocytes is inhibited (10). LPC-progeny can express hepatocyte- or cholangiocyte-specific lineage markers and experimentally have been proven to differentiate into either of these cell types (11-13).

Possibly, adverse effects often seen following treatment could be caused by survival and adaptation of LPC derived CSC.

This would indicate that LPCs could not only play a role in tumour initiation, but also in progression and therapy resistance (14-17). This review will briefly summarize the current knowledge on signalling pathways acting in primary liver tumour biology, specifically their involvement in LPC activation and proliferation, as well as a possible relation between LPCs and CSCs.

5.3. Liver progenitor cells

In case of severe hepatic damage, like in elaborate chronic liver injury, when proliferation of hepatocytes and/or cholangiocytes alone is insufficient to restore the liver mass and function, liver progenitor cells (LPCs) are stimulated to proliferate and replace the damaged cell types (12).

Even though LPCs can most commonly be found in the canals of Hering (18, 19), several other possible locations have been described: intralobular bile ducts, peri-ductal cells and peri-biliary hepatocytes (20). Possibly, the LPC niche also consists of other actors in liver damage, such as hepatic stellate cells and Kupffer cells (21-23). Differential interaction with these cells could account for the different observations concerning LPC location and factors involved in their activation in various models of liver injury (19, 22, 23).The most commonly used markers for identification of LPCs, or determination of cells with LPC like characteristics are Prominin 1 (CD133), epithelial cell adhesion molecule (EpCAM), alfa-fetoprotein (AFP), and (cyto-) keratin 19 (CK19). However, many other stem cell, hepatic and cholangiocytic markers are used to characterize LPCs (Table I:selection of LPC markers and their potential role in hepatocarcinogenesis) (24-26). Although the existence of LPCs and their role in liver injury is generally accepted, and a broad range of markers is being used to identify and/or isolate these cells from livers (13, 19, 27-29), researchers have not yet agreed on a precise set of markers defining the LPC population, therefore filtering out the identity of the “true progenitor cell”, remains a challenge.

5.4. Liver progenitor cells in hepatic carcinogenesis

Several studies have shown that cells with LPC characteristics are part of the tumour niche in primary liver tumours (30-32). Because of their multipotent characteristics there probably is a role for LPCs in HCC and ICC formation, however, due to the dual hepatocytic and cholangiocytic origin, it is CHC that is generally presumed to be a progenitor derived tumour (30, 33).

Currently, there are two major hypotheses on how stem cells influence tumour formation. Firstly, the clonal evolution model, which presumes that a single cell acquires random mutations and gives rise to a group of identical tumour cells, each with equal potential to generate a tumour.

Secondly, the cancer stem cell theory proposes that a tumour consists of a heterozygous cell population, where only certain cells are able to self–renew and differentiate (9).Over the years, CSC have been shown to play a role in the development of certain forms of leukaemia and glioblastoma, as well as in several solid tumours such as breast, gastric and colon cancer (15, 24, 34) and are now being extensively studied in hepatocarcinogenesis (15, 24).The predisposition of primary liver tumours to develop in a background of chronic liver disease in which there is an increased proliferation of progenitor cells (2, 7) increases the likelihood of progenitor cells accumulating and stabilizing enough mutations to obtain a cancerous phenotype. It may thus be possible for LPCs to transform into (hepatic) cancer stem cells and grow into primary liver tumours(15, 24). So far, several pathways have been shown to mediate LPC activation, proliferation and/or differentiation. The balance between Wnt and Notch signalling has been proposed to be crucial for determination of the LPC cell fate. Activation of the Notch pathway is essential for biliary differentiation, as shown by several *in vivo* and *in vitro* experiments (35, 36). Moreover, in case of hepatocyte injury, activation of the canonical Wnt pathway probably prevents activation of the Notch pathway, thus pushing LPC differentiation towards hepatocytes (35, 36). Also, interaction between tumour cells and the extracellular matrix (ECM) is shown to be essential for tumour progression, invasion and metastasis, transforming growth factor-β (TGF-β) mediated epithelial mesenchymal transition (EMT) plays an important role in this interaction (37). Recently TGF-β signalling has also been linked to the presence of LPCs in hepatocarcinogenesis (38).

The Notch, Wnt and TGF-β pathways are also well known to be involved in many tumorigenic processes. For this review we will focus on these three pathways and discuss their role in hepatocarcinogenesis, with special attention to their potential involvement in LPC and/or CSC–mediated tumour initiation and progression (Figure 1).

Table I: selection of LPC markers and their potential role in hepatocarcinogenesis.

Abbreviation	Full name	Role in HCC and/or CC development
CK7	(cyto) keratin 7	Increased expression of these cholangiocytic markers in primary liver tumours indicate poor prognosis (16, 42).
CK19	(cyto) keratin 19	
ALB	Albumin	Hepatocyte-specific marker, up-regulated in ICC, compared to other cholangiocellular tumours like extrahepatic cholangiocarcinoma (43, 44).
OPN	Osteopontin	restricted to cholangiocytes lining the canals of Hering, good LPC marker for lineage studies (12).
OCT4/ Pou5f1	Octamere binding transcription factor/ Pou domain class 5, transcription factor 1	Embryonic transcription factor involved in stem cell self – renewal. Possible prognostic marker for HCC, and up-regulated in chemoresistant liver cancer cells (45).
AFP	Alfa – feto - protein	Fetal serum protein, often but not always re – expressed in HCC and CHC (44, 46)
LIF	Leukemia inhibitory factor	Cells are pushed to differentiate during decreased LIF levels. LIF is elevated in LPCs and known to induce acute phase proteins in hepatocytes (47).
Sox 9	SRY-related HMG box transcription factor 9	Transcription factor involved in cholangiocyte-specific development (48).
CD133	Prominin1	Cancer stem cell marker, up-regulated in most primary liver cancers. Associated with more aggressive phenotype and therapy resistance (49-51).
CD34	CD34 antigen	Cancer cell marker mainly expressed in early hematopoietic cells
CD44	CD44 antigen	Up-regulated in most primary liver cancers, regulation associated with more aggressive phenotype and treatment resistance (51).
CD56/ NCAM	Neural cell adhesion molecule	Shift from E- cadherin to NCAM expression indicates epithelial mesenchymal transition
CD117	c-KIT	Proto oncogene, up-regulation due to mutation occurs in many tumours. C-Kit inhibition is also reported to slow LPC expansion and tumour formation in rodents (52).

5.5. Wnt/β- catenin pathway

The canonical Wnt signalling pathway directs essential cell regulatory mechanisms such as cell proliferation and cell polarity, but also plays an important role during embryonic development (39-41).

A key player in the canonical Wnt signalling pathway is β-Catenin, which also plays a crucial role in intracellular junctions by forming a receptor complex with epithelial cadherin (E -cadherin) (39).Upon binding of Wnt to its receptor Frizzled, β-catenin switches from being part of a destruction complex to the formation of a "Wnt-signalosome" that prevents β-catenin degradation. This allows the latter to migrate to the nucleus where it binds to the T-cell factor/lymphoid enhancer factor and induces transcriptional activation of Wnt-responsive genes (39, 53). This β-catenin signalling has been shown to be necessary for mouse LPC activation upon injury in rodents (54) and to regulate the hepatocytic specification of LPCs (35).

In HCC cell lines, activation of the Wnt/β-catenin signalling pathway not only increases EpCAM accumulation in both the cytoplasm and the nucleus (53), but also increases the $EpCAM^{+}AFP^{+}$ and the Oval Cell marker 6 $(OV6)^{+}$ population. These represent cell populations with strong LPC features which also demonstrate tumorigenic and invasive capacities (41, 55).

Canonical signalling probably also plays a role in chemoresistance, which is strongly linked to LPC proliferation (56, 57), as shown by the increased EpCAM expression in patients with reduced sensibility to interferon α/5-fluorouracil combination therapy (57). In addition, blocking the Wnt/β-catenin pathway not only inhibits HCC cell growth (53), but also diminishes chemoresistant $OV6^{+}$ colonies (41). Interestingly, canonical and non-canonical Wnt pathways seem to have opposing effects on tumour growth (58-60).

The canonical pathway (mediated by Wnt1-3) mediates growth and regeneration and is reported activated in well differentiated HCC cells while it is repressed in poorly differentiated HCC cell lines (41, 54, 60).

Oppositely, activating the non-canonical pathway (including Wnt 5a and 11) has been shown to inhibit HCC and ICC growth (58-60), possibly by antagonizing the canonical pathway, and promoting cell motility and invasion (60).

This could indicate an important role in the growth and migration pattern of the tumour, caused by interaction between these two pathways during hepatocarcinogenesis.

5.6. Transforming growth factor-β pathway

TGF-β is involved in various cellular functions, such as cell growth, differentiation and apoptosis, in adult as well as in embryonic stages (61). Binding of TGF-β to its receptor results in phosphorylation of the receptor eventually followed by the translocation of Smad proteins (SMAD2/3) to the nucleus in a complex with SMAD4 (coSMAD), where they can regulate transcription by binding to Smad-binding elements in co-operation with a plethora of Smad interacting proteins (62, 63). However, TGF-β also uses non-Smad signaling pathways such as the phosphoinositide 3-kinase/Akt/mTOR pathway, the p38 and Jun N-terminal kinase / mitogen-activated protein kinase pathway to transduce its signals (64).

In addition to these non-canonical pathways, TGF-β signalling is regulated at many levels by processes such as endocytosis of the receptor complex, or by molecules like inhibitory Smads6/7 and the bio-activity of the ligands through proteolytic cleavage by their protease (mainly furin) (62).

Like its regulation, the role of TGF-β in tumour formation is rather complicated. In healthy tissue, it acts as a tumour suppressor controlling the cell cycle, inducing apoptosis and regulating autophagy. During tumourigenesis, cells switch their response to TGF-β, making it a potent inducer of cell motility, invasion and metastasis, as well as guardian of stem cell maintenance (65). In liver carcinogenesis, TGF-β has been shown to have both tumour suppressing and promoting effects (24, 61) and its expression is decreased in early, while increased in later stages of tumourigenesis (24, 66, 67).

TGF-β signalling is also a master regulator of initiating and maintaining EMT, the process directing cancer cells towards invasion and metastasis (37).

In HCC cells, inhibition of TGF-β has been reported to upregulate E-Cadherin and thereby lower migration and invasion potential (68). However, in human fetal hepatocytes (cells carrying progenitor cell features, like EpCAM and CK19 as well as hepatoblast features like AFP), TGF-β even induces apoptotic, growth inhibitory signals, as well as pro-invasive, mesenchymal characteristics such as neuronal Cadherin, Snail and Vimentin (68). What is more, during EMT, TGF-β signalling results in dissociation of β-catenin from the E-Cadherin/β-catenin membrane complex resulting in cytoplasmatic and nuclear accumulation of β-catenin and subsequent activation of the Wnt pathway (69). Possibly, this upregulation of the Wnt pathway, due to TGF-β dysregulation causes a larger population of activated LPCs in HCC patients (70) and in mice following partial hepatectomy (71). Furthermore, in patients, high nuclear β-catenin accumulation is correlated with higher vascular invasion grades and increased recurrence after transplantation (70).

These data suggest an important, but contradictory role for TGF-β signalling in hepatocarcinogenesis, possibly regulating the activation and differentiation of LPCs, through regulation of the Wnt- signalling pathway. Because of the important role of TGF-β in EMT, its regulation is decisive for the tumours invasive and metastatic potential.

5.7. Notch pathway

The Notch pathway is important in stem cell self-renewal, differentiation, and plays a special role in the control of many binary cell fate choices in embryonic and adult cells (72). In the liver, notch signalling promotes differentiation of LPCs towards the cholangiocytic lineage rather than to hepatocytes (73). Furthermore, Notch is involved in several fundamental cell regulatory processes such as proliferation, apoptosis and EMT (72).

Binding of Delta or Jagged ligand to the Notch receptor, causes cleavage of the extracellular C-terminal peptide. Notch intracellular domain (NICD) is then cleaved by γ-secretase, releasing it into the cytoplasm so it can migrate to the nucleus, bind to CSL, recruit co-activators such as mastermind–like, and induce Notch-dependent gene transcription. The two major targets are the Hairy and Hes-related repressor protein families of transcription factors (72, 74).

Like the Wnt and TGF-β pathway, aberrant Notch signalling is well described in many different kinds of cancer, such as breast, lung, colorectal, pancreatic and hepatic cancer (24, 74). However, deregulation of the Notch pathway has been described as both oncogenic and tumour suppressive, depending on tissue type and circumstances (74-76).For example, the effect of Notch signalling on hepatocarcinogenesis can be determined by its effect on several players in cell cycle control such as p53 (76), cyclin-A, -D1 and -E (75).

Induction of p53 in HepG2 cells, leads to an increased expression of NICD and downregulation of the cells proliferative capacity, but not the other way around. Moreover, in cells expressing mutant p53, not able to induce NICD up-regulation, administration of recombinant NICD protein did cause reduced proliferation (76).

In a different HCC cell line, SMMC7721, NICD over-expression by retroviral transfection did cause increased p53 levels, as well as decreased levels of proteins involved in cell cycle control, like phosphorylated forms of the retinoblastoma protein, thus also causing inhibition of growth and proliferation (75). Unfortunately neither of these studies investigated the LPC properties of the used cells, before nor after p53 or NICD induction.

In accordance, Notch pathway inhibition by DAPT (γ-secretase inhibitor) in adult mice after conditional deletion of retinoblastoma protein family genes in the liver, which causes proliferation of the progenitor compartment, resulted in an increased number of HCC nodules (77).

Also, over-activation of NICD inhibits cell proliferation in tumour cell lines derived from these retinoblastoma-deficient mice, but not in HepG2 cells (77). These data suggest a differential role for the Notch pathway in progenitor cells compared to hepatocytes, further supported by recent findings of hepatocyte-specific NICD overexpression causing development of HCC with 100% penetrance after 12 months (78) and ICC after partial hepatectomy (79).

Finally, Notch signalisation has also been related to therapy resistance; delta-like ligand-induced activation of the Notch pathway seems to mediate tumour resistance to anti-angiogenic therapy by activating escape mechanisms in the tumour causing the formation of new vessels circumnavigating the therapy-induced blockage (80, 81).

5.8. Role of hypoxia in hepatic carcinogenesis and progenitor cell activation

In the presence of oxygen, HIFα is quickly hydroxylated by prolyl hydroxylase domain proteins, causing degradation. However, in hypoxic conditions, shortage of hydroxyl–groups leads to HIFα stabilisation and migration to the nucleus where it regulates processes supporting cell survival under hypoxic conditions, for example by increasing (neo) angiogenesis (82). Primary liver tumours, especially HCC, often develop in a background of chronic liver disease, characterised by fibrogenesis, eventually leading to cirrhosis. This process is accompanied by increased hypoxia, caused by sinusoidal capillarisation and formation of fibrotic septa increasing resistance to blood flow and thus decreasing oxygen delivery to liver cells. In addition, the fast growing liver tumours quickly outgrow the existing liver vascularisation, thus creating hypoxic conditions (7, 83, 84).

Current treatment strategies for advanced stage liver cancer - such as anti-angiogenic treatment or TACE - often aim to deprive the tumour from its blood and nutrient supply (4).

However, therapy resistance to TACE and anti-angiogenic treatment has been attributed to induction of hypoxic conditions and activation of HIF (3, 7, 85), by adversely increasing cancer cell survival and tumour growth.

Recently, a significant increase in stem cell marker expression has been seen *in vitro* after exposure of HCC cultures to hypoxia (86). Possibly, the decreased oxygen levels in tumour cells stimulate dedifferentiation towards a progenitor phenotype. Potentially increased proliferation and altered differentiation of LPCs in HCC also cause the phenotypic switch to CHC in prolyl hydroxylase domain 2 heterozygous mice, which are characterised by increased HIFα stabilisation (3, 7) and in patients, after receiving TACE treatment(6).

These findings have raised many questions about the future of these therapies, since monotherapies are often insufficient in treatment of HCC and can even induce more aggressive disease. It is of vast importance to consider alternative therapeutic strategies that prevent this massive hypoxic response. For example, a recent study has shown a better outcome in mice with HCC, after treatment with anti-placental growth factor, causing vascular normalisation, instead of blocking neo angiogenesis, and thus causing less hypoxia (3). Also, administration of EF24, could synergistically enhance the antitumor effects of sorafenib, reduce metastasis and overcome sorafenib resistance through inhibiting HIFα by sequestering it in the cytoplasm and promoting degradation by up-regulating the Von Hippel-Lindau tumour suppressor in five different cell lines and in both xenograft and orthotopic mouse models for HCC (87). Possibly, HIF-dependent alterations to the Wnt, Notch and/or TGF- β pathways are responsible for the observed reaction of tumour tissue to hypoxia inducing therapies.

Both *in vitro* and *in vivo* experiments have shown crosstalk between the Wnt and HIF pathways, depletion of β- Catenin resulted in more severe hepatic injury in a mouse model for liver perfusion while an increased Wnt signalisation resulted in a marked decrease of hepatic injury compared to control (88).

In this study, Wnt1 overexpression resulted in a significant higher response of HIF sensitive genes and HIFα protein levels, Whileβ-Catenin/T-Cell factor target gene expression was significantly reduced after ischemia, without a decrease in total β-Catenin. An observation further supported in HCC cells *in vitro*, where a direct interaction between HIFα and β-Catenin was shown, enhancing HIF signalling and driving EMT (89).

So, in hypoxic conditions, HIFα competes with the lymphoid enhancer factor for binding of transcriptional activator β-Catenin thus inhibiting the canonical Wnt pathway responsible for hepatocyte proliferation and instead promoting adaptation, survival and EMT through HIF signalisation (88, 89). This further demonstrates the potency for intratumoural hypoxia to push LPC differentiation towards a more aggressive, therapy resistant cancerous offspring. Furthermore, the EMT of hepatocytes could also contribute to dedifferentiation of hepatocytes towards a stem/progenitor like phenotype as seen in vitro (90). EMT in hypoxic conditions is probably accomplished by HIF-mediated activation of the TGF–β pathway (91, 92).

Next to the β-Catenin-induced intensification, Notch1 signalling has been shown not only essential for HIF and snail mediated EMT (93, 94), but also capable of inducing EMT in normoxic conditions by directly targeting Snail in breast cancer cell lines (94). However, in an HCC cell line a direct interaction between NICD and Snail in the cytoplasm has been shown to result in ubiquitinilation and degradation of Snail (95), again, showing the complex nature of these cell-type specific interactions.

5.9. Conclusions

Despite the increase in scientific interest, the role of LPCs in cancer progression is still unclear. These bipotential progenitor cells could shift to a cancerous phenotype and give rise to HCC, ICC and CHC.

These cells could thus not only be involved in regulating tumour initiation and growth, but also in the invasive and metastatic potential. Likely, specific interactions between several pathways involved in regulation of LPCs can be modulated by intrinsic as well as extrinsic factors and is capable of driving tumourigenesis and determining its phenotype.

Of the 3 main liver tumours potentially derived from LPCs, CHC is most suitable to study the role of bipotential cells during tumour formation, since it consists of both hepatocyte- and cholangiocyte-like cells (96). We discussed a role for altered regulation of Notch, Wnt, HIF and TGF–β signalling in primary liver tumour development.

Interactions between these pathways could possibly force a group of progenitor -or cancer stem cells to behave differently, causing a tumour to exhibit both HCC and ICC like characteristics.

There also is a potential role for hypoxia in the determination of cell fate in LPCs, possibly not only by triggering conversion of its tumourigenic offspring to a more malignant, mixed phenotype (6, 7), but also by inducing therapy resistance (80, 97). As discussed here, the major target of altered signalisation could be EMT, a major process in malignant conversion, provoking hepatocytes to exhibit more stem/progenitor- like features and thus increasing the pool of cancer cells with an LPC signature. These findings are of particular interest when using therapies altering signalisation of one or more of these pathways, triggering changes which could potentially lead to more aggressive tumours. More specifically, inhibiting the involvement of the Notch, Wnt or TGF-β pathway could be the key to altering the massive response to hypoxia and would allow us to reduce the adverse effects so often caused by hypoxia-inducing therapy.

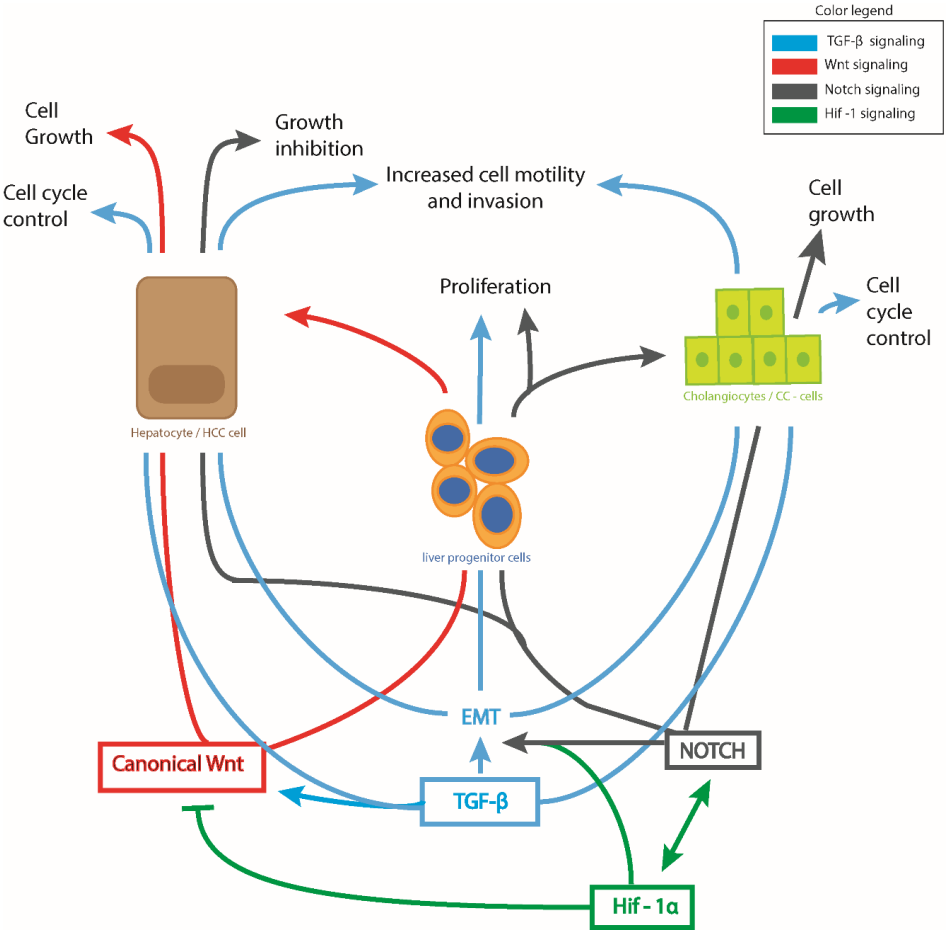

Figure 1: Schematic representation of the role of Wnt, Notch, TGF-β and Hif-1a signalisation on hepatocytes, cholangiocytes and liver progenitor cells in hepatocarcinogenesis.

This figure shows the cel growth promoting effects of the Wnt and Notch pathways on hepatocytes and cholangiocytes respectively, as well as their differential role on liver progenitor cells. Also, the complicated dual role of TGF- β as guardian of cell cycle control, as well as its tumour promoting and invasion and metastasis inducing potential in all cell types is visualised. Finally, the complex interactions between these three pathways, and the possible influence of the HIFpathway is visually represented.

5.10. References

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5.11. Addendum/corrigendum

Official gene symbols were not consequently used in this manuscript, correct nomenclature of used abbreviations is provided in table A1.

Table A1: gene symbols for used abbreviations

Gene symbol	Short	Full
TGFB1	TGF-β	Transforming growth factor beta
PROM1	CD133	Prominin 1
EPCAM	EpCam	Epithelial cell adhesion molecule
AFP	AFP	Alpha feto-protein
KRT19	CK19	(Cyto) keratin 19
KRT7	CK7	(Cyto) keratin 7
ALB	ALB	Albumin
SPP1	OPN	Secreted Phosphoprotein 1 - Osteopontin
POUF1	OCT4/ Pou5f	Pou Class 5 homeobox 1
LIF	LIF	Leukemia inhibitory factor
SOX9	Sox 9	SRY- box 9
CD34	CD34	CD34 molecule
CD44	CD44	CD44 molecule
NCAM1	CD56/NCAM	Neural cell adhesion molecule
KIT	CD117/C-Kit	KIT proto-oncogene receptor tyrosine kinase

Chapter 2

Aims

1. GENERAL AIMS

In this thesis, we describe the work performed to further unravel the role of hypoxia in the expression of LPC characteristics in primary liver cancer and to empower our hypothesis that the Notch signalling pathway plays a crucial role in hypoxia-mediated phenotypic changes in hepatocarcinogenesis (Figure 9). We first evaluated the effect of increased HIFα stabilisation on tumour phenotype and the expression of LPC characteristics at different time points in DEN-induced hepatocarcinogenesis. To determine whether increased hypoxic signalling induces an early signature, we next evaluated the expression of hypoxic, LPC and Notch markers during early DEN-induced hepatocarcinogenesis in PHD2 haplodeficient (PHD2+/-) and wild type (WT) mice. To examine a potential therapeutic role for Notch inhibition to counter the effects of increased hypoxic signalling in HCC, we evaluated tumour growth and expression of LPC characteristics in a xenograft mouse model, which was placed in a hypoxic environment and given a GSI or placebo. Lastly, we attempted to validate a mouse model for inducible biliary specific Notch 1 over-expression; this will allow us to further define the cell and receptor specific effects of Notch signalling in liver disease and cancer.

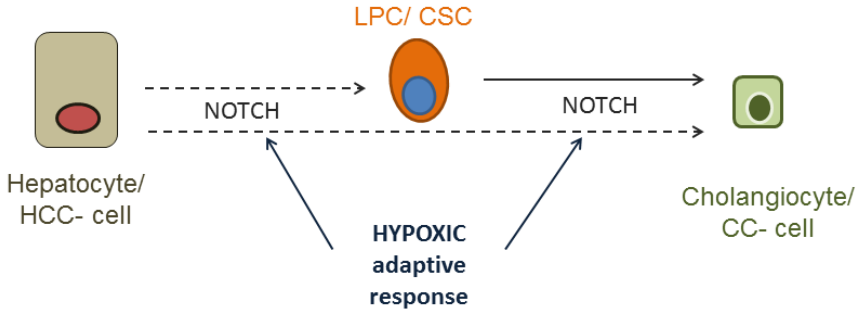

Figure 9: Hypothesis on how the Notch signalling pathway might play a crucial role in hypoxia-mediated effects on tumour phenotype.

We hypothesize that activation of the hypoxic adaptive response mediates phenotypic changes by activating the Notch Signalling pathway. Possibly, Notch signalling in HCC- cells can cause de- or trans differentiation (dotted lines) towards a more LPC- or cholangiocyte – like phenotype.

2. SPECIFIC AIMS

2.1. Determine the impact of prolyl-hydroxylase domain inhibition on the expression of liver progenitor cell characteristics in the pathogenesis of hepatocellular carcinoma

In hypoxic conditions, PHDs can no longer hydroxylate HIFα, resulting in translocation of HIFα to the nucleus and transactivation of pro-survival genes. In primary liver cancer activation of the hypoxic response can occur at different time points in hepatocarcinogenesis. Treatment for advanced HCC is mostly based on depriving the tumour of its oxygen supply.

However, in tumours recurring in HCC patients that underwent the hypoxia inducing TACE treatment followed by transplantation, the more aggressive HCC-CC phenotype, accompanied by increased expression of LPC characteristics, was observed in recurring tumours (1, 2). Moreover, an *in vitro* study has shown that de- or transdifferentiation of HCC- cells can be induced by exposure of HCC cells to reduced oxygen tension (3).

Since the activation of the hypoxic adaptive response is linked to increased expression of progenitor cell/cancer stem cell characteristics and poor prognosis in cancer (4, 5), we aimed to investigate if these effects are time- dependent by inducing increased HIFα stabilisation through PHD inhibition at different time points in the pathogenesis of HCC.

We assessed the time-dependent effect of PHD inhibition and increased HIF signalling by administering the pan-PHD inhibitor dimethyloxaloylglycine (DMOG) at three different time-points in hepatocarcinogenesis: during tumour initiation, during tumour growth and during tumour progression. Elucidating the time points at which activation of the hypoxic pathway could be safe or has detrimental effects with respect to tumour outcome may allow us to anticipate and adapt current hypoxia inducing therapeutic strategies.

We aimed to address these issues in **chapter 3.1** and results were published: *E. Bogaerts, F. Heindryckx, L. Devisscher, A. Paridaens, Y-P. Vandewynckel, A. Van den Bussche, X. Verhelst, L. Libbrecht, L.A. van Grunsven, A. Geerts, H. Van Vlierberghe. Time-Dependent Effect of Hypoxia on Tumor Progression and Liver Progenitor Cell Markers in Primary Liver Tumors.Plos One (2015;*10(3): e0119555) (6).

Interestingly, our lab has previously used PHD2 haplodeficient mice to mimic a hypoxic reaction by increasing HIFα stabilisation. Our group has shown that these mice also show an increased incidence of cholangiocellular lesions after DEN-induced hepatocarcinogenesis. This was associated with a higher expression of LPC markers in livers of DEN-injected PHD2+/- mice compared to livers of DEN-treated wild type (WT) mice(7). This phenotype was also observed in human tumours recurring after transplantation preceded by hypoxia inducing TACE treatment(1).

As PHD2 is the main oxygen sensor in the liver, we aimed to investigate if the effects we observed upon early DMOG treatment could be mediated by PHD2. Moreover, elucidating the kinetics of phenotypic changes during tumour initiation and early development in PHD haplodeficient livers could reveal critical markers and events involved in the observed phenotypic switch at later timepoints.

We therefore also aimed to determine if the effects of PHD2 haplodeficiency in advanced stage hepatocarcinogenesis were preceded by an early LPC signature.These issues were addressed in **chapter 3.2**and results were published: *Bogaerts E, Paridaens A, Verhelst X, Carmeliet P, Geerts A, Vlierberghe HV, Devisscher L. Effect of prolyl hydroxylase domain 2 haplodeficiency on liver progenitor cell characteristics early in mouse hepatocarcinogenesis. Excli Journal (2016;15:687-698)(8).*

2.2. Determine the role of the Notch signalling pathway in the hypoxia-mediated phenotypic switch in hepatocellular carcinoma

Notch signalling is an important regulator of LPC-differentiation, favouring progression towards a cholangiocytic phenotype, and has been shown important during hepatocarcinogenesis.

Several reports have shown that hypoxia-mediated effects on proliferation, migration, invasion and therapy resistance in cancer are moderated by interactions between HIF and NOTCH signalling (9-11). Moreover, overexpression of NOTCH ligands was also shown to induce HCC, high in LPC characteristics, as also observed in patients after TACE treatment and in DEN treated PHD2+/- mice.

To further unravel potential interactions between hypoxic and Notch signalling in our mouse models, we first aimed to determine the effect of PHD inhibition on the expression of Notch receptors, ligands and downstream targets. We therefore examined the mRNA expression of actors of the Notch pathway in DEN mice treated with DMOG at different time points, assessed in **chapter 3.1**and in DEN treated PHD2 haplodeficient and WT mice at different time points, described in **chapter 3.2**.

In these studies, we observed that increased expression of markers for hypoxia coincided with an increased expression of LPC and Notch markers. We hypothesise that the Notch pathway could be involved in the dedifferentiation of HCC towards a LPC or biliary phenotype.

To further evaluate the role of Notch signalling on hypoxia-induced effects on tumour phenotype, we next investigated the effect of Notch inhibition in a xenograft mouse model submitted to decreased oxygen tension in **chapter 3.3**:“Gamma secretase inhibition dampens hypoxia-induced tumour growth and decreases the expression of liver progenitor cell characteristics”.

Here HepG2 xenograft mice were placed in a hypoxic or normoxic environment and treated with either a gamma secretase inhibitor or placebo. We determined the effect of hypoxic conditions on tumour growth and phenotype and evaluated the therapeutic potential of reducing Notch-signalling by inhibiting gamma secretase activity on these hypoxia-induced effects (manuscript in preparation).

2.3. Determine the effect of increased biliary Notch1 signalling in liver disease and cancer.

The Notch signalling pathway is important in cell fate decisions and activation of the NOTCH1 pathway has been linked to induction of liver tumours with a biliary phenotype. However, Notch 1 upregulation and inhibition studies have led to controversial results, which could indicate that the effects of NOTCH1 are cell – specific. Possibly, differential effects of Notch1 in the biliary and hepatocytic lineage could account for conflicting results between Notch1 upregulation and antibody-mediated inhibition studies. Therefore, we created a mouse model for Notch1 upregulation in the biliary compartment.

Our efforts to validate a mouse model for inducible, biliary specific Notch1 upregulation and the effects on liver injury are assessed in **chapter 3.4**.:"Development of a mouse model for inducible Notch1 over-activation in the biliary compartment and the effect on liver injury."

For this study we used the tamoxifen inducible osteopontin-CreERT2 mice (provided by Prof.Lemaigre, UCL), which we crossed to Gt(ROSA)26Sortm1(Notch1)Dam/J mice (Jackson laboratories), to obtain biliary specific, inducible Nicd and green fluorescent (GFP) overexpression.To evaluate the effect of biliary Nicdoverexpression on LPC-mediated cholangiocyte repair, OpnCre+;RosaNicd+/+ and OpnCre-;RosaNicd+/+ mice were submitted to the 3,5-diethoxycarbonyl-1,4-dihydrocollidine(DDC) diet to induce cholestatic liver injury, characterised by a distinct ductular reaction (12).

In future experiments, the tamoxifen regimen will be further validated and the effect of biliary Notch1 overexpression in LPC-mediated hepatocytic repair, will be analysed using the choline deficient ethionine supplemented(CDE) diet.

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Chapter 3

Results

1. TIME-DEPENDENT EFFECT OF HYPOXIA ON TUMOUR PROGRESSION AND LIVER PROGENITOR CELL MARKERS IN PRIMARY LIVER TUMOURS

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1.1. Abstract

Background & Aims

Expression of liver progenitor cell (LPC) characteristics has been proposed as a negative prognostic marker in primary liver tumors. Hypoxia has been linked to activation of the Notch pathway which is responsible for activation and proliferation of LPCs and hypoxia-induced LPC activation has been shown in hepatocellular carcinoma. Our aim was to elucidate the time-dependent effects of hypoxia on the LPC niche in hepatocellular carcinoma which could aid in determining a safe time frame for use of hypoxia inducing therapies.

Methods

We used dimethyloxaloylglycine to mimic a hypoxic reaction in mice by stabilizing hypoxia-inducible factor 1 alpha at three distinct time points in diethylnitrosamine-induced hepatocarcinogenesis. LPC, metastasis and Notch pathway markers were determined by quantitative PCR and (immune)histochemistry (heamatoxillin-eosin, reticulin, Sirius red and cytokeratin 19 staining).

Results

Activating the hypoxia inducible pathway early in hepatocarcinogenesis resulted in an increased incidence of both cholangioma and hepatocellular lesions, associated with high expression of LPC, metastatic and Notch pathway markers.

Adversely, activating the hypoxic response during tumor development resulted in decreased incidence of hepatocellular lesions and increased cholangioma incidence, with an unaltered gene expression profile of LPC-, Notch pathway -and metastatic markers. A hypoxic insult at advanced stages of hepatocarcinogenesis severely increased the expression of LPC characteristics, however without increased expression of actors of the Notch pathway and metastatic markers and minor changes in incidence of hepatocellular and cholangioma lesions.

Conclusion

Our results indicate that increased hypoxia at the onset of tumor development has detrimental effects on tumor progression; patients with HCC developed in a background of fibrosis/cirrhosis might therefore represent a more difficult treatment group. In contrast, hypoxia during tumor development appears to favor tumor outcome, highlighting the importance of early detection. Finally, hypoxia in advanced stages resulted in increased expression of LPC characteristics indicating poor outcome.

1.2. Introduction

Primary liver tumors, especially hepatocellular carcinoma (HCC), often develop in a background of chronic liver disease, characterized by fibrosis and eventually cirrhosis. This process is accompanied by increased hypoxia, caused by sinusoidal capillarization and formation of fibrotic septa, increasing resistance to blood flow and thus decreasing oxygen delivery to liver cells (1).

In addition, fast growing liver tumors quickly outgrow the existing liver vascularization and newly formed intra-tumoral vessels are often structurally and functionally abnormal (2). Ideally, applied anti-angiogenic treatment inhibits further extension of this poorly structured blood supply, depriving tumor cells of oxygen resulting in growth arrest (3, 4). However, this intra-tumoral hypoxia, can also result in inhibition of prolyl hydroxylase domains (PHD), leading to stabilization of the hypoxia inducible factor 1 alpha (HIF-1α) resulting in transactivation of a plethora of genes such as the pro-angiogenic vascular endothelial growth factor alpha (Vegfa), and members of the glycolytic pathway such as glucose transporter 1 (Glut1) and phosphofructokinase (Pfk) aiding tumor cell survival (2, 4). Therapy resistance to sorafenib has been linked to increased HIF signalization and anti-angiogenic treatment has been identified to cause increased local invasion and metastasis, worsening tumor progression (5-8).

Liver progenitor cells (LPCs) reside in the canals of Hering and are activated upon severe acute or chronic hepatic injury (9). These bipotential progenitor cells proliferate and migrate towards the site of injury to replace hepatocytes and/or cholangiocytes and restore liver function.

Interest in the role of LPCs in liver disease pathogenesis has recently expanded (9-15) and the knowledge that Notch and Wnt signaling drive LPC differentiation towards cholangiocytes or hepatocytes respectively has opened new perspectives into the regulation of hepatic cell differentiation(10, 15).

Several other pathways, including the HIF-1α-pathway have been linked to differential LPC behavior in liver disease and cancer (14). For example: exposure of HCC cells to hypoxia significantly increased stem cell marker expression *in vitro* which could account for the observed dedifferentiation in tumors with low oxygen supply (16). Interestingly, PHD2 haplodeficient mice, in which the HIF-dependent pathway is continuously activated, show increased cholangiocarcinoma (CC) burden, coinciding with increased expression of liver progenitor cell (LPC) markers after diethylnitrosamine (DEN)-induced HCC induction (17). Additionally, TACE treatment is also able to switch tumor phenotype from HCC to mixed HCC-CC, with increased expression of LPC markers, a more aggressive character and worse prognosis compared to HCC (18, 19).

Treatment-induced hypoxia may thus increase the expression of stem/progenitor characteristics, which can mediate tumor progression, invasion, metastasis, therapy resistance, early post-operative recurrence and induce a phenotypic switch (5, 6, 8, 17-24). Elucidating the time points in hepatocarcinogenesis at which activation of the hypoxic pathway has detrimental effects with respect to tumor outcome may allow us to anticipate and adapt current therapeutic strategies. Therefore, we assessed the time dependent consequences of elevated HIF signaling on tumor progression and LPC activation by using the PAN-PHD inhibitor dimethyloxaloylglycine (DMOG) in an orthotopic HCC mouse model.

1.3. Materials and methods

Primary tumor induction and dimethyloxaloylglycine (DMOG) mediated activation of the HIF pathway

Ethics statement: All experiments were evaluated and approved by the Ghent University, faculty of health and medicine's ethical commission for animal testing (ECD 12/57) and all efforts were made to minimize animal discomfort.

Weekly intraperitoneal (IP) DEN (Sigma –Aldrich, Bornem, Belgium) injections (35mg/kg) are known to induce neoplastic regions after 16 weeks, HCC nodules after 20 weeks and HCC with 100% penetrance from 25 weeks on (2). For this study we administered DEN for 22 weeks in 5-week-old male 129S2/svPasCrl mice, control mice received weekly doses of saline equivalent to DEN counterparts.

Dose and interval of DMOG, which has been shown to induce HIF-1α stabilization (25), was first tested for its ability to effectively induce functional HIF-1α by measuring the transactivation of Vegfa, Glut1 and Pfk. Mice received a single IP DMOG injection (4,8mg/20g) followed by euthanasia after 3 and 7 days. Livers were removed and sections were lysed for RNA extraction and qPCR. Results showed that biweekly DMOG injections effectively induce HIF activation (Figure 1A) and this treatment strategy was further applied.

DEN -treated mice received DMOG or PBS for five weeks at three different time points during tumor development: at early (1-5 weeks), intermediate (Int, 16-22 weeks) and advanced (Adv, 22-27 weeks) stages.

For comparability between intermediate and advanced treatment groups and to reduce bias by acute DMOG effects, we chose to sacrifice mice from these groups 7 days after the final DMOG injection. Saline control mice received DMOG from week 16 to 22 or from week 22 to 27 and were sacrificed after respectively 22 or 27 weeks.

Sacrification was preceded by anesthesia of the mice with isoflurane (Florene, Abbott, Hoofddorp, the Nederlands) in oxygen for weighting and blood sampling from the ophthalmic artery. After cervical dislocation, the liver was prelevated and weighed. Part of the liver was emerged in RNA later (Ambion, Gent, Belgium) and snap frozen, remaining tissue was incubated in 4% phosphate buffered formaldehyde (KP4078.9010 Klinipath, Olen, Belgium) and imbedded in paraffin, as previously described (1, 2, 7).

Immunohistological analyses

Hematoxilin-eosin (H&E) staining was performed as previously described (26) and sections were analyzed by a pathologist for general morphology and neoplasticity based on the following characteristics: enlarged cells with normal nucleus to cytoplasm ratio (n/c), small cells with increased n/c, enlarged pleomorphic nuclei, and binucleation.Sirius red staining was performed as routinely described (26) to assess fibrosis which allows distinction between areas of ductular proliferation and cholangioma characterized by presence of typical cholangiofibrosis (7, 17).Reticulin staining was performed to evaluate the presence of HCC nodules (26), which are absent for reticulin.

HIF-1α stabilization was evaluated through immunohistochemistry, using a rabbit anti – HIF-1α antibody (sc-10790, 1/400 in PBS, RRID: AB_2116990, Santa Cruz biotechnology, INC, California USA).

Cytokeratin 19 (CK19) staining was performed to visualize structures of the cholangiocytic lineage, including LPCs, using monoclonal rabbit anti-CK19 (1/200 in TBS, ab133496, RRID:AB_11155282, abcam, Cambridge, UK). Epithelial cell adhesion molecule (Epcam) expression was examined using a goat polyclonal antibody raised against the transcriptionally active intracellular domain of Epcam (sc-23788, 1/300 in PBS, RIDD: AB_2098653, Santa Cruz biotechnology, INC, California, USA).

LSAB-horseradish peroxidase-mediated visualization (K0690, DAKO, Heverlee, Belgium) was performed for all protocols. Overall immunoreactivity was calculated using Cell D software (Olympus Imaging Solutions, Münster, Germany) to assess increased expression of all cells of the cholangiocytic lineage. Since cholangiocytes organize in ductular structures and LPCs occur as singular cells, 5 portal areas were centred at a magnification of 400 and all CK19+ single cells were counted.

Quantitative real time PCR (qPCR)

RNA was extracted from 20 mg of frozen liver tissue preserved in RNA-later, according to the manufacturer's guidelines (Rneasy Mini Kit, Quiagen, Venlo, the Nederlands).

cDNA was obtained from 1µg RNA using the iScript cDNA synthesis kit (Bio-Rad, Nazareth-Eke, Belgium) and real time quantitative PCR (RT-qPCR) analyses were performed using a SYBR green mix (Sensifast Bioline Reagents Ltd, London, UK).

Primer sets are listed in Table SI, their efficiency was calculated from the slope of a standard curve using the following formula: E= 〖10〗 ^(-1/slope)-1. All reactions were run in duplicate and normalized to reference genes that showed stable expression in all samples. The comparative Ct method was used to compare gene expression between groups.

Statistics

Data were analysed using SPSS21 software (IMB corp, Armonk NY, USA). Kolmogorov-Smirnov test was used to test for normality. Student's T-test was then performed in case of normality; the Mann-Whitney-U test was used for not normally distributed data. P-values ≤0, 05 where considered significant. All data are presented as average ±SEM.

1.4. Results

Time dependent effect of HIF-1α stabilization on DEN-induced tumorigenesis

We first analyzed whether 4,8mg/20g biweekly or weekly DMOG injections are required to maintain stable activation of the HIF-pathway in livers, by performing qPCR analysis of HIF sensitive genes like Vegfa, Glut1 and Pfk.

There was an increased expression of HIF sensitive genes, for at least 3 but not 7 days, significant for Vegfa and Pfk after DMOG induction, compared to PBS control (Figure 1A). Further treatment regimes were therefore carried out by biweekly DMOG injections.

To assess the effect of HIF-1α stabilization early on in tumorigenesis, DMOG was administered during the first 5 weeks of DEN treatment (Early). Samples were taken after 22 weeks, and we observed that early DMOG treatment had no effect on relative liver weight (Figure 2A). Sirius red staining revealed cholangioma formation in 37,5% and reticulin staining showed premalignant HCC lesions in 62,5% of mice compared to respectively 0 and 50 % of the mice receiving PBS (Figure 2B, C).

To evaluate the activation of the hypoxic pathway during intermediate stages, DMOG was injected from week 16 to week 22 (intermediate, Int.) during DEN induction. Samples were taken at the end of week 22, Int. DMOG also did not influence relative liver weight (figure 2A). Sirius red and reticulin staining in Int. DMOG-treated animals showed cholangioma formation in 50% of DMOG injected mice and no HCC lesions, compared to no cholangioma lesions and 50% HCC lesions in PBS control mice (Figure 2B, C).

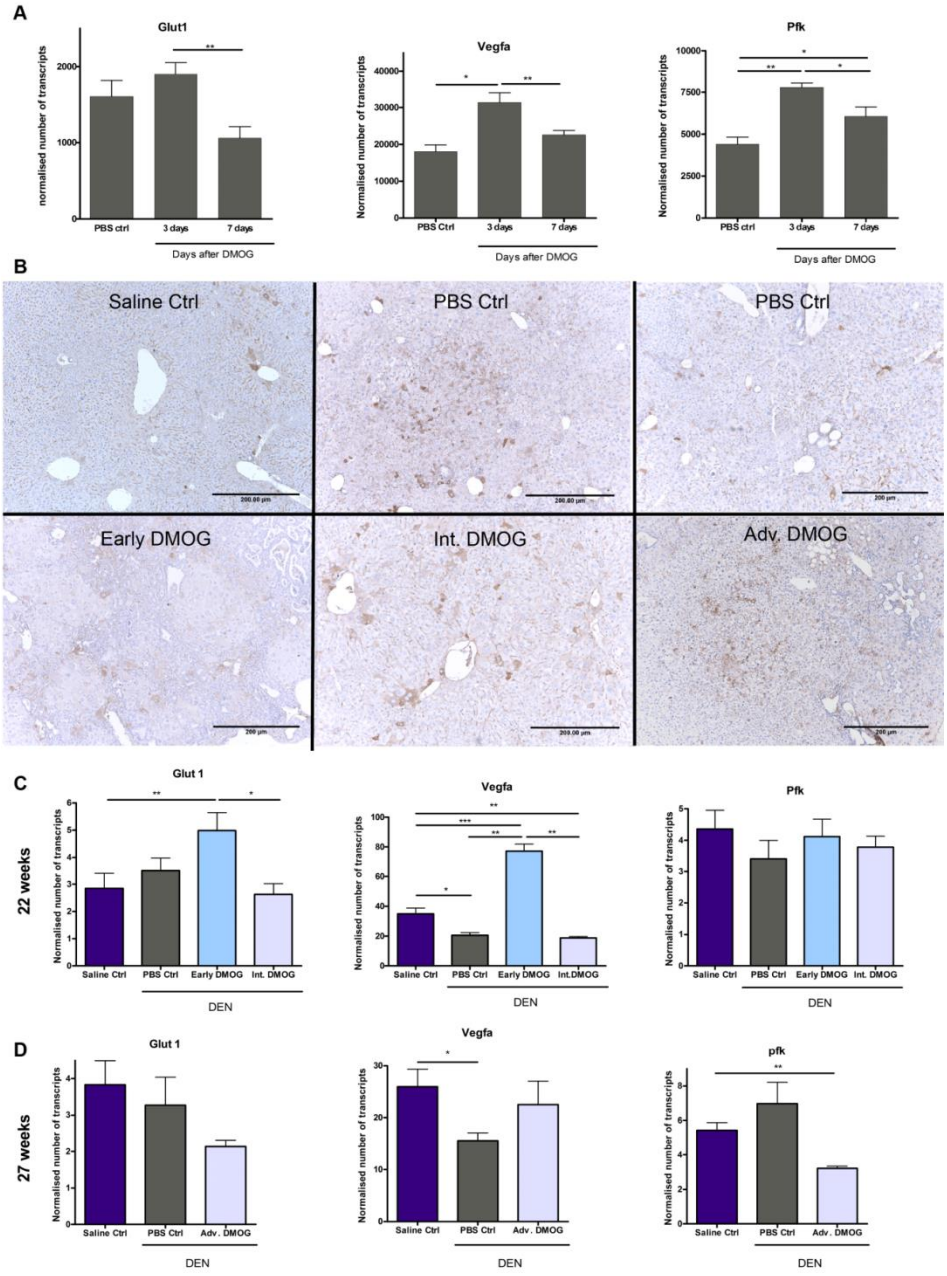


Figure 1.Hypoxia inducible factor expression.

A: Expression of HIF-1α target genes, characteristic for stabilization of HIF-1α, 3 and 7 days after single DMOG injection **B.** Representative images of HIF-1α staining, showing HIF stabilization in all DEN groups, mostly located in and around cholangioma and HCC lesions and near portal areas. **C.** mRNA expression of HIF-1α markers .Scale bars: 200µm, *: p<0,05; **p<0,01

For the effect of HIF-1α stabilization after the final DEN injection, during tumor growth, DMOG (or PBS) was administered from week 22 to 27 (advanced, Adv.) and samples were taken after 27 weeks. Adv. DMOG resulted in a significantly increased relative liver weight compared to PBS counterparts (Figure 2A). Sirius red staining showed cholangioma lesions in 66,7% of DMOG and 50% of PBS induced animals and reticulin staining showed HCC lesions in 40% of DMOG and 50% of PBS treated livers (Figure 2B, C).

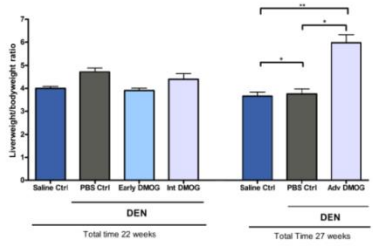
This suggests that, Early Int. and Adv. DMOG result in increased cholangioma formation and Early DMOG even increases HCC formation, while Int. DMOG inhibits HCC formation.

Immunohistochemistry and qPCR analysis was then performed to assess HIF-1α stabilization and activity after Early, Int. and Adv. DMOG treatment. While HIF-1α immunopositivity was limited in saline control livers, in DEN treated livers there was some cytoplasmic presence of the HIF-1α protein in hepatocytes and cholangiocytes around the portal area, but immunopositivity was mostly observed in and around hepatocellular and cholangioma lesions (Figure 1B).

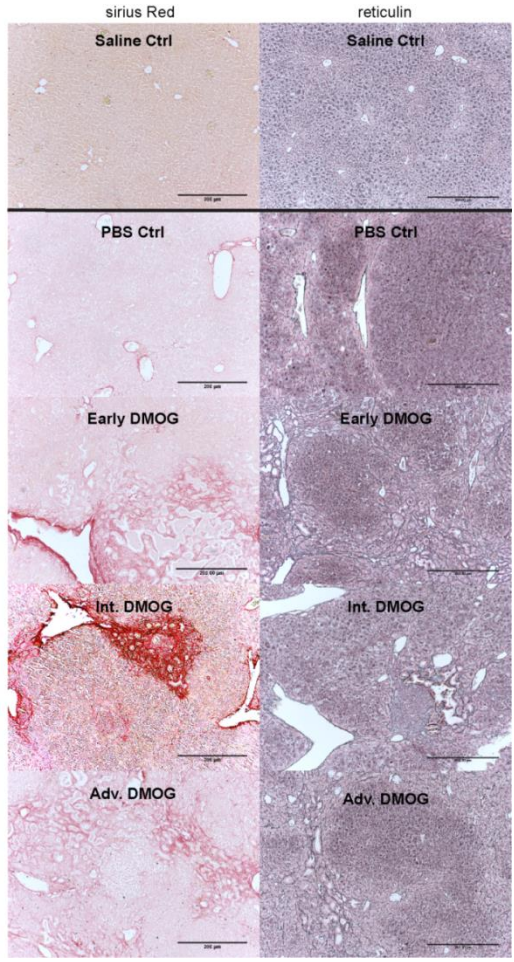
HIF-1α activity was determined through qPCR analysis for downstream HIF target genes. As expected, there was no increased expression in Int. –and Adv. DMOG groups compared to their PBS control groups (Figure 1C). Strangely, these DEN groups also show no increased or even a decreased expression of HIF-dependent genes compared to saline control.

However, we do see significantly increased mRNA expression of HIF target genes Glut1 and Vegfa in DEN livers after Early DMOG treatment compared to all other groups (Figure 1C).

A



B



C

Prevelance of cholangioma and (pre) malignant HCC lesions

	% cases with cholangioma lesions	% cases with (pre neoplastic) hepatocellular lesions
Early DMOG	37,5	62,5
Int. DMOG	50	0
PBS Ctrl	0	50
Adv. DMOG	66,7	40
PBS Ctrl	40	50

Figure 2: General parameters.

A: Liver/ bodyweight ratios for all groups**B**: Prevalence of cholangioma and hepatocellular lesions, showing percentage of mice showing one or more cholangioma or (premalignant) HCC lesions**C:** Representative images of Sirius red and reticulin staining showing cholangioma lesions in all DMOG groups except for PBS control after 22 weeks, and HCC lesions in all DEN groups except for the Int. DMOG group.

Scale bars: 200µm, *: p<0,05; **p<0,01

DMOG: dimethyloxaloylglycine; DEN: diethylnitrosamine

Time dependent effect of hypoxia on LPC characteristics in DEN treated mice

Tumor sections were analyzed for overall CK19 immunopositivity, which is a marker for biliary epithelial cells, including LPCs. All animals receiving DEN showed increased immunopositivity for CK19 after 22 weeks compared to saline controls. (Figure 3A and B left graph). Mice treated with DMOG at advanced stages showed significantly enhanced CK19 immunopositivity compared to PBS control (Figure 3A and B right graph). CK19+ single cells (Figure 3C) were numerous in livers of DEN treated groups compared to saline controls at 22 weeks ($p<0,05$), but no significant difference was seen between treatment regimes (Figure 3D upper graph). DMOG treatment from week 22-27 did however result in a significant increase in CK19+ single cells compared to both PBS and saline control groups (Figure 3D lower graph).

Next, we examined sections for Epcam immunopositivity, which is a marker for biliairy epithelial cells, including LPCs(27) as well as tumor cells(28). In saline control mice, staining was limited to cholangiocytes and some membranous staining of hepatocytes (Figure 4A). In DEN treated mice immunopositivity was seen in the cytoplasm of hepatocytes, mostly around portal areas and in regions containing cholangioma -and hepatocellular lesions (Figure 4A). These results were in line with those of CK19, with a positive trend to increased Epcam expression for all DEN mice, significant for all DMOG groups and for mice that received PBS from week 22 to 27 compared to saline control (Figure 4B). Futhermore, Adv. DMOG treatment also resulted in a significantly increased Epcam immunopositivity compared to PBS control after 27 weeks (Figure 4B).

Since progenitor cell markers have been proposed as markers of poor prognosis in HCC, we next examined the mRNA expression of liver progenitor cell markers cytokeratin 7 (CK7), CK19, CD44, alpha-fetoprotein (Afp), Epcam and prominin1 (Prom1).

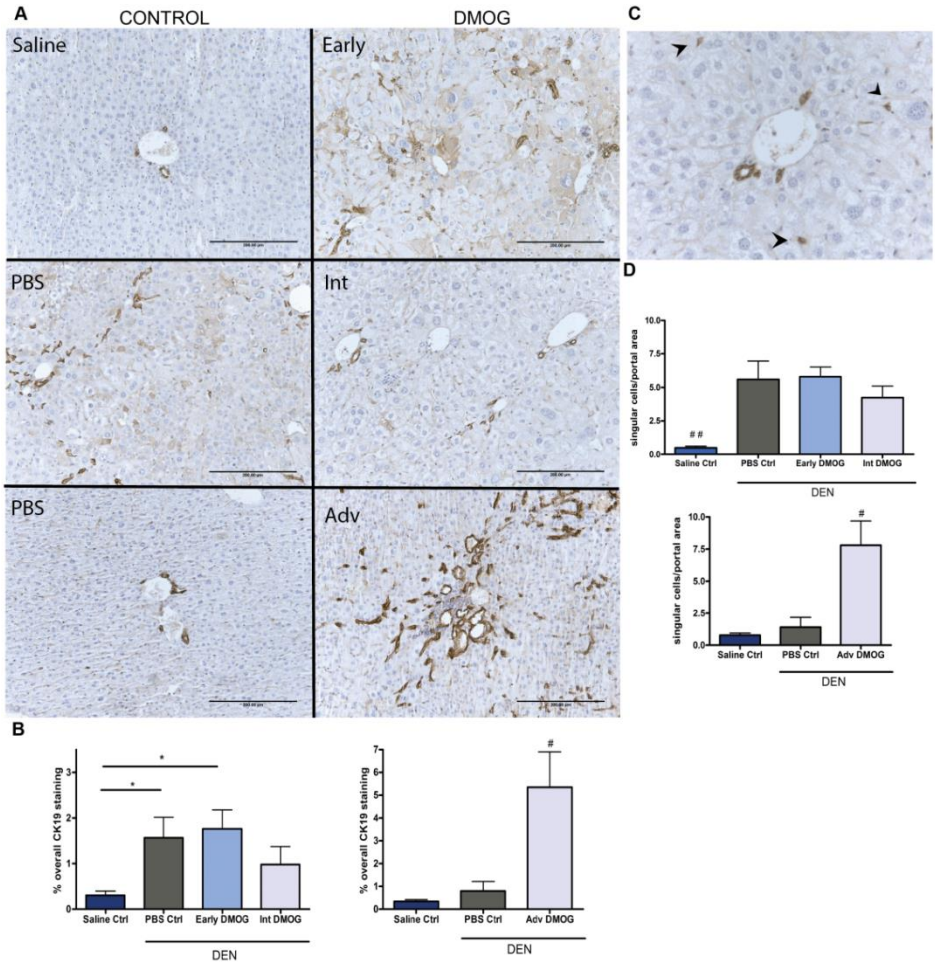


Figure 3.immunohistochemistry for cytokeratin 19.

A: Representative images of CK19 staining, early DMOG and PBS counterparts show cytoplasmatic staining in hepatocytes **B:** quantified % of overall CK19 staining after 22 and 27 weeks **C:** Image of portal area arrowheads point to single cells **D:** Average number of single cells per portal area for each group

Scale bars: 40µm, *p<0,05, **p<0,01 and#p<0,05, ##p<0,01 compared to all other groups

In DEN mice receiving DMOG at early stages, there was a non-significant increased expression of CK7, CK19, Epcam and Prom1, compared to PBS control, which was not seen in mice treated with DMOG at intermediate stages (Figure 5A).

Suggesting a protective role for Int. DMOG and a previously unreported effect of early hypoxia. Afp expression was significantly increased after both early and intermediate DMOG treatment compared to PBS control (Figure 5A).

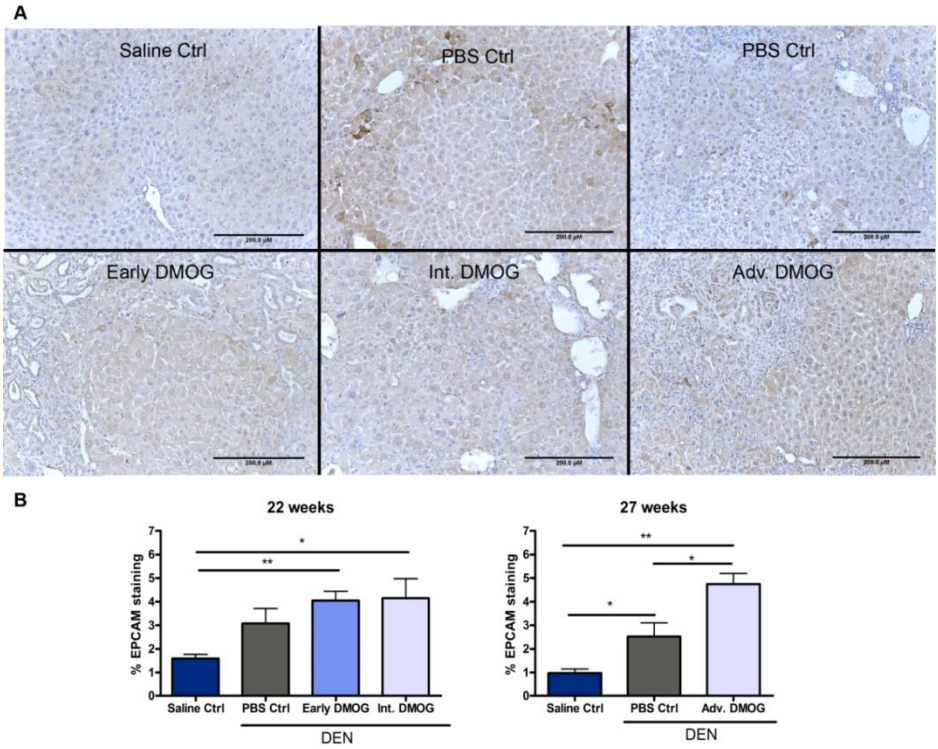


Figure 4.Epithelial Cell Adhesion Molecule.

A. representative images of Epcam staining showing presence around cell membranes of hepatocytes and in cytoplasm of cholangiocytes in saline control livers (upper left). In DEN treated livers, cytoplasmic expression in hepatocytes around portal areas, cholangioma –and hepatocellular lesions was increased.**B.**quantified % of Epcam intracellular domain staining after 22 and 27 weeks. Scale bars: 40µm *:p<0,05; **p<0,01

Adv. DMOG resulted in a non-significant increase of all markers, except for CK7 where a non-significant decreased expression was seen, compared to PBS control. Both DEN groups showed increased expression of LPC markers compared to saline control (all significant for Adv. DMOG group, significant for Prom1, CK7 and CK19 for PBS group), (figure 5B).

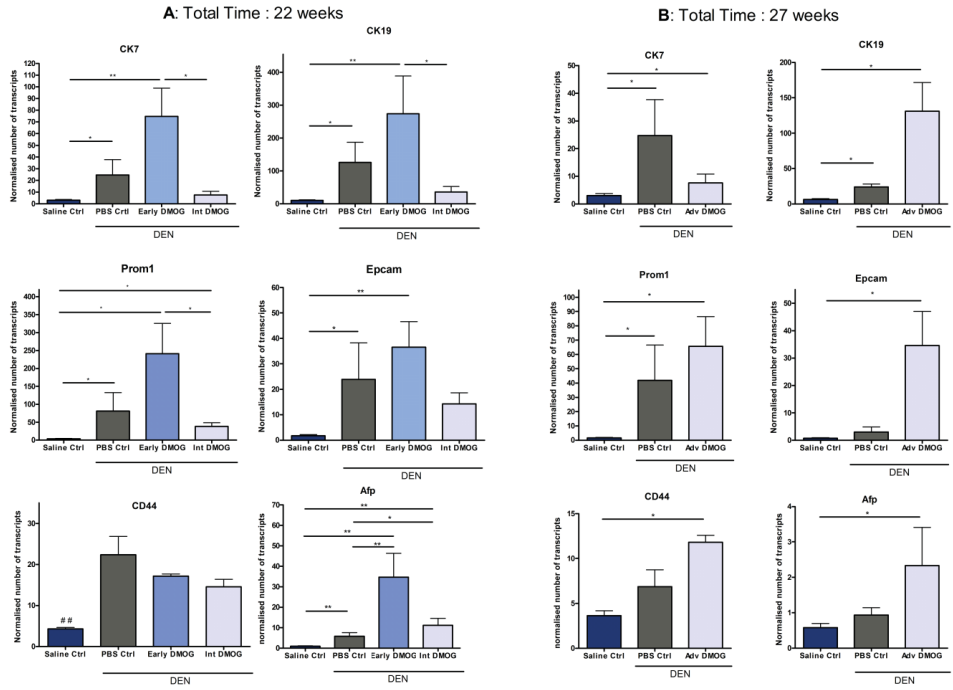


Figure 5.mRNA expression of LPC markers

A: mRNA expression of LPC markers after early and Int. DMOG **B:** mRNA expression of LPC markers after Adv. DMOG. *:p<0,05; **p<0,01; ##<0,01 compared toall other groups

Since Notch signaling is known to be involved in the differentiation of LPCs to cholangiocytes, and has also been suggested to mediate hypoxia-induced therapy resistance and increased invasion/metastasis (14), we examined mRNA levels of Notch 1, Notch 2 and Notch3 receptors, the ligand Jagged1 and main target gene: hairy enhancer of split 1 (Hes1); and matrix metalloproteinase 9 (Mmp9) and Integrin alpha 5 (ItgaV) as markers for metastasis (4).

Interestingly, Notch and metastasis markers were only up-regulated in mice that received DMOG at early stages (Figure 6). Table 1 summarizes the main findings for all groups.

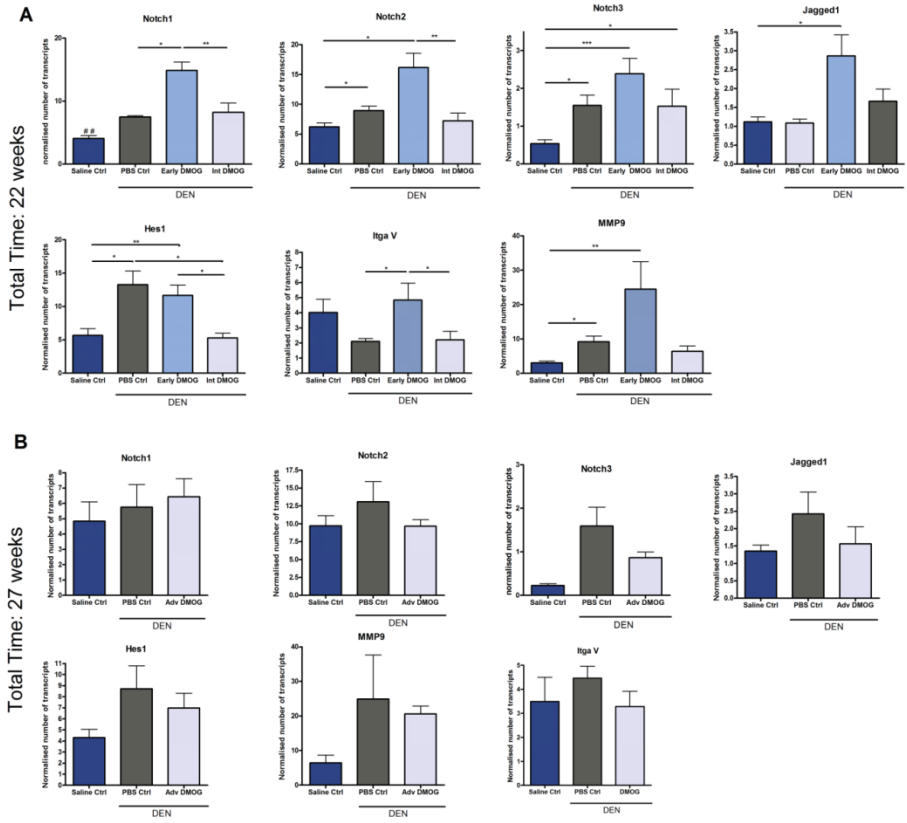


Figure 6.mRNA expression of Notch and metastasis markers.

A: mRNA expression of Notch and metastasis markers after early and Int. DMOG **B:** mRNA expression of Notch and metastasis markers after Adv. DMOG in DEN-induced hepatocellular carcinoma. *p<0,05 ; **p<0,01 , ## p<0,01 compared to all other groups

Table 1. Summary of groups and major findings.

Timing DMOG	Early week 1 -5	intermediate week 16 -22	advanced week 22 -27
DEN induction	22 weeks		
Total time	22 weeks		27 weeks
DEN control group	22w DEN + PBS (week16-22)		22w DEN + PBS (week 22 -27)
General parameters			
relative liver weight	⇵ 1,039 ± 0,02798	⇵ 1,167 ± 0,06543	↑* 1,321 ± 0,07186
Tumor type			
Hepatocellular lesions	↑	↓	↓
Cholangioma	↑	↑	↑

Liver progenitor cell markers				
	CK19+ single cells	↓↑ 1,036 ± 0,1279	↓ 0,7589 ± 0,1511	↑* 4,179 ± 1,012
	CK19 mRNA	↑ 2,172 ± 0,9123	↓ 0,2844 ± 0,1336	↑ 5,711 ± 1,423
	CK7 mRNA	↑ 3,020 ± 0,9846	↓ 0,3080 ± 0,1281	↓ 8,932 ± 2,806
	Prom1 mRNA	↑ 2,973 ± 1,044	↓ 0,4728 ± 0,1257	↑ 1,972 ± 0,4273
	Epcam mRNA	↑ 1,337 ± 0,4107	↓ 0,5967 ± 0,1828	↑ 14,29 ± 3,995
	CD44 mRNA	↓ 0,8260 ± 0,1985	↓ 0,6499 ± 0,08237	↑ 4,075 ± 1,909
	Afp mRNA	↑** 5,978 ± 2,008	↑* 1,912 ± 0,5875	↑ 2,913 ± 1,338
Notch markers				
	Notch1 mRNA	↑* 2,096 ± 0,02613	↓↑ 1,185 ± 0,08566	↑ 1,460 ± 0,2858
	Notch2 mRNA	↑ 1,807 ± 0,2696	↓↑ 0,8063 ± 0,1477	↓↑ 0,8674± 0,05704
	Notch3 mRNA	↑ 1,543 ± 0,2627	↓↑ 0,9848 ± 0,2933	↓ 0,6119 ±0,09868
	Jagged1 mRNA	↑ 2,633 ± 0,5159	↑ 1,529 ± 0,2996	↓ 0,8272 ± 0,2529
	Hes1 mRNA	↓↑ 0,8795 ± 0,1181	↓* 0,4431 ± 0,04125	↓↑ 0,9004 ± 0,2051
Metastatic markers				
	MMP9 mRNA	↑ 2,688 ± 0,8184	↓↑ 0,8170 ± 0,2145	↓↑ 0,8541 ± 0,1239
	ItgaV mRNA	↑* 3,063 ± 0,9327	↓↑ 0,9311 ± 0,2445	↓↑ 0,7502 ± 0,3067

First row: ↓↑: No change compared to DEN control, ↑: Increase compared to DEN control, ↓: Decrease compared to DEN control.
Second row: fold changes ±Standard deviation, compared to PBS control .
*: p<0,05, **: p<0,01

1.5. Discussion

In the present study, we show that pan-PHD inhibition in early and advanced stages of hepatocarcinogenesis induces increased expression of LPC characteristics, while PHD inhibition in intermediate stages has a tendency to decrease the expression of LPC characteristics.

Furthermore, the early, but not intermediate or advanced-stage HIF-1α stabilization, concurred with increased expression of actors of the Notch pathway and metastatic markers. These results indicate an important time-dependent effect of hypoxic stimuli in HCC and a previously undescribed detrimental delayed effect of an early hypoxic event on tumor development. Currently LPCs are being intensively studied for their role in various liver diseases and have recently also been implicated in the pathogenesis of primary liver tumors. Increased expression of LPC characteristics serves as a marker for poor prognosis (21, 23, 24, 29). Moreover, since LPCs highly express multi drug resistance proteins, they are also implicated in therapy resistance (31). Furthermore, different studies have shown a phenotypic switch from HCC to HCC-CC following hypoxic stimuli, coinciding with increased expression of progenitor cell markers (6, 17, 18). Since activation of the hypoxic pathway could alter LPC behavior in hepatocellular carcinoma, we studied the effect of increased activation of HIF on DEN-induced hepatocarcinogenesis.

We used DMOG to induce the hypoxic response, aimed at mimicking oxygen deprivation at different time points in tumor development. The early HIF-1α stabilization reflects patients with chronic liver disease, characterized with fibrotic strands and hypoxia prior to tumor development.Additionally, early HIF-1α stabilization may also mimic hypoxia-inducing treatment strategies affecting recurrent tumor behavior.

The intermediate DMOG induction relates to patients undergoing anti-angiogenic treatment for early stage cancer. Lastly, the group receiving DMOG at advanced stages resembles patients receiving treatment for advanced stage HCC.

Immunohistochemistry for HIF-1α expression showed few hepatocytes expressing HIF-1α in saline groups, and cells expressing the HIF-1α protein in DEN groups were mostly residing in the portal area, and in and around hepatocellular- and cholangioma lesions, coinciding with CK19 and Epcam immunopositive regions.

Our aim was not to show HIF-1α stabilization following DMOG at specific time points but to evaluate its effect on tumorigenesis and LPC activation on the long term. We therefore chose to euthanize mice 7 days after the final DMOG injection, which is confronted with an attenuation of HIF-1α stabilization and transcriptional activation as shown in by the single DMOG injection experiment. Activation of the HIF pathway in DEN groups was examined through qPCR analysis of HIF target genes, no difference could be detected between intermediate and advanced DMOG and their respective PBS controls, confirming that acute effects of PHD inhibition were eliminated by euthanizing animals 7 days after the final DMOG injection.

Strangely, saline groups had an equal Glut1 and Pfk and even an increased Vegfa mRNA expression compared to intermediate and advanced DMOG groups and their PBS controls. Since saline mice had also received DMOG, possibly a variety of feedback loops could be differentially regulated in DEN compared saline mice (31, 32), which should be further investigated.

Interestingly, the DEN group that received DMOG at early stages, did show significantly increased expression Vegfa and Glut1 compared to other groups. HIF induction early in DEN-induced hepatocarcinogenesis also caused increased formation of cholangioma and HCC lesions as well as a massive upregulation of LPC features and metastatic markers on the RNA level, compared to groups that received DMOG or PBS at intermediate stages.

This massive delayed effect of hypoxia has not previously been described and indicates that early hypoxia could readily prepare cells for later tumor growth and growth–induced hypoxia, resulting in tumors with a more aggressive phenotype.

Thus, monitoring the extent to which the hypoxic pathway is activated after hypoxic treatment for recurring tumors or as a result of inflammation and fibrosis in chronic liver disease could have prognostic value when these patients (re)develop HCC later on.

Indeed, there is evidence of a phenotypic switch in tumors recurring after TACE, which induces a massive hypoxic response (18, 19).

Mice receiving DMOG during tumor development (at intermediate stages) displayed no HCC lesions and no altered expression of LPC or metastatic markers compared to PBS control mice. While the increased formation of cholangioma lesions should be monitored, these benign intrahepatic bile duct adenomas usually do not require treatment (33). Taken together, this could point to a safe therapeutic window for hypoxia inducing treatment after early detection.

Administering the pan-PHD inhibitor DMOG in advanced stages of tumor development resulted in a significantly increased relative liver weight, a slight decrease in HCC and a minor increase in cholangioma lesions coinciding with a significantly increased expression of LPC markers and number of CK19+ single cells. This observation is in line with previous reports showing that treatment-induced hypoxia is linked to an increased expression of stem/progenitor characteristics (16, 34, 35). However, while this hypoxia-induced LPC signature did not coincide with increased expression of metastatic markers, HCC lesions with a cholangiocytic signature have been linked to poor prognosis and early recurrence (20-24, 30, 36). Furthermore, while the increased relative liver weight compared to PBS counterparts is at least partly caused by the increased amount of cholangioma and its accompanying cholangiofibrosis, it could also be a sign of increased tumor burden.

Notch signalization is involved in LPC proliferation and pushes LPC differentiation towards cholangiocytic structures. Since we observed an increased incidence of cholangioma lesions in DEN livers after a hypoxic insult, RNA expression of Notch related genes was analyzed. Increased mRNA expression of actors of the Notch pathway was seen in livers of mice receiving DMOG early in hepatocarcinogenesis coinciding with high expression of LPC- and metastatic markers.

This suggests a role for Notch-mediated increased proliferation of LPCs and differentiation towards cholangiocytes in the pathogenesis of HCC after early hypoxic stimuli, thus contributing to the development or recurrence of aggressive, more invasive tumors with a mixed phenotype.

Indeed, pharmacological inhibition of the Notch pathway has already been proven to be effective in reducing the amount of chemo-resistant cancer stem cells in breast and colon cancer (37, 38). Surprisingly, DMOG administration at both intermediate and advanced stages did not lead to increased expression of actors of the Notch pathway.

While CK19 and CK7 positive liver tumors have been proposed to be progenitor cell derived (37), in vitro experiments have shown that HCC cells are capable of trans differentiating towards a cholangiocytic phenotype (39, 40). The fact that the stem cell marker Prom1 is only marginally up-regulated compared to the pronounced cholangiocytic markers CK7 and CK19, and there does not appear to be any Notch involvement in tumors undergoing hypoxia at advanced stages, might reflect this HCC trans-differentiation rather than LPC involvement. However, while whole liver analysis showed no Notch pathway activation, individual cell populations should be analyzed for more clarity on Notch involvement.

The present study underlines that early hypoxic stimuli have detrimental effects on tumor progression with an increased expression of poor prognostic markers later on.

Activation of the HIF pathway at advanced stages of tumorigenesis resulted in severely increased expression of LPC characteristics without Notch activation. Hypoxic treatment at intermediate stages of DEN-induced hepatocarcinogenesis appears to have the least detrimental effect on tumor progression and reflects the advantages of early tumor diagnosis with most favorable treatment options/effects.

Supplementary data

Table SI: Genes and primersets

Gene ID	short	Full name	Forward primer	Reverse primer	Marker
14433	GAPDH	glyceraldehyde 3 phosphate dehydrogenase	CATGGCCTTCCGTGT TCCTA	GCGGCACGTCAG ATCCA	Reference
15288	HMBS	hydroxymethyl-bilane synthase	AAG GGC TTT TCT GAG GCA CC	AGT TGC CCA TCT TTC ATC ACT G	Reference
15452	HPRT	hypoxanthine guanine phosphoribosyl transferase	GTT AAG CAG TAC AGC CCC AAA	AGG GCA TAT CCA ACA ACA AAC TT	Reference
66945	SDHA	succinate dehydrogenase complex, subunit A	CTTGAATGAGGCTGA CTGTG	ATCACATAAGCTG GTCCTGT	Reference
11576	AFP	alpha – fetoprotein	AGCTTCCACGTTAGA TTCCTCC	ACAAACTGGGTA AAGGTGATGG	LPC
16669	CK19	Cytokeratin 19	GTTCAGTACGCATTG GGTCAG	GAGGACGAGGTC ACGAAGC	LPC
110310	CK7	Cytokeratin 7	AGGAGATCAACCGA CGCAC	CACCTTGTTCGTG TAGGCG	LPC
12505	CD44	CD44 antigen	TCGATTTGAATGTAA CCTGCCG	CAGTCCGGGAGA TACTGTAGC	LPC
19126	Prom1	Prominin 1	CTCCCATCAGTGGAT AGAGAACT	ATACCCCCTTTTG ACGAGGCT	LPC
17075	Epcam	Epithelial cell adhesion molecule	GCGGCTCAGAGAGA CTGTG	CCAAGCATTTAGA CGCCAGTTT	LPC
16402	ITGAV	Integrine alpha 5	CAATTGCTGCTCCCT ATGGT	GATTTGAGATGG CACCGAAT	Metastasis
17395	MMP9	Matrix metalloproteïnase 9	GAGACGGGTATCCCT TCGAC	TGACATGGGGCA CCATTTGAG	Metastasis
16449	JAG1	Jagged 1	ATGCAGAACGTGAAT GGAGAG	GCGGGACTGATA CTCCTTGAG	NOTCH
18128	NOTCH1	Notch 1	GATGGCCTCAATGGG TACAAG	TCGTTGTTGTTGA TGTCACAGT	NOTCH
18129	NOTCH2	Neurogenic locus notch homolog protein 2	ATGTGGACGAGTGTC TGTTGC	GGAAGCATAGGC ACAGTCATC	NOTCH
18131	NOTCH3	Neurogenic locus notch homolog protein 3	AGTGCCGATCTGGTA CAAGTT	CACTACGGGGTTC TCACACA	NOTCH
15205	HES1	Hairy enhancer of split 1	ACGTGCGAGGGCGT TAATAC	ACGTGCGAGGGC GTTAATAC	NOTCH
22339	VEGFa	Vascular endothelial growth factor A	ACTCGGATGCCGACA CGGGA	CCTGGCCTTGCTT GCTCCCC	Hypoxia
20525	Glut1	Glucose transporter 1	GCT TAT GGG CTT CTC CAA ACT	GT GAC ACC TCT CCC ACA TAC	Hypoxia
18642	Pfk	Phosphofructokinase	GCCGGCTCAGTGAG ACAAG	TGGCACCTTCAGC AACAATG	Hypoxia

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1.7. Addendum/Corrigendum

Materials and methods

The number of mice per group should be added to the materials and methods (Table A1)

TableA1: Total number of analysed mice/group

22 weeks				27 weeks		
Saline Ctrl	PBS Ctrl	Early DMOG	Int. DMOG	Saline Ctrl	PBS Ctrl	Adv. DMOG
8	8	6	5	7	6	5

Official gene symbols were not consequently used in this manuscript, correct nomenclature of used abbreviations is provided in table A2.

Table A2: Gene symbols

Gene ID	Gene symbol	short	Full name	Marker
14433	*Gapdh*	GAPDH	glyceraldehyde 3 phosphate dehydrogenase	Reference
15288	*Hmbs*	HMBS	hydroxymethyl-bilane synthase	Reference
15452	*Hprt*	HPRT	hypoxanthine guanine phosphoribosyl transferase	Reference
66945	*Sdha*	SDHA	succinate dehydrogenase complex, subunit A	Reference
11576	*Afp*	AFP	alpha – fetoprotein	LPC
16669	*Krt19*	CK19	Cytokeratin 19	LPC
110310	*Krt7*	CK7	Cytokeratin 7	LPC

12505	*Cd44*	CD44	CD44 antigen	LPC
19126	*Prom1*	PROM1	Prominin 1	LPC
17075	*Epcam*	EPCAM	Epithelial cell adhesion molecule	LPC
16402	*Itga5*	ITGAV	Integrine alpha 5	Metastasis
17395	*Mmp9*	MMP9	Matrix metalloproteïnase 9	Metastasis
16449	*Jag1*	JAG1	Jagged 1	NOTCH
18128	*Notch1*	NOTCH1	Notch 1	NOTCH
18129	*Notch2*	NOTCH2	Neurogenic locus notch homolog protein 2	NOTCH
18131	*Notch3*	NOTCH3	Neurogenic locus notch homolog protein 3	NOTCH
15205	*Hes1*	HES1	Hairy enhancer of split 1	NOTCH
22339	*Vegfa*	VEGFa	Vascular endothelial growth factor A	Hypoxia
20525	*Slc2a1*	Glut1	Glucose transporter 1	Hypoxia
18642	*Pfk*	Pfk	Phosphofructokinase	Hypoxia

Results

Y-Axes of graphs illustrating qPCR data show the normalised gene expression.

The legend of Figure 2 should be adjusted to:

Figure 2: General parameters.
A: Liver/ bodyweight ratios for all groups. **B**: Representative images of Sirius red and reticulin staining showing cholangioma lesions in all DMOG groups except for PBS control after 22 weeks, and HCC lesions in all DEN groups except for the Int. DMOG group. **C:** Prevalence of cholangioma and hepatocellular lesions, showing percentage of mice showing one or more cholangioma or (premalignant) HCC lesions.
Scale bars: 200µm, *: p<0,05; **p<0,01
DMOG: dimethyloxaloylglycine; DEN: diethylnitrosamine

2. EFFECT OF PROLYL HYDROXYLASE DOMAIN 2 HAPLODEFICIENCY ON LIVER PROGENITOR CELL CHARACTERISTICS EARLY IN MOUSE HEPATOCARCINOGENESIS

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2.1. Abstract

Activation of the hypoxia-inducible factor (HIF)-pathway in hepatocellular carcinoma (HCC) induces therapy resistant tumours, characterized by increased liver progenitor cell (LPCs) characteristics and poor prognosis. We previously reported corresponding results in mice with HCC in which hypoxia was mimicked by prolyl hydroxylase domain (PHD) inhibition. Here, we aimed at investigating whether induction of LPC characteristics occurs during the onset of hepatocarcinogenesis and if this is associated with activation of Notch signalling. Dietheylnitrosamine (DEN) was used to induce hepatic tumours in PHD2 haplodeficient (PHD2+/-) mice which were euthanized at 5, 10, 15 and 17 weeks following DEN during neoplastic transformation, before tumour formation. Neoplasia and mRNA expression of LPC and Notch markers were evaluated by histology and qPCR on isolated livers. PHD2 haplodeficiency resulted in enhanced expression of HIF target genes after 17 weeks of DEN compared to wild type (WT) littermates but had no effect on the onset of neoplastic transformation. The mRNA expression of Afp and Epcam was increased at all time points following DEN whereas CK19, Prom1 and Notch3 were increased after 17 weeks of DEN, without difference between PHD2+/- and WT mice. MDR1 mRNA expression was increased in all DEN treated mice compared to saline control with increased expression in PHD2+/- compared to WT from 15 weeks. These results indicate that the effects of PHD2 haplodeficiency on the expression of LPC and Notch markers manifest during tumour nodule formation and not early on during neoplastic transformation.

2.2. Introduction

With an estimated overall five year survival of less than 20%, liver cancer is the 2nd leading cause of cancer related death worldwide (1). Liver tumours often arise in a background of chronic liver disease characterised by inflammation, sinusoidal capillarisation and the formation of fibrous septa.

Moreover, when tumours outgrow their vascular supply, newly formed vasculature is often structurally and functionally anomalous during further tumour growth. These processes contribute to a reduced liver oxygenation early during tumour development and later, during tumour growth (2, 3).

Insufficient oxygen supply results in hypoxia, a situation known to inhibit prolyl hydroxylase domain (PHD) enzyme activity, causing stabilisation of the hypoxia inducible factor (HIF). HIF stabilisation and its nuclear translocation results in the transactivation of genes involved in cell-survival by, amongst others, stimulating (neo-) angiogenesis (through induction of pro-angiogenic factors, such as the vascular endothelial factor or Vegf), and boosting the anaerobe glucose metabolism (via Glucose transporter 1 or Glut1, phospho-fructokinase or Pfk) (2, 4, 5). Activation of the hypoxia inducible pathway is known as the 'hypoxic adaptive response' and has extensively been investigated in tumorigenesis as a mediator of tumour growth, therapy resistance and metastasis (6-8)

Previous studies have shown that activation of the hypoxic pathway can induce therapy resistance and is related to poor prognosis in primary liver tumours (8-13).

Furthermore, in humans, pre-operative trans-arterial chemoembolization, which has been shown to induce a hypoxic adaptive response, has been linked to higher recurrence rates and a phenotypic switch from hepatcellular carcinoma (HCC) to hepato-cholangiocarcinoma (HCC-CC), with increased expression LPC characteristics (14-16).

In accordance, we previously reported that inhibiting PHDs, (using a pan-PHD inhibitor or PHD2 haplodeficient mice (PHD2+/-), in murine diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC), results in a more aggressive mixed hepato-cholangiocarcinoma (HCC-CC) phenotype high in LPC characteristics, coinciding with increased expression of markers for metastasis and actors of the Notch signalling pathway (2, 4).

Possibly, PHD inhibition during carcinogenesis can readily prime future tumour cells to react differently to later hypoxic stimuli, and give rise to more aggressive mixed phenotype cancers, with increased LPC characteristics and an increased risk for therapy resistance and metastasis (5).

LPCs are bipotential cells that reside in the canals of Hering in the liver, were they act as facultative adult stem cells (17). In healthy liver, loss of hepatocyte or cholangiocyte cell mass can easily be replaced by the immense self-replicative capacity of the parenchyma. However, in situations of severely reduced liver function, like in chronic liver disease, the progenitor cell compartment is activated (18).

LPCs then proliferate and migrate to the site of injury where they differentiate to replenish the lost cell mass by a series of tightly organised interactions controlled by the Notch and Wnt signalling pathways. Activation of the Notch pathway drives LPC's towards a cholangiocytic phenotype, while Wnt-induced inhibition of Notch signalisation results in hepatic differentiation (18).The Notch pathway not only plays a pivotal role in the cell-fate determination of LPCs, it is also shown to be an important mediator of hepatocarcinogenesis. Interestingly, activation or inhibition of the different Notch receptors can have both pro- and anti-oncogenic effects (19-22).

In our previous studies, mRNA expression of actors of the Notch signalling pathway was increased in DEN-induced HCC in which PHDs were inhibited. The Notch pathway could thus play a role in PHD inhibition-mediated expression of LPC characteristics, which would be an attractive therapeutic target.

Since we observed increased expression of LPC characteristics by inhibiting PHD proteins during HCC development, which was associated with increased mRNA expression of actors of the Notch pathway, we aimed to investigate if the effect of PHD2 haplodeficiency on liver tumour phenotype in advanced DEN-induced HCC is preceded by altered LPC and/or Notch expression at early stages of hepatocarcinogenesis.

A better understanding of the effect of hypoxic conditions early during tumour initiation and development, mimicked by PHD2 haplodeficiency, a situation readily present during chronic liver disease and tumour relapse, could allow us to pinpoint critical markers and events involved in the observed hypoxia-induced phenotypic switch, therapy resistance and metastasis.

2.3. Materials and methods

Induction of hepatocarcinogenesis in PHD2 haplodeficient mice

PHD2+/- mice were obtained from the Vesalius Research Center (KUleuven, Leuven, Belgium). A heterozygous couple was used for breeding and offspring was genotyped using the following primers in a concentration of 10μM: ACCTATGATCTCAGCATTTGGGAG, TCAGGACAGTGAAGCCTAGAAACT and AAATTCTAATCGTAGCTGATGTGAGC (2).

To investigate the effect of PHD2 haplodeficiency on early hepatocarcinogenesis in mice, 5 week old PHD2+/- and wild type (WT) littermates (129S6 background) received weekly intraperitoneal DEN injections (35mg/kg, Sigma –Aldrich, Bornem, Belgium).

This induces microscopic neoplastic cells after 15 weeks, macroscopic nodule formation at 20 weeks and HCC after 25 weeks, which was previously reported by our group (23). These mice were euthanised after the 5th, 10th, 15th and 17th week of DEN, before HCC nodules could form (23). As we have previously shown that there is no difference between WT and PHD2+/- healthy mice (2), we administered weekly saline injections for 17 weeks to PHD2+/- mice as controls.

Mice were euthanised at indicated time-points by cervical dislocation, the liver was prelevated and divided for histology and qPCR analysis, respectively submerged in 4% formaldehyde (Klinipath, Olen, Belgium) for paraffin embedding and stored at -80°C in RNA later (Ambion, Thermo Fisher scientific, Gent Belgium) for RNA extraction.

All experiments were approved by the ethical committee for animal experiments at the faculty of medicine and health sciences of Ghent University Belgium (ECD13/61).

Histological evaluation

General morphology of liver tissue was assessed using Haematoxylin-Eosin, Sirius Red and Reticulin stainings on 5µm sections of paraffin embedded tissue as routinely described. Neoplasticity was defined as enlarged cells with normal nucleus to cytoplasm ratio (n/c), small cells with increased n/c, enlarged pleomorphic nuclei, and binucleation, (pre) neoplastic hepatocytic lesions were identified by loss of reticulin staining and sirius red staining was performed to identify potential cholangiocytic lesions marked by cholangiofibrosis, as previously described (4).

Cytokeratin 19 immunohistochemistry (1/200 in TBS, ab133496, RRID:AB_11155282, abcam, Cambridge, UK) was used to visualize structures of the cholangiocytic lineage as well as LPCs.

Overall CK19 immunoreactivity was measured using Cell D software (Olympus Imaging Solutions, Münster, Germany) and to evaluate the LPC response, 5 portal areas were centred at a magnification of 400 and all CK19 positive single cells were counted.

Quantitative real time PCR (qPCR)

RNA was extracted from 20 mg of frozen liver tissue preserved in RNA-later, according to the manufacturer's guidelines (Rneasy Mini Kit, Quiagen, Venlo, the Nederlands).

cDNA was obtained from 1µg RNA using the iScript cDNA synthesis kit (Bio-Rad, Nazareth-Eke, Belgium) and quantitative PCR (qPCR) analyses were performed using a SYBR green mix (Sensifast Bioline Reagents Ltd, London, UK), using the primersets listed in table1.

All reactions were run in duplicate; the comparative Cq method was used to determine the gene expression which was normalised to reference genes that showed stable expression in all samples, as also previously described (2, 4).

Table 1:primersets

short	Full name	Forward primer	Reverse primer
Gapdh	glyceraldehyde 3 phosphate dehydrogenase	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
Hmbs	hydroxymethyl-bilane synthase	AAGGGCTTTTCTGAGGCACC	AGTTGCCCATCTTTCATCACTG
Hprt	hypoxanthine guanine phosphoribosyl transferase	GTTAAGCAGTACAGCCCCAAA	AGGGCATATCCAACAACAAACTT
Sdha	succinate dehydrogenase complex, subunit A	CTTGAATGAGGCTGACTGTG	ATCACATAAGCTGGTCCTGT
Afp	alpha – fetoprotein	AGCTTCCACGTTAGATTCCTCC	ACAAACTGGGTAAAGGTGATGG
CK19	Cytokeratin 19	GTTCAGTACGCATTGGGTCAG	GAGGACGAGGTCACGAAGC
Prom1	Prominin 1	CTCCCATCAGTGGATAGAGAAC	ATACCCCCTTTTGACGAGGCT
EpCam	Epithelial cell adhesion molecule	GCGGCTCAGAGAGACTGTG	CCAAGCATTTAGACGCCAGTTT
Mdr1	Multi drug resistance protein 1	AGCCGTAAGAGGCTGAGGCCG	TCACGTGCCACCTCCGGGTT
Jag1	Jagged 1	ATGCAGAACGTGAATGGAGAG	GCGGGACTGATACTCCTTGAG
Notch1	Notch 1	GATGGCCTCAATGGGTACAAG	TCGTTGTTGTTGATGTCACAGT
Notch2	Neurogenic locus notch homolog protein 2	ATGTGGACGAGTGTCTGTTGC	GGAAGCATAGGCACAGTCATC
Notch3	Neurogenic locus notch homolog protein 3	AGTGCCGATCTGGTACAAGTT	CACTACGGGGTTCTCACACA
Hes1	Hairy enhancer of split 1	ACGTGCGAGGGCGTTAATAC	ACGTGCGAGGGCGTTAATAC
Vegfa	Vascular endothelial growth factor A	ACTCGGATGCCGACACGGGA	CCTGGCCTTGCTTGCTCCCC
Glut1	Glucose transporter 1	GCTTATGGGCTTCTCCAAACT	GTGACACCTCTCCCACATAC
Pfk	Phosphofructokinase	GCCGGCTCAGTGAGACAAG	TGGCACCTTCAGCAACAATG

Statistical analysis

Data were analysed using SPSS23 software (IMB corp, Armonk NY, USA) and graphs were illustrated using Graphpad prism 6 software (Graphpad software, inc; San Diego CA, USA). Kolmogorov-Smirnov test was used to test for normality. Student's T-test was then performed in case of normality; the Mann-Whitney-U test was used for not normally distributed data. P-values ≤0.05 were considered significant. All data are presented as mean ±SEM.

2.4. Results

PHD2 haplodeficiency does not alter the onset of neoplastic transformation

We first assessed the effect of PHD2 haplodeficiency on HIF stabilisation by assessing the activation of HIF target genes. In early hepatocarcinogenesis, we did not observe a significant activation of the hypoxic pathway compared to saline control mice, in either genotype. However, HIF downstream targets showed a peak RNA expression after 17 weeks of DEN compared to other time-points (Figure 1A, C) and in PHD2+/- mice compared to WT mice at the same time-point. (Figure 1B, C). To assess general morphology and neoplasia, haematoxylin-eosin, sirius red and reticulin stainings were performed.

Neoplastic cells and reticulin free hepatocytic plates could be observed from 10 weeks onwards (Figure S1) and neoplastic nodules were observed from 15 weeks onwards (Figure S1). Sirius red staining was evaluated as previously described (4), and showed no cholangiocytic lesions (Figure S1). No difference was observed between PHD2+/- and WT livers at the indicated time points.

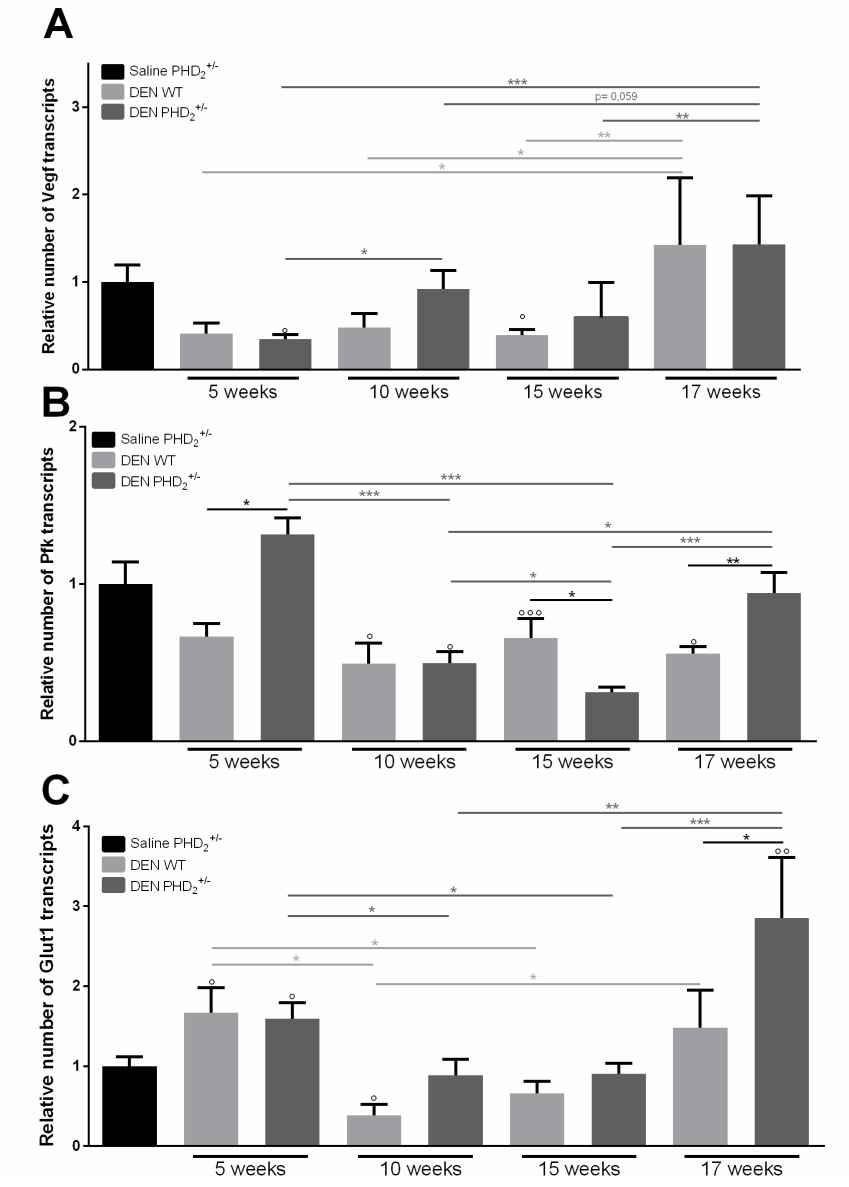


Figure 1: mRNA expression of HIF target genes

A. vascular endothelial growth factor alpha (Vegfa), B. phosphofructokinase (Pfk) and C. glucose transporter 1 (Glut1) in $PHD_2^{+/-}$ and WT mice, euthanised at different time points in hepatocarcinogenesis.

°: p<0.05, °°: p<0.01 and °°°:p<0.001 compared to saline control mice

*:p<0.05, **:p<0.01 and ***:p<0.001

Neoplastic transformation coincides with increased expression of LPC characteristics during early hepatocarcinogenesis

To evaluate the effect of PHD2 haplodeficiency on the expression of LPC characteristics, we performed qPCR analysis of Cytokeratin 19 (CK19), Prominin 1 (Prom1), Epithelial cell adhesion molecule (Epcam), Alpha fetoprotein (Afp) and multi drug resistance protein 1 (MDR1). In the early pathogenesis of DEN-induced HCC, the mRNA expression of Epcam and Afp was continuously upregulated in all DEN treated mice compared to saline control (Figure 2A,B), strengthening the evidence for these characteristics as good markers of carcinogenesis (24, 25).

While no time dependent, PHD2 haplodefiency related effect could be observed concerning Epcam mRNA expression (Figure 2A), Afp expression was significantly increased after 15 weeks in PHD2+/- mice compared to WT livers. However, this increased expression was not maintained after 17 weeks of DEN induction (Figure 2B).

Like Afp and Epcam, MDR1 mRNA expression was increased in all groups that received DEN compared to saline control (Figure 2E). Furthermore, MDR1 expression was significantly increased after 15 and 17 weeks of DEN, compared to all earlier time points in PHD2+/- mice and compared to WT counterparts, and differed significantly between 15 and 17 weeks of DEN in WT livers (Figure 2E).

Comparison of CK19 immunopositivity between PHD2+/- and WT mice at different time points in hepatocarcinogenesis showed an increased number of central vein concentrated CK19+ single cells after 15 and 17 weeks of DEN (Figure 3A) and a tendency towards increased CK19 expression in PHD2+/- mice at week 15 and 17 compared to earlier time points and compared to WT counterparts (Figure 3B).

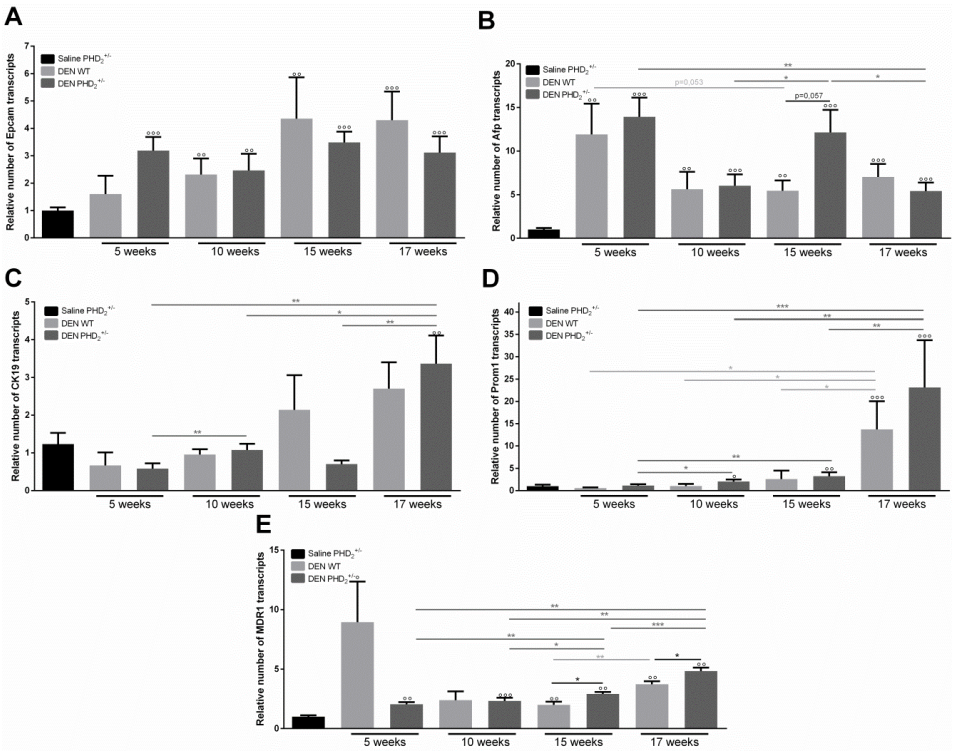


Figure 2: mRNA expression of LPC markers

A. epithelial cell adhesion molecule (Epcam), **B**. alpha feto-protein (Afp), **C**. cytokeratin 19 (CK19),

D. prominin 1 (Prom1) and **E**. multi drug resistance protein 1 (MDR1) in PHD2+/- and WT mice

°: p<0.05, °°: p<0.01 and °°°:p<0.001 compared to saline control mice

*:p<0.05, **:p<0.01 and ***:p<0.001

Induction of the hypoxic adaptive response coincides with increased expression of Notch3 mRNA in early hepatocarcinogenesis

The Notch pathway plays a pivotal role in the cell-fate determination of LPCs and could also play a role in the increased expression of LPC characteristics observed after PHD inhibition. We therefore investigated the mRNA expression of Notch markers in early DEN-induced hepatocarcinogenesis in PHD2+/- and WT counterparts. We performed qPCR analysis of Notch receptors Notch1, 2 and 3, Notch ligand Jagged 1 (Jag1) as well as the main Notch effector gene Hairy enhancer of split 1 (Hes1).

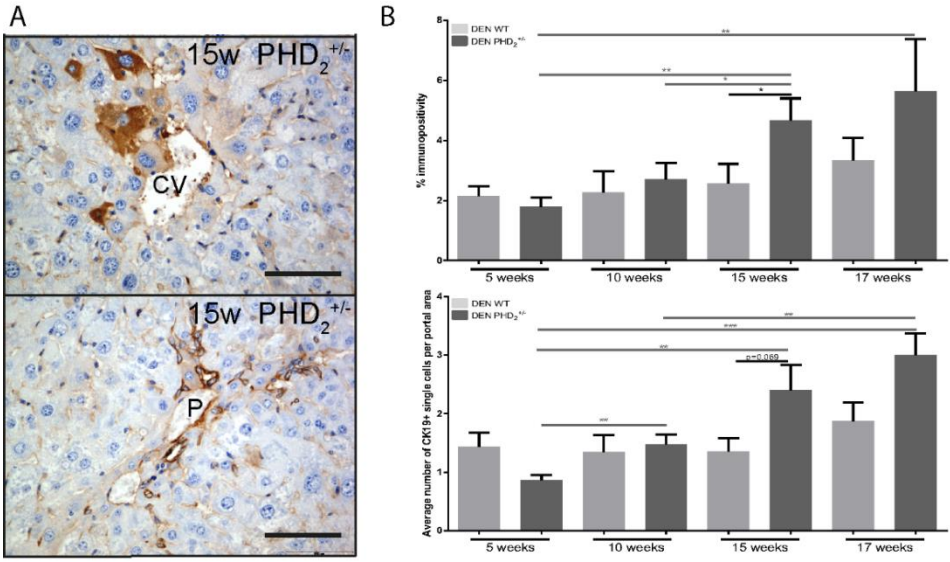


Figure 3: cytokeratin 19 immunohistochemistry

A. CK19 immunopositive hepatocytes around the central vein after 15 week of DEN.**B.**percent immunopositivity and average number of CK19 positive cells per portal area in PHD2+/- and WT counterparts at different time points in hepatocarcinogenesis.

CV: Central vein, P: Portal vein, Scale bars: 10µm

*:p<0.05, **:p<0.01 and ***:p<0.001

DEN treatment did not induce consistent effects on Notch1 and Notch2 mRNA expression (Figure 4A,B). Expression of Notch3, Hes1 and Jag1 mRNA was significantly upregulated compared to saline control after 17 weeks of DEN in both WT and PHD2+/- livers which coincides with increased expression of markers for hypoxia and HIF stabilisation (Figure 1, 4C,D,E). However, no difference could be observed between PHD2+/- and WT mice. After 17 weeks mRNA expression of Notch3 and Jag1 was also significantly upregulated in PHD2+/- livers compared to same genotype livers at earlier time points (Figure 4C,D).

2.5. Discussion

We have previously shown that in the DEN mouse model for hepatocarcinogenesis, PHD inhibition results in a mixed HCC-CC phenotype, high in LPC characteristics, which has been associated with a worse prognosis (2, 4).

In this study we aimed to investigate the effect of continuous PHD inhibition in early stages of hepatocarcinogenesis. We therefore used $PHD_2^{+/-}$ mice that were euthanised at different time points to unravel the dynamics of PHD_2 haplodeficiency during early hepatocarcinogenesis, before nodule formation.

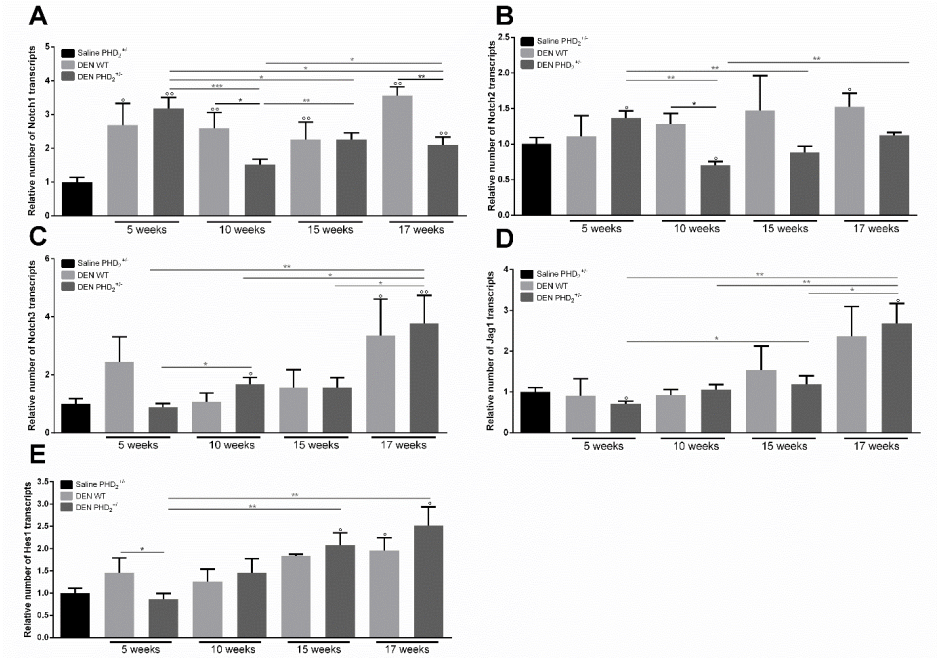


Figure 4: mRNA expression of Notch receptors and Notch target genes

A. Notch1, **B.** Notch2, **C**. Notch3, **D**. jagged 1 (Jag1) and **E**.hairy transcriptor of split 1 (HES1) in PHD2+/- and WT mice, euthanised at different time points in hepatocarcinogenesis.

°: p<0.05, °°: p<0.01 and °°°:p<0.001 compared to saline control mice

*:p<0.05, **:p<0.01 and ***:p<0.001

We observed that PHD2 haplodeficiency did not result in altered liver morphology or onset of neoplastic transformation compared to WT controls. After 17 weeks of DEN, we observed a peak expression of HIF target genes, which was more pronounced in $PHD_2^{+/-}$ livers and coincided with the start of nodule formation as shown by histology.

This allows the assumption that PHD haplodeficiency only affects gene expression of HIF target genes in the presence of hypoxia.

We found that Afp and Epcam mRNA expression was continuously upregulated, in all DEN treated mice, at all observed time points. Indeed, Afp and Epcam have been shown to be expressed in hepatocytes during embryogenesis and in cirrhotic and cancerous livers (24-28).

The mRNA expression of CK19, Prom1 peaked after 17 weeks of DEN and coincided with increased expression of HIF target genes. Inherently to its microscopic structure, the liver can be divided in 3 zones, reflecting the level of oxygenation, with the hepatocytes around the central vein most prone to oxygen deprivation. The effects of PHD2 haplodeficiency will thus be most apparent in those cells. We did observe CK19+ hepatocytes around the central vein from 15 weeks onwards, further indicating that expression of CK19 could be related to increased HIF stabilisation. While it is unclear if CK19 and Prom1 expressing cells are progenitor cell derived (29), or dedifferentiated hepatocytes (30), recent studies have shown that increased CK19 and Prom1 expression in HCC is related to prognosis (29, 31, 32) and recurrence (15, 32).

Multi Drug resistance (MDR) proteins, which are inherently expressed by stem – and progenitor cells (33) are drivers of therapy resistance and have been shown upregulated in hypoxic conditions (13, 34), attributing to the observed poor prognosis for liver cancer with an increased progenitor and/or hypoxic signature.

Interestingly, MDR1 mRNA expression was increased in all DEN treated mice, compared to saline control and in $PHD_2^{+/-}$ livers compared to WT counterparts from 15 weeks onwards, indicating that MDR1 could possibly be a marker for decreased PHD activity in early hepatocarcinogenesis. mRNA expression of MDR1 has, to our knowledge, not yet been mapped over time in animal models for hepatocarcinogenesis and its value as a potential marker for ongoing tumorigenesis and increased hypoxic signalling has not yet been explored.

Activation of the Notch signalling pathway has been shown to be involved in liver - and other cancers, with contradictory proposed roles (20, 35, 36).

Furthermore, while the distribution and prevalence of different Notch receptors have been reported in human healthy and diseased liver(37) and in a murine model for experimental HCC (21), little is known about the expression of different Notch receptors in relation to phenotype and prognosis in experimental or human liver cancer.

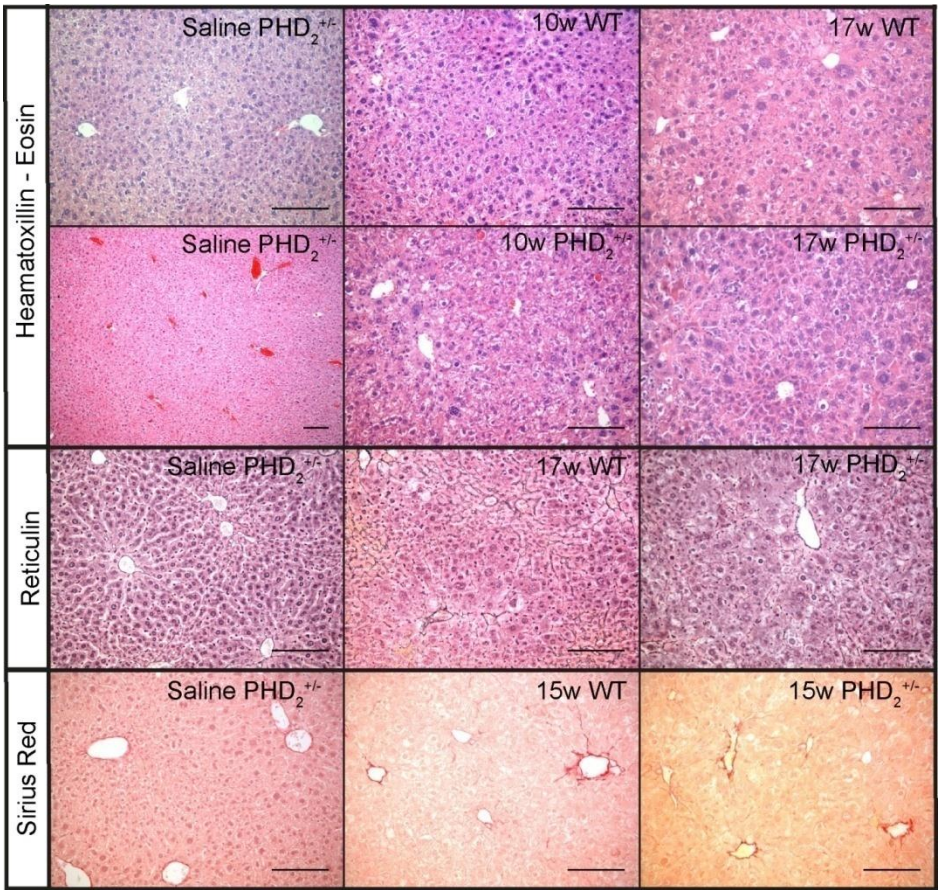
Inhibiting Notch 2 decreased HCC cell proliferation (22) and tumour burden (21), while Notch 1 and 2 overexpression in the liver resulted in spontaneous HCC development with biliary or LPC characteristics (19, 36, 38).These observations are similar to the phenotype observed after PHD inhibition in HCC mice in previous studies (2, 4) and as in human tumours recurring after TACE treatment (14, 39). In this study we could not show altered Notch1 or 2 receptor mRNA expression during early hepatocarcinogenesis. Yet, we previously showed increased Notch2 mRNA expression, following PHD inhibition, associated with increased hepatocellular and cholangiocellular tumour burden at end stage DEN-induced carcinogenesis (2, 4).

In PHD2+/- and WT mice, Notch3 expression peaked after 17 weeks of DEN, like CK19 and Prom1 mRNA expression, indicating that expression of these markers coincides with nodule formation. This is in line with previous data, were Notch3 overexpressing HCC cells were shown to have increased aldehyde dehydrogenase activity (40), characteristic for LPCs (41) and inhibition was shown to overcome therapy resistance in HCC cells, increasing sorafenib toxicity(42).

In conclusion, we used PHD2+/-mice to evaluate the effect of increased HIF stabilisation during early hepatocarcinogenesis. However, increased HIF signalling was only observed during nodule formation, coinciding with increased mRNA expression of LPC characteristics and Notch3. We hypothesise that previously observed effects of increased HIF signalling on tumour phenotype manifest during tumour growth rather than development and are not preceded by an early LPC or Notch signature.

Further elucidating possible mechanisms involved in this process could help to develop new therapeutic strategies to improve prognosis of patients with tumours growing in a fibrotic background, or receiving hypoxia- inducing therapies.

Supplementary material



FigureS1: representative images for Haematoxylin-Eosin, Reticulin and Sirius red stainings showing different groups at different time points. We observe neoplastic cells from 10 weeks onwards and loss of reticulin from 15 weeks onwards in PHD2+/- and WT livers. Sirius red staining showed no presence of cholangiocytic lesions. Scale bars 200µm

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2.7. Addendum/Corrigendum

Materials and methods

Final primerconcentration for genotyping was 400nM

A table showing the exact number of mice analysed for each group should be added to the materials and methods section (Table A1).

Table A1: Total number of mice/group

PHD2+/-					Wild type			
Saline	5w DEN	10w DEN	15w DEN	17w DEN	5w DEN	10w DEN	15w DEN	17w DEN
11	8	8	8	7	5	5	6	8

Official gene symbols were not consequently used in this manuscript, table A2 provides the correct gene symbol for the used abbreviations.

Table A2:gene symbols for used abbreviations

short	Gene symbol	Full name
Gapdh	*Gapdh*	glyceraldehyde 3 phosphate dehydrogenase
Hmbs	*Hmbs*	hydroxymethyl-bilane synthase
Hprt	*Hprt*	hypoxanthine guanine phosphoribosyl transferase
Sdha	*Sdha*	succinate dehydrogenase complex, subunit A
Afp	*Afp*	alpha – fetoprotein
CK19	*Krt19*	Cytokeratin 19
Prom1	*Prom1*	Prominin 1
EpCam	*Epcam*	Epithelial cell adhesion molecule
MDR1	*Abcb1b*	Multi drug resistance protein 1
Jag1	*Jag1*	Jagged 1
Notch1	*Notch1*	Notch 1
Notch2	*Notch2*	Neurogenic locus notch homolog protein 2
Notch3	*Notch3*	Neurogenic locus notch homolog protein 3
Hes1	*Hes1*	Hairy enhancer of split 1
Vegfa	*Vegfa*	Vascular endothelial growth factor A
Glut1	*Slc2a1*	Glucose transporter 1
Pfk	*Pkf*	Phosphofructokinase

Results

Y-Axes of graphs illustrating qPCR data show the normalised gene expression.

3. GAMMA SECRETASE INHIBITION DAMPENS HYPOXIA-INDUCED TUMOUR GROWTH AND DECREASES THE EXPRESSION OF LIVER PROGENITOR CELL CHARACTERISTICS IN HEPATOCELLULAR CARCINOMA.

Eliene Bogaerts, Annelies Paridaens,Anja Geerts, Hans Van Vlierbergheand Lindsey Devisscher

3.1. Abstract

Background

Treatment for advanced hepatocellular carcinoma is based on decreasing tumour vascularisation, thus reducing oxygenation, to dampen tumour growth. However, adaptive responses allow cells to respond to low oxygen tension by addressing a transcriptional cascade of pro-survival factors, something that has extensively been investigated in carcinogenesis. In hepatocellular carcinoma, increased hypoxic signalling has been related to increased expression of liver progenitor cell characteristics, associated with poor prognosis. Progenitor cell fate is regulated by the Notch signalling pathway and several reports have indicated that hypoxic and Notch signalling pathways cooperate in the induction of a more aggressive, therapy resistant phenotype, correlated with poor prognosis in cancer.

Methods

Our aim was to examine the effect of hypoxia on tumour growth, Notch and progenitor cell characteristics and the therapeutic potential of Notch inhibition to inhibit these effects. We therefore used a HepG2 xenograft mouse model, a hypoxic unit (10,5% O_2) and a gamma secretase inhibitor. Tumour size and tumour expression of markers for hypoxia, proliferation, Notch activation and liver progenitor cell characteristics were assessed after 2 weeks of treatment, in normoxic and hypoxic conditions.

Results

Hypoxia, accompanied by an enhanced hypoxic gene signature, significantly increased tumour growth, Notch activation and cytokeratin 19 expression in HepG2 xenografts. Treatment with the gamma secretase inhibitor decreased Notch activation and this was associated with reduced tumour growth in tumours grown in hypoxic conditions.

Gamma secretase inhibition was also associated with reduced expression of hypoxic and liver progenitor cell markers compared to control treatment, both in hypoxic and normoxic tumours.

Conclusions

Our data show that *in vivo* activation of the hypoxic adaptive response results in increased HCC tumour growth and cytokeratin 19 expression and that this is accompanied by Notch pathway activation. In addition, we provide promising pre-clinical evidence for the therapeutic potential of gamma secretase inhibition as an adjuvant to counteract the potential side-effects of hypoxia inducing therapies.

3.2. Introduction

Growing tumours are often insufficiently vascularised, resulting in low intratumoural oxygen concentrations and activation of the hypoxic adaptive response (1), a cellular adaptation mechanism that allows cells to rapidly respond to changes in oxygen tension. Upon insufficient oxygen supply, the hypoxia inducible factors 1 and 2 alpha (HIFα) are stabilised and migrate to the nucleus where a plethora of pro-survival genes are transactivated (2), which is crucial for many developmental and physiological processes (2, 3). However, in pathological conditions, like in carcinogenesis, activation of this hypoxic adaptive response can have detrimental effects on prognosis (2, 4). Indeed, in hepatocellular carcinoma (HCC), activation of the hypoxic adaptive response is linked to therapy resistance and increased invasive and metastatic potential (5). Furthermore, an increased hypoxic signature was linked to increased expression of liver progenitor cell (LPC) characteristics in primary liver tumours, which has been linked to worse prognosis (6-10).

Several reports have shown that hypoxia-mediated effects on proliferation, migration, invasion and therapy resistance in cancer are moderated by interactions between the hypoxia inducible factor (HIF) and NOTCH signalling (11-13).

The NOTCH pathway is important in stem cell self-renewal, and plays a specific role in binary cell fate decisions (14). The role of NOTCH signalling has been studied in HCC (15, 16) and overexpression of NOTCH signalling has been shown to induce hepatic tumours with increased expression of LPC markers (17-20), which was linked to poor prognosis.

There are 4 NOTCH receptors and 2 types of ligands described in mammals: the NOTCH 1, NOTCH 2, NOTCH 3 and NOTCH 4 receptors, and the jagged and Delta ligands. Ligand binding to the N-terminal extracellular domain of the receptor triggers cleavage of the C-terminal NOTCH intracellular domain (NICD) (14, 16, 21-24). NICD cleavage is a two-step process; the second step is mediated by the presenilin-gamma-secretase complex. Upon its release into the cytoplasm, NICD migrates to the nucleus, binds to CSL (CBF1/Su(H)/Lag-1), and recruits co-activators to induce NOTCH-dependent gene transcription, of which the transcription factor hairy and enhancer of split-1 (HES1) is one of the main target genes (14, 24).

Importantly, primary hepatocytes and HCC cells have been shown able to dedifferentiate towards a more LPC-like phenotype in conditions of increased stress, like hypoxia (25-28). As current treatment strategies for advanced stage HCC are based on depriving the tumour from its oxygen supply, we investigated the effect of hypoxia on tumour growth, NOTCH activation and LPC characteristics and the therapeutic potential of decreasing NOTCH signalisation by gamma secretase inhibition (GSI) in HepG2 transplanted xenografts as a model for HCC growth.

3.3. Materials and methods

Animal experiments

HepG2 cells, derived from human HCC (ATCC, France), were cultured in dulbecco's modified eagle medium (Life Technologies, Belgium) supplemented with 10% foetal calf serum in 5% CO2 at 37°C and were passaged at ±80% confluency. 32 homozygous Crl:NU-Foxn1nu nude mice (Charles river, France) were subcutaneously injected in the right flank with 7,5x106 HepG2 cells in 100 µL matrigel (BD, Belgium), 1/1 in DMEM.

Tumour volume was measured throughout the experiment using a calliper and tumour volume was calculated using the formula $\frac{\text{Major axis X Minor axis}^2}{2}$(25).

When average tumour volume reached 300mm³, mice were divided into two groups, the first received daily intragastric treatment with a GSI (LY411,575, sigma Aldrich, Belgium) at 3mg/kg, as previously described (29, 30) in a 1% methylcellulose (MC, sigma, Belgium) solution. The other mice served as controls and received equal volumes of the 1% MC solution.

Sixteen hours after the first GSI treatment, half the mice of each treatment group were placed in either the hypoxic unit, which was kept at 10,5% (3), by controlling the nitrogen inflow rate (continuously monitored by an oxygen sensor, Biospherix, United states), to obtain a hypoxic response, or at 21% O2 in normal airflow. We thus obtained 4 groups: 21% O2+MC, 21% O2+GSI, 10,5% O2+MC and 10,5% O2+GSI.

Fourteen days after the first GSI treatment, mice were weighed and euthanized by cervical dislocation. Tumours were prelevated, weighed and divided for histological, RNA and protein analysis, as previously described (22). As GSI's have been described to induce gastro-intestinal toxicity (31) we also sampled colon tissue, which was fixed in a 4% PBS buffered formaldehyde solution (klinipath, Belgium) and embedded in paraffin for histological analysis.

All animal experiments were approved by the Ethical Review Board for the use of experimental animals of Ghent University, faculty of medicine and health sciences (ECD approval 15/92).

Protein analysis

For histological analyses, hematoxilin-eosin (H&E) staining was performed on 5µm paraffin embedded sections as routinely described.

Endoglin, a marker for endothelial cell activation, was used to evaluate tumour vascularisation (goat polyclonal anti mouse endoglin, 1/50, R&D systems, United Kingdom). The LSAB-horseradish peroxidase–mediated visualisation (Dako, Belgium) was used and overall intratumoural immunoreactivity was calculated using Cell D software (Olympus Imaging Solutions, Germany).

Total protein extract was obtained by homogenizing tumour tissue in RIPA buffer (PBS, 0.5% NP-40, 0.5% sodium deoxycholate, 5.5% β-glycerophosphate, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol and complete protease and phosphatase inhibitors (Roche Diagnostics, Vilvoorde, Belgium). Total protein yield was determined using Bradford reagent (Biorad, Temse, Belgium).

To evaluate NOTCH activation, we determined the amount of NICD in tumour lysates using the Pathscan Cleaved NOTCH1 (Val1744) Sandwich ELISA kit according to manufacturer's instructions (Cell Signalling, The Netherlands), absorbance at 450nm was normalised to total protein concentration for each sample.

Quantitative polymerase chain reaction (qPCR)

RNA was extracted from frozen tumour tissue preserved in RNA-later, according to the manufacturer's guidelines (Rneasy Mini Kit, Quiagen, Venlo, Nederland). cDNA was obtained from 1µg RNA using the iScript cDNA synthesis kit (Bio-Rad, Nazareth-Eke, Belgium) and real time quantitative PCR (RT-qPCR) analyses were performed using a SYBR green mix (Sensifast Bioline Reagents Ltd, London, UK).

Primer sets are listed in Table 1, their efficiency was calculated from the slope of a standard curve using the following formula: E=10(-1/slope)-1.

All reactions were run in duplicate and normalized to reference genes that showed stable expression in all samples. The comparative Cq method was used to compare gene expression between different groups.

Statistics

Data were analysed using SPSS21 software (IMB corp, Armonk NY, USA). Kolmogorov-Smirnov test was used to test for normality. Student's T-test was performed in case of normality; the Mann-Whitney-U test was used for not normally distributed data. P-values ≤0,05 where considered significant. All data arepresented as the mean of all cases from two independent experiments (n=6 - 8/group) ±SEM.

Table 1: Primersets

Gene symbol	Full name	Forward prirmer	Reverse primer
GAPDH	glyceraldehyde 3 phosphate dehydrogenase	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
HMBS	hydroxymethyl-bilane synthase	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
HPRT	hypoxanthine guanine phosphoribosyl transferase	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
SDHA	succinate dehydrogenase complex, subunit A	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
SLC2A1 (GLUT1)	glucose transporter 1	AAATGCTTGTGGATTGAGGG	GTCGAAGTCTAAGCCGTTGC
VEGFA	vascular endothelial growth factor alpha	TCCTCACACCATTGAAACCA	GATCCTGCCCTGTCTCTCTG
PCNA	proliferating cell nuclear antigen	GCGTGAACCTCACCAGTATGT	TCTTCGGCCCTTAGTGTAATGAT
KRT19	cytokeratin 19	AACGGCGAGCTAGAGGTGA	GGATGGTCGTGTAGTAGTGGC
SOX9	sex determining region Y (SRY)-box9	CTCTGGAGACTTCTGAACGA	TTGAAGATGGCGTTGGG
EPCAM	epithelial cell adhesion molecule	ATAACCTGCTCTGAGCGAGTG	TGCAGTCCGCAAACTTTTACTA
PROM1	prominin 1	AGTCGGAAACTGGCAGATAGC	GGTAGTGTTGTACTGGGCCAAT
HES1	hairy and enhancer of split	ACGTGCGAGGGCGTTAATAC	GGGGTAGGTCATGGCATGA

3.4. Results

Decreased oxygen tension enhances tumour growth, NOTCH signalization and expression of LPC characteristics in HCC xenografts

We first analysed the effect of hypoxic housing on tumour growth. Relative tumour weight and mRNA expression of proliferating cell nuclear antigen (PCNA) were increased in mice housed in hypoxic conditions (Figure1A,B) and this was associated with increased expression of HIFtarget genes, vascular endothelial growth factor alpha (*VEGFA*) and Glucose transporter protein 1 (*GLUT1*) (Figure1C,D).

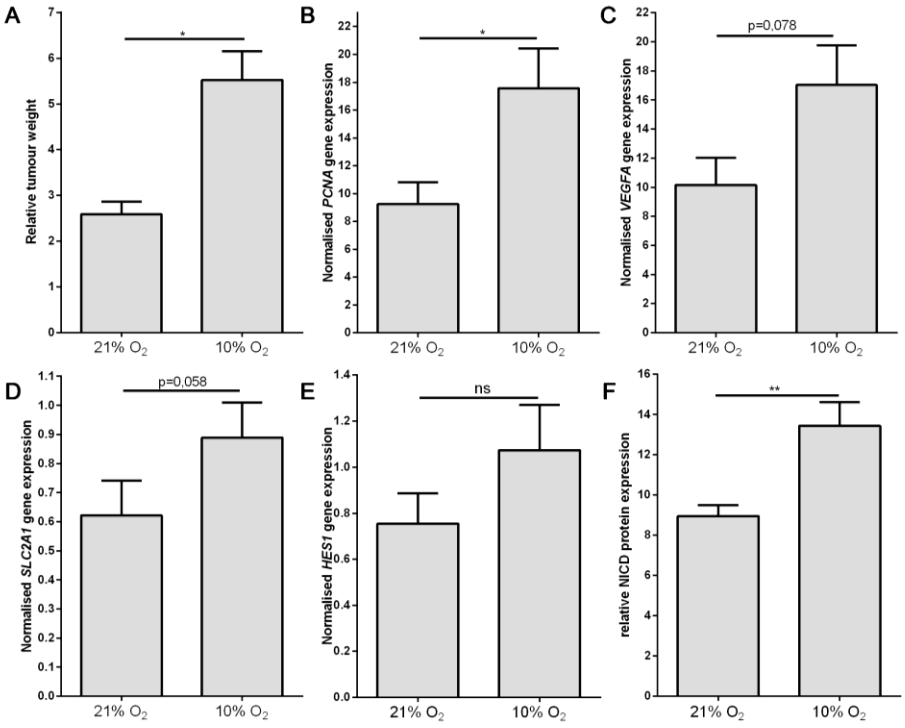

Figure 1: activation of the hypoxic adaptive response, increased tumour growth and NOTCH activation in tumours from mice housed in hypoxic conditions.

Relative tumour weight (**A**), and *PCNA* mRNA expression (**B**), HIF target genes: *VEGFA* (**C**), GLUT1 (*SLC2A1*, **D**) and NOTCH target gene *HES1* (**E**) mRNA expression and NICD protein levels (**F**) in tumours grown in normoxic (21% O2) or hypoxic (10,5% O2) mice.

*:P<0,05; **:p<0,01, ns: not significant).

The mRNA expression of *HES1*, the main NOTCH effector gene (14, 24), and NICD protein levels were increased in tumours from mice housed in hypoxic conditions, indicating NOTCH pathway activation (Figure 1E,F).

Since both activation of the hypoxic response and increased NOTCH signalling have been related to increased expression of LPC characteristics in HCC, we also determined the mRNA expression of LPC characteristics in tumour tissue of mice housed in normoxic and hypoxic conditions. mRNA expression was significantly increased for cytokeratin 19 (*KRT19*) but this increase was not significant for Prominin1 (*PROM1*), Epithelial cell adhesion molecule (*EPCAM*) and SRY-box9 (*SOX9*) (Figure 2).

In conclusion, hypoxic housing results in the development of tumours with increased growth, an increased hypoxic signature and higher expression of LPC characteristics.

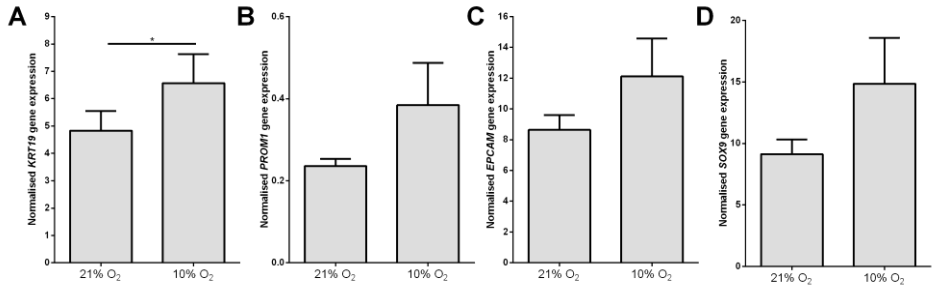

Figure 2: mRNA expression of LPC markers is increased in tumours grown in mice housed in hypoxic conditions

mRNA expression of *KRT19* (A), *PROM1* (B), *EPCAM* (C) and *SOX9* (D)(*:P<0,05)

Gamma secretase inhibition reduces NOTCH pathway activation and tumour growth in mice housed in hypoxic conditions.

To evaluate the therapeutic potential of NOTCH inhibition on the effects of hypoxic housing, we used daily GSI treatment.

This resulted in effective inhibition of the NOTCH pathway in tumours from mice housed in hypoxic conditions, as seen by significantly decreased expressions of *HES1* mRNA and NICD protein levels (Figure 3A,B). Importantly, GSI treatment resulted in significantly smaller relative tumour sizes and decreased *PCNA* mRNA expression compared to MC-control treatment in hypoxic conditions (Figure 3C,D). This effect of GSI treatment on NOTCH inhibition and tumour reduction was not observed under normoxic conditions (Figure 3A-D). Taken together, this data shows that GSI treatment reduces Notch signalling and tumour growth in hypoxic but not normoxic conditions.

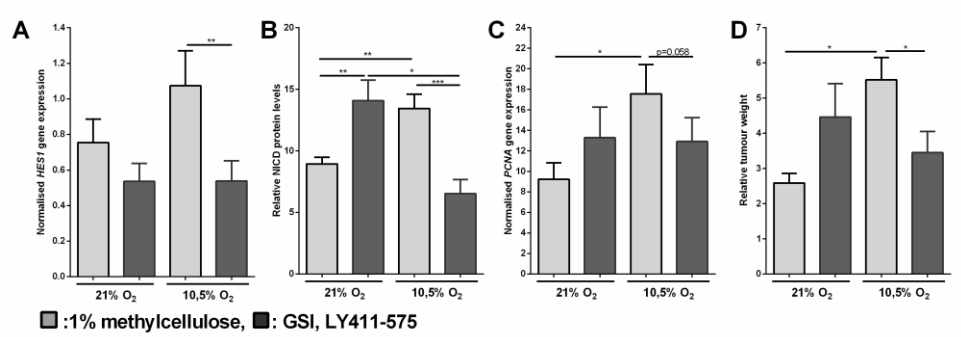

Figure 3: GSI treatment decreases NOTCH signalling and reduces tumour growth in hypoxic conditions

mRNA expression of *HES1*(**A**), protein levels of NICD (**B**), *PCNA* mRNA expression (**C**) and relative tumour weight (**D**) are significantly decreased in GSI treated tumours compared to control in the 10,5% O2 but not the 21% O2 groups. (*:P<0,05; **:p<0,01; ***:p<0,001)

Since previous studies have reported GSI-induced gastro-intestinal toxicity (29, 31), we analysed body weight, as an indicator of general wellbeing, and evaluated H&E stained sections of colon tissue but did not observe any difference between MC and GSI groups after 14 days of treatment (Figure 4), indicating that the administered dose was not toxic.

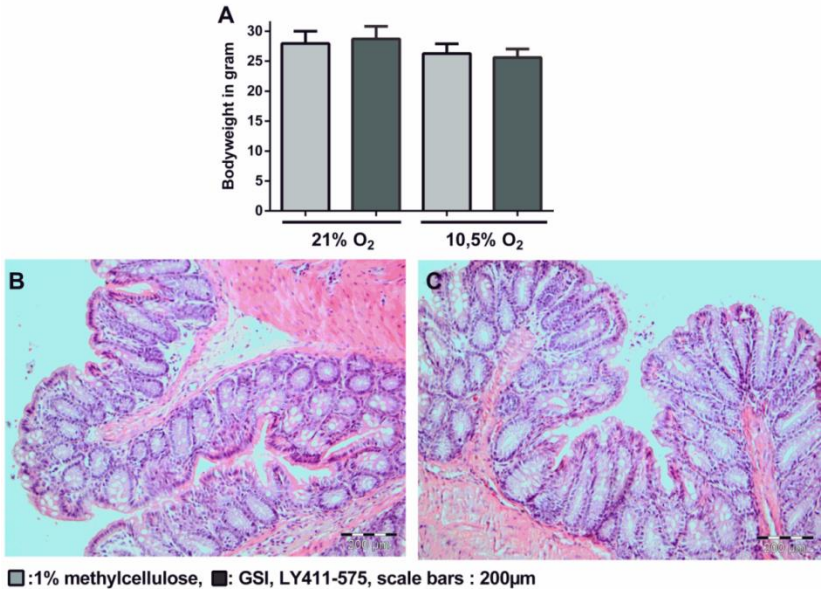

Figure 4: GSI treatment was not toxic

Bodyweight after 14 days of GSI treatment (**A**) and H&E sections of MC (**B**) and GSI (**C**) treated mice

Gamma secretase inhibition decreases activation of the hypoxic adaptive response and expression of liver progenitor cell characteristics in HCC xenografts

Since NOTCH and hypoxia are interconnected and have both been associated with an LPC-like phenotype in liver cancer, we analysed if the effect of GSI treatment also affected the expression of HIF target genes, endoglin and LPC markers in our xenograft model. The mRNA expression of *GLUT1* and *VEGFA* and endoglin immunopositivity was lower in GSI compared to MC control treated tumours both under normoxic and hypoxic conditions (Figure 5A-E).

LPC markers, *KRT19* and *SOX9*, were also decreased in tumour tissue of GSI treated mice compared to control treated groups, in both hypoxic and normoxic conditions (Figure 5F,G). *EPCAM* and *PROM1* mRNA expression was not significantly altered upon GSI treatment (Figure 5H,I).

Overall, GSI treatment appeared to decrease the expression of LPC characteristics and markers for hypoxia in both hypoxic and normoxic conditions.

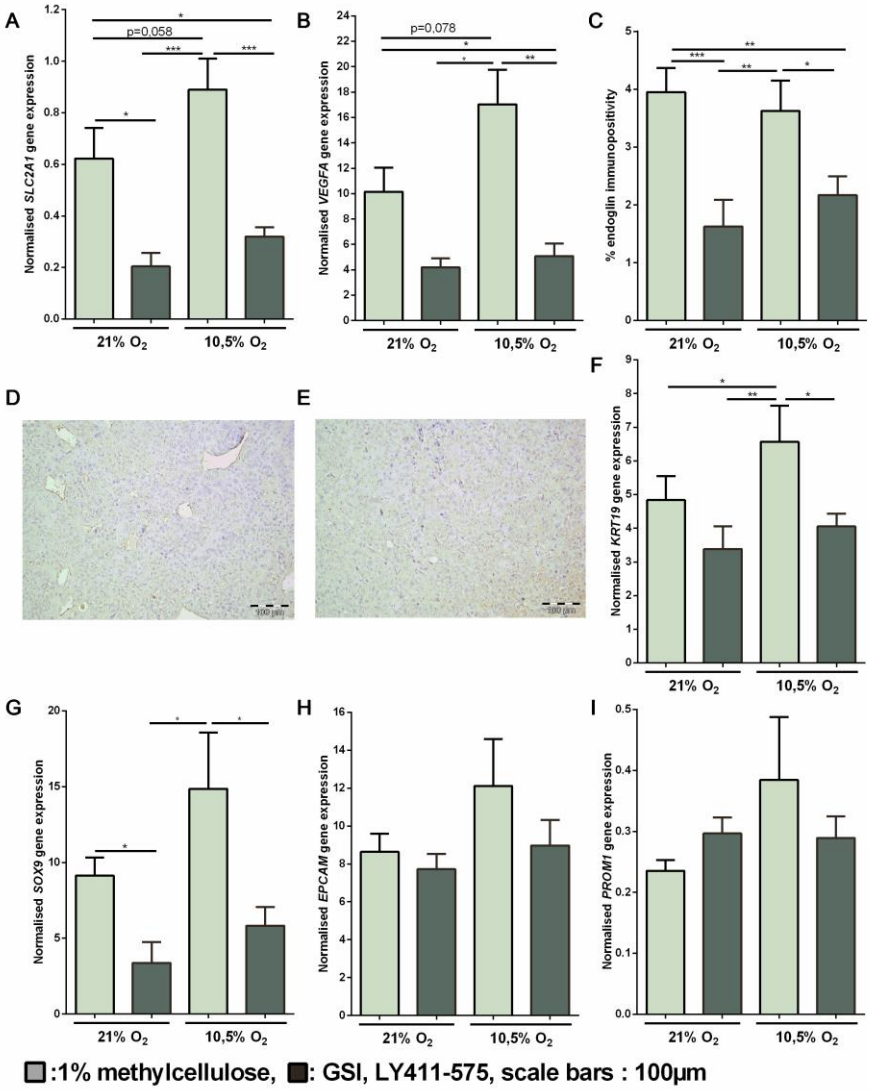

Figure 5: GSI treatment impedes activation of the hypoxic adaptive response, reduces tumour vascularisation and decreases expression of LPC markers.

mRNA expression of *GLUT1* (*SLC2A1*, **A**) and *VEGFA* (**B**), quantified percent of tumoural endoglin staining (**C**) and representative images of endoglin stained control (21%O2 +MC)(**D**) and GSI (10%O2+ GSI) treated tumour tissue (**E**). mRNA expression of *KRT19* (**F**), *SOX9*(**G**), *EPCAM* (**H**) and P*ROM1* (**I**). (*:p<0,05; **p<0,01; ***:p<0,001)

3.5. Discussion

In HCC, increased HIFα stabilisation has been linked to increased expression of LPC characteristics and poor prognosis (6, 7, 9, 22, 32, 33), and over activation of the NOTCH signalling pathway was shown to induce hepatic tumours with an LPC signature (16, 17, 20). Several reports have indicated that the HIF and NOTCH signalling pathways might cooperate in the induction of a more aggressive and therapy resistant phenotype, correlated with poor prognosis in cancer (11, 13, 34-36). To examine the therapeutic potential of NOTCH inhibition to prevent hypoxia-induced effects on tumour phenotype we used a HepG2 xenograft mouse model subjected to 10,5% O_2 housing conditions.

We observed an activation of the hypoxic adaptive response and increased relative tumour size and increased expression of LPC marker *KRT19* in tumours from mice housed in hypoxic conditions. Our data of increased *HES1* and NICD expression confirmed previous reports of increased expression of NOTCH receptors and target genes upon increased HIFα stabilisation (11, 13, 35, 36). Both HIF and NOTCH signalling have independently been associated with an enhanced LPC signature (8, 17, 20, 22, 28).

In human patients, hypoxia inducing transarterial chemoembolization treatment prior to transplantation was shown to induce an increased LPC signature in recurring tumours (8, 9, 33). Moreover, liver specific increased NOTCH signalling was shown to induce hepatic tumours with LPC characteristics (16).

In a previous study we also observed increased expression of LPC characteristics in tumours with increased HIFα stabilisation, coinciding with a higher tumour burden and increased expression of NOTCH receptors and ligands (22).

The association of NOTCH pathway activation and expression of LPC characteristics upon increased hypoxic signalling aligns with our observations regarding tumour size, *KRT19*expression and NOTCH pathway activation in hypoxic mice. We therefore evaluated the therapeutic potential of NOTCH inhibition, using a GSI, known to inhibit the presenilin-gamma secretase complex, thereby inhibiting cleavage of NICD and subsequent activation of the NOTCH signalling pathway.

GSI treatment resulted in significantly decreased *HES1* and NICD expression, associated with impeded tumour growth in hypoxic mice. The expression of HIF downstream targets (*VEGFA* and *GLUT*) and LPC markers *KRT19* and*SOX9* were reduced upon GSI treatment, both under normoxic and hypoxic conditions. The previously reported gastro-intestinal toxicity upon GSI treatment (29, 31) was not observed in our treatment regime.

In line with the proposed link between HIF and NOTCH, the NOTCH signalling pathway has previously been shown to be involved in neo-angiogenesis in tumours, with contradicting effects (37). We observed decreased endoglin expression in tumours from GSI treated mice in both hypoxic and normoxic conditions. As we did not observe significant effects of GSI treatment on NOTCH pathway activation in normoxia, the observations concerning HIF targets, LPC markers and endoglin expression could result from, perhaps more potent, off-target effects of GSI treatment.

Indeed GSI's have been shown to cleave/activate other membranous proteins involved in carcinogenesis, like the epithelial– and neuronal adhesion molecules (CDH1 and CDH2)(38). CDH1 is expressed by epithelial cells and is an important regulator of cell-cell contact to maintain tissue integrity. Adversely, CDH2 promotes cell- matrix contact, potentiating cell motility and invasion and is expressed by stem/progenitor cells (like LPCs), mesenchymal cells and cancer cells that underwent epithelial to mesenchymal transition (39).

Cleavage of the intracellular domain of cadherins releases β-catenin and activates the Wnt signalling pathway(39). Wnt signalling was shown to promote HCC growth and is activated in well differentiated HCC(34, 40). Interestingly, in hypoxic conditions, it was shown that HIFα can bind β-catenin to increase its stability, inhibiting the Wnt pathway, increasing HIF signalling and promoting cellular adaptation (41, 42). As Wnt signalling is also known to counteract the Notch signalling pathway in LPC differentiation (43), this could explain the observed discrepancies between the effects of GSI treatment in normoxic and hypoxic conditions.

On the other hand, previous studies have shown that different NOTCH receptors and ligands can have antagonistic effects on tumour progression and angiogenesis (44-46). Thus, it is possible that the effects we observed in both hypoxia and normoxia result from inhibition of a different NOTCH receptor. Future research using receptor specific antibodies are necessary to further unravel this issue.

3.6. Conclusions

Our data show that *in vivo* activation of the hypoxic adaptive response results in increased HCC tumour growth and *KRT19* expression and that this is accompanied by NOTCH pathway activation.

Our data show the therapeutic value of GSI on the growth of HCC subjected to hypoxic threats, which might indicate that GSIs could serve as an adjuvant in HCCs with a (treatment-induced) increased hypoxic signature.

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4. DEVELOPMENT OF A MOUSE MODEL FOR INDUCIBLE NOTCH1 OVER ACTIVATION IN THE BILIARY COMPARTMENT AND THE EFFECT ON LIVER INJURY

Eliene Bogaerts, Stefaan Verhulst, Annelies Paridaens, Sander Lefere, Frederik.Lemaigre, Lindsey Devisscher, Leo A. van Grunsven and Hans Van Vlierberghe

4.1. Introduction

The Notch pathway is important in stem cell self-renewal, and plays a special role in the control of many binary cell fate choices in embryonic and adult cells (1). There are 4 Notch receptors and 2 types of ligands described in mammals: the NOTCH1, NOTCH2, NOTCH3 and NOTCH4 receptors and the jagged (JAG) and Delta ligands. In short, ligand binding to the N- terminal extracellular Notch domain triggers cleavage of the C- terminal Notch intracellular domain (NICD). Upon its release into the cytoplasm, NICD migrates to the nucleus, associates with the CSL (CBF1/Su(H)/Lag-1) transcription complex, and recruits co-activators, such as mastermind–like, to induce Notch-dependent gene transcription (1-3).

The role of Notch signalling has been extensively studied in liver disease (3, 4). NOTCH1 and 2 have shown to be upregulated in the biliary compartment during cholestatic liver disease (5-7). Using a hepatocyte nuclear factor1β:Cre mouse, researchers also demonstrated that NOTCH2 overexpression in the biliary compartment induces severe ductular reactions, by increasing the proliferative capacity of the targeted cells (8). Furthermore, upregulation of Notch1 and Notch2 in hepatocytes and hepatoblasts have been shown to induce hepatocellular tumours, high in biliary characteristics in mice (8-10). However, Notch1 inhibition drastically decreased HCC but increased the cholangiocellular burden in a mouse model for HCC, while Notch 2 inhibition only decreased the HCC load (11). These findings imply a different effect of Notch1 on the biliary and hepatocytic compartment.

The biliary compartment consists of cholangiocytes and liver progenitor cells (LPCs). LPCs are bipotential stem cells that can differentiate towards hepatocytes and cholangiocytes (12-14). Upon severe acute or chronic injury, when hepatocytes and cholangiocytes can no longer restore liver function by self-replication, LPCs are activated.

The Notch signalling pathway plays a pivotal role in the regulation of the cell fate of LPCs, which is described by Boulter et al. (5). Cholangiocyte damage attracts portal myofibroblasts carrying JAG1 ligands, which activate the Notch signalling pathway in LPCs, resulting in biliary differentiation (5). However, during hepatocyte damage, macrophage produced WNT, induced by phagocytosis of hepatocytic debris, inhibits NOTCH signalling, and pushes LPCs towards the hepatocellular lineage (5). While interest in the role of LPCs in liver injury, disease and carcinogenesis has expanded in the last decade, the exact role for LPCs and of different Notch receptors in liver pathology has not yet been determined (15).

In vitro, NOTCH 2 and 4 have been shown essential for LPC proliferation, while NOTCH 3 was shown to induce hepatocytic differentiation, but no NOTCH1 mediated effects are described (16). As the aforementioned discrepancies between NOTCH 1 inhibition and upregulation studies could be caused by cell specific effects, we aimed to investigate the effect of NOTCH 1 upregulation in the biliary compartment, and the effect on liver injury and repair.

4.2. Materials and methods

Mouse strains

For inducible Cre expression in the biliary compartment of the liver, transgenic mice carrying a tamoxifen inducible CRE (iCre) controlled by an Osteopontin (Opn) enhancer- promotor (17) were used. These mice were crossed with mice homozygously carrying the conditional Nicd1 and IRES coupled green fluorescent protein (GFP) sequence in the murine Rosa26 locus (Rosa26-LoxP-STOP-LoxP-Nicd1- GFP, from now on shortened as RosaNicd+/+,strain 008159, Jackson laboratory, Maine USA) to obtain OpnCre+;RosaNicd+/+ and OpnCre-; RosaNicd+/+ littermate control mice. Mice were genotyped using the following primers: CAGGATCTGCACACAGACAGG and GAAATTGCCCTTTTCCTTGC, in a final concentration of 200 nM,for the OpnCre construct,

and TAA GCC TGC CCA GAA GAC TC, GAA AGA CCG CGA AGA GTT TG and AAA GTC GCT CTG AGT TGT TAT, in a final concentration of 500nM, for the RosaNicd construct.

Tamoxifen trial

Tamoxifen was dissolved in corn oil and administered to 5 week old OpnCre+;RosaNicd+/+ and OpnCre-;RosaNicd+/+ (n=3 per group) mice intraperitoneally, thrice at a concentration of 250mg/kg with a 36hour interval. All mice were euthanized 2 weeks after the final tamoxifen injection. mRNA expression of the *iCre*-recombinase and *GFP* expression was evaluated to verify genotype and to determine efficient iCre-recombinase-mediated Lox excision. We also assessed the ratio of full length *Notch1* to *Nicd*- mRNA and evaluated the mRNA expression LPC and cholangiocyte markers to further characterise the effect of biliary Nicd overexpression.

Induction of liver injury

3 week old male OpnCre+;RosaNicd+/+ and OpnCre-;RosaNicd+/+ mice received 3 250mg/kg tamoxifen injections in 36-hour intervals. After 3 weeks, allowing time for full elimination of tamoxifen, mice received a diet containing 0,1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), to induce cholestatic liver injury and an LPC response (18), for 3 weeks, after which they were euthanized (OpnCre+;RosaNicd+/+: n=8; OpnCre-;RosaNicd+/+: n=11)(Figure 1).

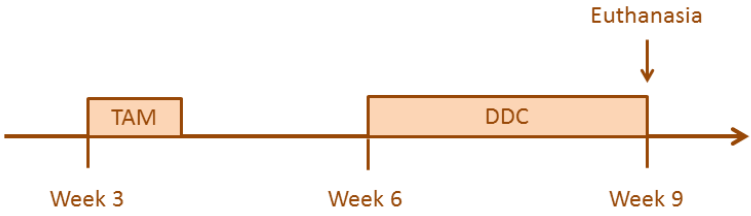

Figure 1: Timeline for DDC- administration

Tamoxifen injections occurred when mice were 3 weeks of age, 3 weeks after the first tamoxifen dose, the DDC diet was started for 3 weeks, after which mice were euthanized and sampled.

Sampling

Mice were weighed and anaesthetised using ketamine (100mg/kg) and xylazine (10mg/kg). Blood was sampled from the ophthalmic vein and animals were then euthanized by cervical dislocation. The liver and spleen were excised and weighed and the liver was emerged in 4% PBS buffered formaldehyde (Klinipath, Olen, Belgium) for subsequent histological evaluation and in RNA later (ambion, Thermo Fisher scientific, Ghent, Belgium) for RNA extraction and qPCR analysis.

Quantitative real time PCR (qPCR)

RNA was extracted from 20 mg of frozen liver tissue, according to the manufacturer's guidelines (Aurum total RNA kit, Biorad, Eke Belgium).

cDNA was obtained from 1µg RNA using the sensifast cDNA synthesis kit (GC biotech Alphen aan den Rijn, The Nederlands) and real time quantitative PCR (qPCR) analyses were performed using a SYBR green mix (Sensifast Bioline Reagents Ltd, London, UK).All reactions were run in duplicate; the comparative Cq method was used to compare gene expression between different groups, which were normalised to reference genes that showed stable expression in all samples (19, 20). A list of all used primer sets is included in Table 1.

Histology

General morphology of liver tissue was assessed using Haematoxylin- Eosin and sirius red stainings on 5µm sections of paraffin embedded tissue. Cytokeratin 19 immunohistochemistry (1/200 in TBS, ab133496, RRID:AB_11155282, abcam, Cambridge, UK) was used to visualize structures of the cholangiocytic lineage as well as LPCs as previously described (19).

Table 1: Primersets

Gene Symbol	Full name	Forward primer	Reverse primer
Gapdh	glyceraldehyde 3 phosphate dehydrogenase	CATGGCCTTCCGTGTTCCTA	GCGGCACGTCAGATCCA
Hmbs	hydroxymethyl-bilane synthase	AAG GGC TTT TCT GAG GCA CC	AGT TGC CCA TCT TTC ATC ACT G
Hprt	hypoxanthine guanine phosphoribosyl transferase	GTT AAG CAG TAC AGC CCC AAA	AGGGCATATCCAACAA CAAACTT
Sdha	succinate dehydrogenase complex, subunit A	CTTGAATGAGGCTGACTGT G	ATCACATAAGCTGGTCC TGT
GFP	green fluorescent protein	AAGCTGACCCTGAAGTTCAT CTGC	CTTGTAGTTGCCGTCGT CCTTGAA
iCre	inducible cre- recombinase	TCGCCCTTCTGACTCCAATG	GGTCTTGGTCCTGCCAA TGT
Krt19	cytokeratin 19	GTTCAGTACGCATTGGGTCA G	GAGGACGAGGTCACGA AGC
Prom1	prominin 1	CTCCCATCAGTGGATAGAG AACT	ATACCCCCTTTTGACGA GGCT
Epcam	epithelial cell adhesion molecule	GCGGCTCAGAGAGACTGTG	CCAAGCATTTAGACGCC AGTTT
Notch1	Notch 1	GATGGCCTCAATGGGTACA AG	TCGTTGTTGTTGATGTC ACAGT
Nicd	Notch intracellular domain	GGACATGCAGAACAACAAG G	CAGTCTCATAGCTGCCC TCA
Hes1	hairy enhancer of split 1	ACGTGCGAGGGCGTTAATA C	ACGTGCGAGGGCGTTA ATAC
Acta2	alpha smooth muscle actin	CCA GCA CCA TGA AGA TCA AG	TGG AAG GTA GAC AGC GAA GC
Tnf	tumour necrosis factor alpha	CATCTTCTCAAAATTCGAGT GACAA	TGGGAGTAGACAAGGT ACAACCC
Vcam1	vascular cell adhesion molecule	TGCCGAGCTAAATTACACAT TG	CCTTGTGGAGGGATGT ACAGA

Statistical analysis

Data from DDC fed mice was analysed using SPSS23 software (IMB corp, Armonk NY, USA). Kolmogorov-Smirnov test was used to test for normality. Student's T-test was then performed in case of normality; the Mann-Whitney-U test was used for not normally distributed data. P-values ≤0, 05 were considered significant. All graphs were illustrated using Graphpad prism 6 software (Graphpad software, inc; San Diego CA, USA), data are presented as mean ±SEM.

4.3. Results

Successful tamoxifen-induced activation of the RosaNicd gene construct

iCre- recombinase mRNA expression was higher in all OpnCre+ ;RosaNicd+/+ mice, compared to OpnCre-;RosaNicd+/+ mice (Figure2A) and *GFP* mRNA expression was induced in OpnCre+;RosaNicd+/+ livers only (Figure2B), indicating successful LoxP recombination and stop codon excision.

This was also confirmed in the increased *Nicd* expression for unchanged full-length *Notch1* expression (Figure 2C,D), which is better reflected by the decreased *Notch1/Nicd* ratio (Figure 2E) and increased expression of the major Notch target gene hairy enhancer of split 1 (*Hes1*) (Figure 2F).

LPC markers prominin1 (Prom1) and cytokeratin 19 (Krt19) were also increased in OpnCre+;RosaNicd+/+ mice, compared to OpnCre-; RosaNicd+/+ mice upon tamoxifen administration. These data show that tamoxifen has successfully induced iCre mediated LoxP excision.

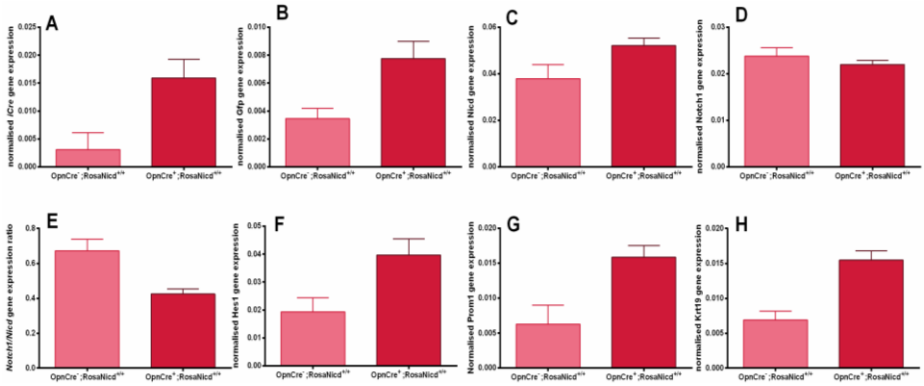

Figure2:mRNA expression of *iCre*- recombinase, *GFP*, Notch and LPC markers in healthy OpnCre-;RosaNicd+/+ and OpnCre+;RosaNicd+/+ livers after tamoxifen injections.

iCre (**A**)and *GFP* (**B**) mRNA expression show evidence of succesfull recombination. Gene expression of *Nicd* (**C**), *Notch1*(**D**), the *Notch1/Nicd* ratio (**E**)and *Hes1*(**F**)show increased Notch signallingin OpnCre+;RosaNicd+/+ livers.qPCR analysis of LPC makers *Prom1* (**G**) and *Krt19* (**H**) also show increased expression in OpnCre+;RosaNicd+/+ livers.

no statistics were performed as n=3/group

■OpnCre+;RosaNicd+/+, ■ OpnCre-; RosaNicd+/+

Effects of biliary Notch1 overexpression in mouse model for cholangiocyte injury

To assess the effect of biliary Notch1 overexpression in cholangiocyte injury, tamoxifen injected mice were fed the DDC diet for 3 weeks. Body weight, liver and spleen weight at euthanasia revealed no significant differences between tamoxifen treated OpnCre-;RosaNicd+/+ and OpnCre+;RosaNicd+/+ mice (Figure 3 A,B,C).

Successful iCre-mediated recombination in OpnCre+;RosaNicd+/+ mice was confirmed by increased *iCre* and *GFP* mRNA expression compared to OpnCre-;RosaNicd+/+ mice. We could however not observe an altered *Notch1* to *Nicd* ratio in full liver lysates (Figure 4F).

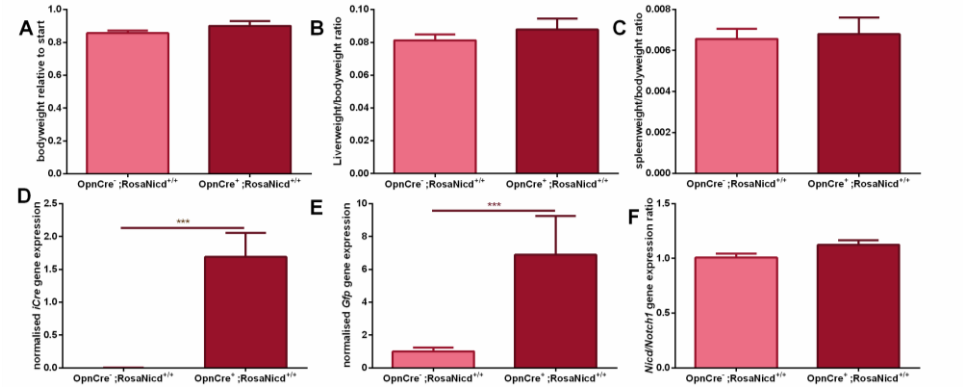

Figure 3: general parameters of DDC treated OpnCre-;RosaNicd+/+ and OpnCre+;RosaNicd+/+ livers

OpnCre+;RosaNicd+/+ mice did not differ from OpnCre-;RosaNicd+/+ mice in **A**. body weight, **B.** relative liver weight and **C**. relative spleen weight. **D.***iCre*- recombinase and **E.** *GFP* mRNA nicely show *GFP* expression is induced in OpnCre+;RosaNicd+/+ mice upon tamoxifen induction**. F.** We did not observe any change in the *Nicd/Notch1*gene expression ratio.

■OpnCre+;RosaNicd+/+, ■ OpnCre-; RosaNicd+/+

Livers of OpnCre+;RosaNicd+/+ and OpnCre-;RosaNicd+/+ DDC fed mice were further evaluated histologically to determine the effect of increased biliary Nicd expression on disease progression. Haematoxylin- Eosin staining showed typical DDC-induced porphyrin plugs associated with a severe ductular reaction (Figure 4 A, B) and sirius red staining showed fibrotic strands in these areas (Figure 4 C, D).

No differences were observed between OpnCre-;RosaNicd+/+ and OpnCre+;RosaNicd+/+ mice.

These data show that, while we were able to confirm the induction of *GFP* mRNA in DDC fed OpnCre+;RosaNicd+/+ mice, the expected increased Nicd expression, nor any effects on general parameters and histology could be detected on whole liver compared to OpnCre-RosaNicd+/+ DDC fed mice. To verify that there was no difference in the level of fibrosis and hepatic stellate cell (HSC) activation, we also performed qPCR analysis for smooth muscle actin alpha (*Acta2*), a marker for activated HSCs and observed no significant difference in mRNA expression between both genotypes (Figure 5A).

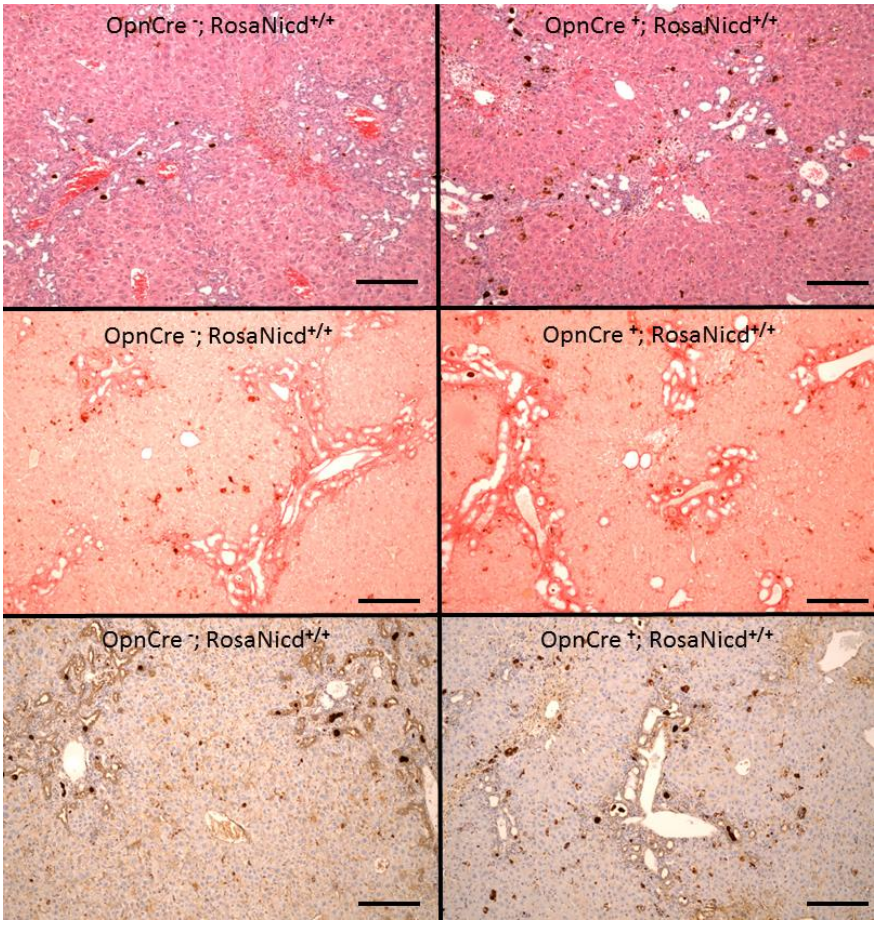

Figure 4: Histology of DDC treated OpnCre-;RosaNicd+/+ and OpnCre+;RosaNicd+/+ livers

Top: heamatoxillin eosin staining did not show any difference between Cre+ and Cre- mice. Brown dots are porphyrin plugs. **Middle**: sirius red stained sections show no difference in fibrosis between iCre positive and iCre negative mice. **Bottom**: cytokeratin 19 immunopositivity is comparable between iCre+ and iCre- livers. Scale bars: 20µm

We next evaluated the mRNA expression of LPC characteristics: *Prom1*, *Krt19* and epithelial cell adhesion molecule (*Epcam*) and an exploratory set of pro-inflammatory markers, which have previously been shown increased upon DDC feeding, vascular cell adhesion molecule (*VCam1*) and tumour necrosis factor-alpha (*Tnf*). LPC marker *Prom1* expression was significantly decreased while we did not observe any effects on the expression of LPC/cholangiocyte characteristics *Epcam* and *Krt19* (Figure 5C,D) while in OpnCre+;RosaNicd+/+ compared to OpnCre-;RosaNicd+/+ controls (Figure 5B). *Vcam1* mRNA (Figure 5E) expression was decreased in OpnCre+;RosaNicd$^{+/+}$ mice, but no effect on *Tnf* mRNA expression was observed (Figure 5F).

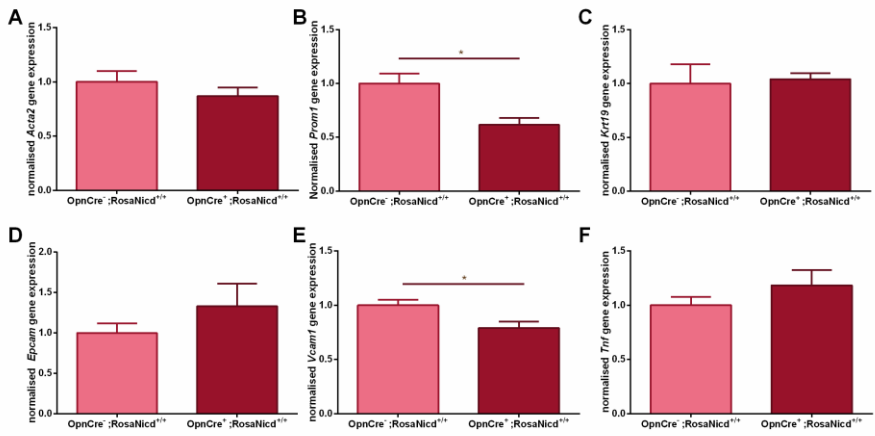

Figure 5: mRNA expression of markers for fibrosis, LPCsand inflammation in OpnCre+;RosaNicd+/+ and OpnCre+;RosaNicd+/+ DDC treated livers

mRNA expression of **A**. *Acta2*, **B**. *Prom1*, **C**. *Krt19*, **D**. *Epcam*, **E.***Vcam1*, and **H**. *Tnf*

■OpnCre+;RosaNicd+/+, ■ OpnCre-; RosaNicd+/+

In conclusion, LPC marker *Prom1* and inflammatory marker *Vcam1* were significantly downregulated in OpnCre+;RosaNicd+/+, which could point to a decreased ductular reaction. However, no effect could be observed on biliary markers (Epcam and Krt19) and inflammatory marker *Tnf.*

4.4. Discussion and future perspectives

The liver distinguishes itself from other organs by its great regenerative capacity: in case of mild or moderate damage or cell – loss, hepatocytes and cholangiocyte can replicate to restore cell mass. However, in case of severe acute or chronic injury, the progenitor cell compartment is activated.

These facultative stemcells can migrate to the site of injury and differentiate into the damaged cell type. Activation and differentiation of LPCs is tightly regulated by Notch and Wnt signalling: in case of severe cholangiocytic damage, the notch pathway is activated in LPCs and drives the differentiation towards a cholangiocytic phenotype. In case of hepatocytic injury, macrophage derived Wnt signalling opposes Notch activation in LPCs, resulting in hepatocytic differentiation (5).

Generation of a mouse model for inducible biliary Notch1 overexpression

In this study, we created a mouse model for inducible Nicd and GFP expression controlled by an osteopontin promotor enhancer sequence (17), resulting in continuous activation of the Notch1 signalling pathway and nuclear GFP expression in cells of the biliary lineage. The specificity of OpnCre mouse model we used for tamoxifen inducible Cre expression in the biliary lineage has previously been examined, using a Rosa26 – lox-stop-lox- yellow fluorescent protein (YFP) reporter sequence. YFP expression was shown restricted to LPCs and cholangiocytes and was not expressed in liver macrophages, stellate cells or hepatocytes. Efficiency of YFP expression was calculated as the ratio of cells co-expressing Sox9 and YFP and was 69 – 84%(17, 21).

For our experiments, this OpnCre mouse was crossed to a RosaNicd mouse, in which the intracellular, transcriptionally active domain of the Notch1 receptor and a reporter GFP are (over)expressed upon excision of the LoxP flanked stop codon.

We first established a protocol for tamoxifen administration to induce *GFP* mRNA expression. Notch overexpression was less apparent, but present in whole liver lysates. Since the bulk of liver cells are hepatocytes, this could obscure a clear *Nicd* upregulation in the biliary compartment.

Future experiments will further evaluate the efficiency of tamoxifen-induced iCre activity and the specificity of Nicd and *GFP* mRNA and protein overexpression by evaluating different cell types separately.

Hepatocytes will first be isolated through density gradient centrifugation, and biliary cells can then be sorted from a rest- fraction (containing endothelial cells, leukocytes, stellate cells,…) through Epcam positivity using fluorescent- and/or magnetic- activated cell sorting (FACS and/or MACS) (22). The efficiency can be calculated by evaluating the percentage of Epcam positive cells that co-express GFP.

Constitutive over-activation of Notch 1 signalling in both hepatocytes and cholangiocytes or hepatocytes only resulted in the formation of tumours with a cholangiocytic signature (9, 10), while antibody-mediated Notch1 inhibition decreased HCC burden in favour of cholangiocytic lesions (11).

To determine if Notch1 signalling has cell-specific effects in the liver, and during hepatocarcinogenesis, we will further evaluate the effects of biliary Notch 1 overexpression on liver physiology and it's potential to induce hepatic tumours by evaluating liver structure of OpnCre+;RosaNicd+/+ mice for neoplastic transformation at different timepoints after tamoxifen induction.

Effects of biliary Notch 1 overexpression on the LPC response in liver injury

While the involvement of LPCs has been shown in different liver diseases, their exact role in the pathogenesis of chronic liver injury has not yet fully been unravelled. It is possible that, in case of chronic injury, the LPC-mediated repair can go haywire, as seen in the ductular reaction, attributing to disease progression.

To further investigate the role of LPCs in disease progression, mice overexpressing Nicd in the biliary lineage were submitted to the DDC diet, resulting in a cholangiocyte damage-mediated LPC response.

While histology and mRNA expression of markers for both LPCs and cholangiocytes, *Epcam* and *Krt19*, showed no effect of increased biliary Notch signalling, we did observe decreased expressions of *Prom1* and pro- inflammatory marker *Vcam1*. Further analyses should confirm or refute a pro-inflammatory response in Cre+ mice.

Possibly, increasing Notch signalling accelerates the differentiation towards a cholangiocytic phenotype, resulting in a faster depletion of Prom1 expressing progenitor cells, in favour of cholangiocytes. During liver injury, the liver progenitor cell niche contains HSCs and inflammatory cells, contributing to disease progression and fibrogenesis. Several studies have shown that reduced pro-inflammatory signalling attenuates the LPC- response (23-25), and it has been suggested that inhibiting proliferation of LPCs also reduces the pro-inflammatory response, in turn attenuating the progression of liver disease (26).

If targeted increased Notch1 signalling can reduce the severity of the LPC response, by inducing faster differentiation, and/or decrease the inflammatory response, this could thus have implications with regards to disease progression and fibrogenesis in cholestatic liver disease.

To determine whether biliary Nicd overexpression-mediated reduced mRNA expression of pro- inflammatory markers and Prom1 affect disease progression, we should further investigate the effects at later timepoints in DDC-induced injury.

However, since the Notch pathway is already over-activated in LPCs in case of biliary damage, further increasing Nicd expression might not affect the pathogenesis of DDC- feeding-induced damage and/or repair.

On the contrary, differentiation of LPCs towards the hepatocyte lineage requires inhibition of Notch signalling. Therefore, we can assume that, in mice with continuously activated Notch signalling in LPCs, the contribution of these LPCs in resolving hepatocytic injury will be impeded.

As there is some controversy to the importance of the LPC response in the progression of liver disease vis-à-vis simply replacing damaged hepatocytes (27, 28), we will further investigate the role of the LPC response in resolving severe hepatocyte injury by using the choline deficient, ethionine supplemented (CDE) diet in OpnCre+;RosaNicd+/+ and OpnCre-RosaNicd+/+ mice. These mice will receive tamoxifen injections at week 3, and CDE diet will start at week 5 after birth. We will investigate the liver after 2 and 4 weeks of CDE diet, and after 2 weeks of recovery. We will then evaluate disease progression through histology, and evaluate mRNA and protein expression of LPC and inflammatory markers.

To summarise, we have shown that our tamoxifen regimen successfully induces continuous Nicd and GFP expression in the liver, however efficiency and specificity are being investigated further. Next we have shown that Nicd overexpression in osteopontin expressing cells has no effect on early pathogenesis of cholestatic liver disease evaluated by histology.

However, we did observe a small decrease in stem cell marker Prom1 and pro-inflammatory marker mRNA expression, which could result in a decreased time- to-progression in DDC-mediated biliary disease and should be further investigated. To evaluate the role of LPCs in hepatocyte-mediated liver injury, we will compare the effect of CDE feeding in OpnCre+;RosaNicd+/+ and OpnCre-;RosaNicd+/+ tamoxifen-induced mice.

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Chapter 4

Discussion

1. THE EFFECT OF PROLYL HYDROXYLASE DOMAIN INHIBITION ON THE EXPRESSION OF LPC CHARACTERISTICS IN THE PATHOGENESIS OF HEPATOCELLULAR CARCINOMA

Liver tumours often arise in a background of chronic liver disease and when tumours outgrow their vascular supply, newly formed vasculature is often structurally and functionally anomalous. Furthermore, current therapeutic options mostly aim at decreasing oxygen and nutrient supply to decrease tumour growth. These factors can all contribute to reduced liver oxygenation and activation of hypoxic adaptive response in HCC. Increased expression of markers for hypoxia has been correlated to increased invasive and metastatic potential, augment therapy resistance and induce increased expression of liver and progenitor cell characteristics, related to poor prognosis in HCC(1).

We first aimed to investigate the effect of increased HIFα stabilisation at different timepoints of hepatocarcinogenesis on the expression of LPC characteristics and prognosis in a mouse model for HCC, as described in **chapter 3.1**(2).

Increased HIFα stabilisation at advanced stages of tumourigenesis resulted in severely increased expression of LPC characteristics. However, markers of Notch signalling and metastasis were not increased at this timepoint. Conversely, HIFα stabilisation at intermediate stages of DEN-induced hepatocarcinogenesis appeared to have the least detrimental effect on tumour progression and phenotype, underlining the importance of early detection and treatment in HCC.

Interestingly, we show that early hypoxic stimuli have detrimental effects on tumour progression, resulting in tumours with an HCC-CC phenotype and increased expression of LPC characteristics and markers for metastasis indicating poor prognosis (3).

This phenotype coincided with increased expression of Notch- related markers that were not observed after DMOG treatment at intermediate or advanced stages. These results show that tumour cells can possibly be primed by hypoxic conditions early on, causing them to be more resistant to growth-or treatment-induced hypoxic conditions at later stages.

Previous studies in our research group have also shown an increased prevalence of the more aggressive mixed HCC-CC phenotype, with increased expression of LPC characteristics, after DEN-induced HCC in PHD2+/- mice (4). As this phenotype strongly resembles the mixed phenotype observed in patients with tumours recurring after resection preceded by hypoxia inducing TACE treatment (5, 6), we wanted to further evaluate early hepatocarcinogenesis in this mouse model, to identify possible predictive markers and characterise events leading to the developments of mixed phenotype tumours at later stages. We therefore analysed DEN-induced HCC in PHD2+/- and WT littermates at early timepoints, before nodule formation in **chapter 3.2**.

PHD2+/- mice are haplodeficient for the main hepatic oxygen sensor PHD2 (4), resulting in a reduced capacity for HIFα hydroxylation in normoxic conditions. However, data presented show that PHD2 haplodeficiency did not lead to increased activation of the hypoxic adaptive response at early timepoints.

Interestingly, at week 17 the expression of markers for hypoxia peaked in both genotypes and expression was higher in PHD2+/- livers compared to WTs. This peak expression of markers for hypoxia coincided with increased expression of LPC markers KRT19 and Prom1, without differences between PHD2+/- and WT mice (3).

We also found increased expression of Notch3 at this time points, which aligns with results from a previous study, where Notch3 overexpressing HCC cells were shown to have increased aldehyde dehydrogenase activity (7), characteristic to LPCs (8). We thus concluded that the observed “hypoxia”-induced phenotypical switch we previously described at later timepoints is not preceded by an early LPC or hypoxic signature.

Interestingly, Notch1 and 2 mRNA expression was decreased compared to WT DEN mice at several timepoints in PHD2+/- mice.As we did not observe any effects of this decreased Notch1 and 2 mRNA expression on downstream marker *Hes1*, it is possible that the stability of the intracellular domains of these receptors was increased through interaction with HIF1α. Indeed previous studies have shown that interaction between HIF1α and the intracellular domain of Notch receptors can increase their transcriptional activity, thus increasing Notch signalling without increasing the amount of receptors(9, 10). This theory should be tested by examining Nicd protein concentrations.

Moreover, the possibility of a negative feedback loop, decreasing the transcription of Notch1 and Notch 2 upon increased Nicd stability should be further explored. Especially since we did observe increased Notch1 and 2 mRNA expression, following early PHD inhibition in chapter 3.1, and increased Notch2 mRNA expression in a previous study using PHD2+/- mice, both associated with increased hepatocellular and cholangiocellular tumour burden at later timepoints after DEN-induced HCC (2, 4).

Possibly, during nodule formation the suggested negative feedback loop is somehow overruled in tumours with increased hypoxic signalling, resulting in increased Notch receptor expression and a peak in Notch signalling, which could, with respect to our hypotheses, potentially drive the phenotypic changes we have previously described.

While the distribution and prevalence of different Notch receptors has been mapped in human healthy liver, primary sclerosing cholangitis, primary biliary cirrhosis and alcoholic liver disease (11) and a murine model for experimental HCC (12), little is known about the expression of different Notch receptors in relation to phenotype and prognosis in experimental or human liver cancer. Further research should thus focus on determining the possibility of a negative feedback loop, decreasing the transcription of Notch receptors 1 and 2. A better understanding of the mechanisms driving these observations could provide important insights in the observed phenotypic switch.

In this observational study, we could not detect an altered LPC signature in PHD2+/- livers before nodule formation as the early neoplastic liver had not yet established the more malignant phenotype at early timepoints. Further research unravelling the events and pathways involved in the phenotypic switch, like studying the involvement of the Notch signalling pathway, could lead to new therapeutic targets and strategies to prevent the hypoxia-induced effects on prognosis in HCC.

Using the DEN mouse model has the important advantage that tumours develop in a background of inflammation and fibrosis (13), which is similar to most human cases of HCC. However, in our study, we did not observe the previously reported F2 fibrosis (13); our model could be improved by combining the DEN injections with carbon tetrachloride injections, to induce a more fibrotic background (14), more adequately mimicking the human situation.

Furthermore, the pan-PHD inhibitor DMOG was administered intraperitoneally, while this ensures fast delivery to the liver via the portal vein, the compound did not just act on liver tumour cells, but healthy hepatocytes and stroma alike.

It has been shown that PHD inhibition in tumour stroma cells can adversely decrease the invasive and metastatic potential of tumours (15). This could help explain why expression of markers for metastasis was not increased in tumours treated at advanced stages, as we also observed increased expression of HIF downstream markers in healthy liver upon DMOG administration. Interestingly, we did not observe this effect on downstream HIF targets in PHD2 haplodeficient mice until the hypoxic response was activated in both genotypes. For our studies, using the haplodeficient mice thus provided the advantage, over chemically induced HIFα stabilisation, that only hypoxic (cancer) cells were affected.

Our results further elucidate the role and dynamics of hypoxia and increased stabilisation of HIFα in HCC. We confirm that the induction of the hypoxic adaptive response in HCC is related to increased expression of LPC characteristics (2, 3, 16) and poor prognosis (2). Moreover, the timing at which a hypoxic stimulus is introduced could determine the outcome (2), and the observed increased expression of biliary characteristics in DEN-induced tumours is not preceded by an early LPC or hypoxic signature, but most likely occurs during tumour growth and progression (3). These observations can fuel further research to unravel the exact mechanism by which HIFα stabilisation affects prognosis and phenotype, to discover new therapeutic targets and/or to optimise current strategies.

2. NOTCH AS A THERAPEUTIC TARGET AGAINST HYPOXIA-INDUCED TUMOUR GROWTH AND EXPRESSION OF LPC CHARACTERISTICS IN HEPATOCELLULAR CARCINOMA

The Notch signalling pathway is involved in liver -and other cancers (17-19). Liver specific overexpression of Notch signalling was shown to induce HCC with increased expression of biliary and LPC markers (18, 20-22), as also observed in HCC with an increased hypoxic signature upon pharmacological or physiological activation of the hypoxic pathway (4-6, 10, 23, 24). Furthermore, several reports have indicated that hypoxia-mediated effects on proliferation, migration, invasion and therapy resistance in cancer are mediated by interactions between HIF and Notch signalling (1, 10, 25-28).Interestingly, previous studies have also shown that Notch 1 and 2 overexpression in hepatocytes results in the development of HCC with a strong LPC signature(18, 20, 29), similar to the phenotype we observed after early or continuous PHD inhibition in HCC mice (2, 4) and as in human tumours recurring after TACE treatment (5, 6).

As the Notch signalling pathway is suggested to be involved in HIF-mediated effects on prognosis, and we, and others, have shown increased expression of Notch receptors, ligands and downstream targets coinciding with increased hypoxic stimuli, we wanted to explore the therapeutic potential of inhibiting the Notch signalling pathway to prevent HIF-mediated effects on growth, prognosis and expression of LPC characteristics. A major downside to our first two studies is that we used PHD inhibition to mimic a hypoxic response, which, as seen in the first study (3), does not always adequately increases HIFα stabilisation and, as seen in the second study (2), can result in a, probably compensatory, decreased HIF signalling after the effects of the inhibitor wear off.

Therefore, in chapter 3.3, we aimed to induce a hypoxic response by decreasing the environmental oxygen pressure (16). Taking animal ethics into account, we used a xenograft mouse model to investigate the therapeutic potential of Notch inhibition, using a GSI, to inhibit the hypoxia-induced effects on tumour prognosis in HCC.

We found that hypoxia-induced a hypoxic adaptive response in HepG2 tumours and resulted in increased tumour growth and activation of the Notch signalling pathway (16). In line with results from PHD inhibition experiments, these tumours also had a higher expression of LPC characteristics (16). GSI treatment decreased relative tumour size and Notch pathway activation in hypoxia and decreased mRNA expression of LPC characteristics and HIF target genes in tumours in both hypoxia and normoxia. These pre-clinical results are promising for the use of GSI's as an adjuvant for tumours with a hypoxic and/or LPC signature, but its merit should be tested further in orthotopic chemical/genetic mouse models for HCC.

There are two major hypotheses concerning the interaction between Notch and HIF: i) activation of the hypoxic pathway increases Notch signalling by stimulating expression of Notch ligands, receptors and target genes (10, 25, 27), by increasing the activity of the gamma secretase complex (30, 31) and/or by stabilising the NICD protein (10) and ii) HIFα requires Notch signalling pathway activation to exert its effects on tumour prognosis (10, 32).

The data presented in chapters 3.1 and 3.2support the first hypothesis. As Notch pathway activation was higher in tumours grown in hypoxic compared to normoxic mice, and GSI inhibition decreased the expression of HIF target genes (and LPC characteristics) in both normoxic and hypoxic conditions, this study shows that both hypotheses have merit (16).

However, while the GSI treatment successfully suppressed both *Hes1* and NICD expression in tumours from mice housed in hypoxia, it resulted in increased NICD protein expression in tumours from normoxic mice (16).

Further research could help determine whether this is due to poor GSI activity or a compensatory increased NICD, Notch receptor or ligand stabilisation or expression or caused by off target interactions. Moreover, while HepG2 tumours from mice treated with the GSI were significantly smaller compared to vehicle treated mice when housed in hypoxia, GSI treatment tended to (non-significantly) increase tumour size in normoxia (16). This suggests that the effect of GSI on tumour growth might be HIF dependent, which could help to explain the controversial results concerning Notch pathway inhibition in cancer (19, 33, 34). These result implicate that measuring tumour oxygenation could be a marker for the effectiveness of GSI's (as an adjuvant) in the treatment of HCC, and possibly other solid tumours in which a strong Notch signature has been observed, like breast, cervical, pancreatic, ovarian and colon cancer (25, 27, 35, 36). A schematic overview of all findings is shown in Figure 1.

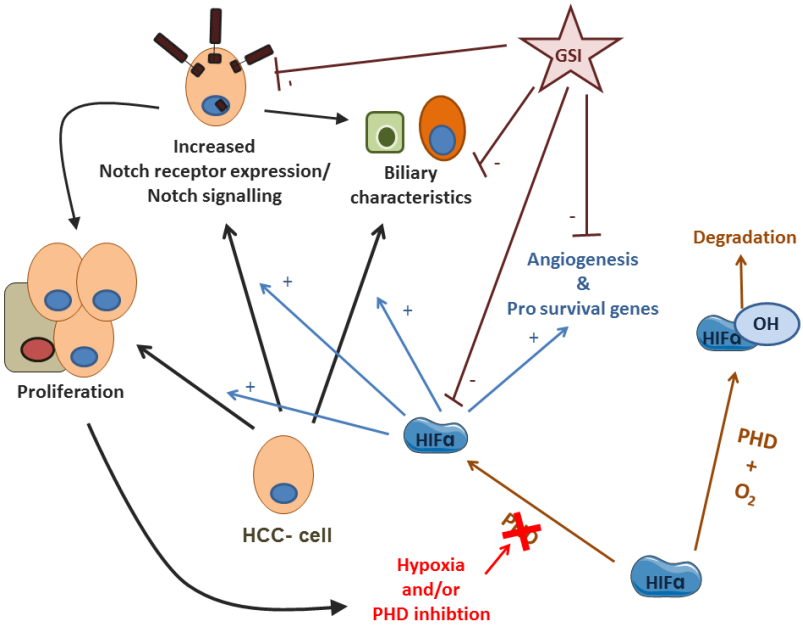

Figure 1: schematic overview of different interactions we observed in this thesis.

Black arrows present events we studied in hepatocarcinogenesis, blue arrows present the effects of increased HIFα stabilisation, purple arrows display the effects of the GSI we used.

3. FUTURE PERSPECTIVES

To unravel the effects of HIF signalling in the pathogenesis of HCC, we first used a PHD inhibitor at different timepoints in hepatocarcinogenesis and found that administering hypoxia- mimicking treatment in early or advanced stages of HCC resulted in tumours with increased expression of LPC characteristics (2). We next used PHD2+/-mice to evaluate the effect of increased HIFα stabilisation during early hepatocarcinogenesis(3). Interestingly, we found that HIF signalling was not increased in PHD2+/- mice until week 17, during nodule formation, when it also peaked in WT mice, coinciding with increased mRNA expression of LPC characteristics (3). We concluded that previously observed effects of PHD inhibition on tumour phenotype manifest during tumour growth and progression rather than in early development and are not preceded by an early LPC or Notch signature(3). Inhibiting different down-stream mediators in the presence of hypoxia and/or adding recombinant proteins possibly involved in HIF-mediated effects, like actors of the notch signalling pathway, in the absence of hypoxia in an *in vitro* setting could help elucidate possible mechanisms contributing to our observations. Continuing research focussing on the interactions at play in HIF-mediated effects on tumour characteristics could help to develop new therapeutic strategies to improve prognosis of patients with tumours growing in a fibrotic background, or receiving hypoxia- inducing therapies.

We have shown increased expression of Notch receptor mRNA in the pathogenesis of DEN-induced HCC in mice with increased HIFα stabilisation (2-4) and inhibiting the Notch signalling pathway resulted in decreased tumour size and LPC mRNA expression in a Xenograft mouse model (16).

We could thus also evaluate the therapeutic potential of Notch inhibition in DEN mice, in which PHDs are inhibited to evaluate its potential to decrease tumour burden and expression of LPC characteristics in an orthotopic model for HCC with biliary characteristics. Furthermore, these long term studies could provide more information concerning GSI safety and, in line with previous studies, this setup would also allow to investigate the effects of the GSI treatment at different timepoints in hepatocarcinogenesis.

The use of gamma secretase inhibitors is accompanied by several adverse effects of which (severe) gastro-intestinal toxicity, resulting in diarrhoea, mostly resulting from off target Notch inhibition (36). Furthermore, GSI's have been shown to cleave other membranous proteins like E-cadherin and N-cadherin, which are also involved in tumourigenesis (37). This lack in specificity not only contributes to the many side effects, it also increases the urge to improve our understanding of the role of different Notch receptors, to enable more specific inhibition of Notch receptors involved in cancer processes only. Indeed, previous studies using specific antibodies in a transgenic model for HCC and an LPC cell line have shown distinct roles for each receptor (12, 38). We too have shown that the mRNA of different Notch receptors is upregulated at different timepoints in the pathogenesis of HCC, and after the shift to a more biliary phenotype.

While the distribution and prevalence of different Notch receptors has been mapped in human healthy liver, primary sclerosing cholangitis, primary biliary cirrhosis and alcoholic liver disease (11) and a murine model for experimental HCC (12), little is known about the expression and activation of different Notch receptors in relation to phenotype and prognosis in experimental or human liver cancer.

To further investigate the role of these different receptors in the pathogenesis of HCC, and their role in hypoxia-mediated effects on prognosis and phenotype, we could use antibodies to specifically inhibit one or more receptors, as it was previously shown that inhibiting different Notch receptors had distinct effects on tumourigenesis in a mouse model for HCC driven by V-AKT and N-RAS mutations (12). Their use in different tumour models and in combination strategies with hypoxia inducing compounds like sorafenib or TACE, will allow us to further evaluate their potential to inhibit hypoxia-induced therapy resistance, metastasis and phenotypic switch(10, 25-27).

Upregulation of Notch1 and Notch2 in hepatocytes and hepatoblasts have been shown to induce hepatocellular tumours, high in biliary characteristics (18, 29, 39). However, Notch1 inhibition drastically decreased HCC but increased the cholangiocellular burden in a mouse model for HCC, while Notch 2 inhibition only decreased the HCC load (12). These findings imply a differential effect of Notch1 on the biliary and hepatocytic compartment. As Notch1 pathway activation was also suggested to be a mediator of HIF-induced effects in tumourigenesis (26, 32), further unravelling the role of Notch1 signalling on the biliary compartment is crucial for the development of targeted Notch inhibition strategies, to overcome hypoxia-mediated effects with minimal adverse effects.

This could be assessed using Notch1 inhibition (using antibodies or shRNA), and/or recombinant NICD proteins in HCC, iCC and LPC cell lines *in vitro* (25, 27). While *in vivo*, this could be approached by upregulating or knocking out Notch1 in specific cell types of the liver (18, 29, 40).

To this effect, we have started the validation of a mouse model for biliary specific increased Notch1 signalling in **chapter 3.4**.

The specificity of OpnCre mouse model we used for tamoxifen inducible Cre expression in the biliary lineage has previously been examined, using a Rosa26–loxP-stop-loxP-YFP reporter sequence and was shown restricted to LPCs and cholangiocytes in the liver (41). Efficiency of YFP expression was calculated as the ratio of cells co-expressing Sox9 and YFP, and was 69 – 84% (41, 42).

For our experiments, this OpnCre mouse was crossed to a RosaNicd mouse, in which the intracellular, transcriptionally active domain of the Notch1 receptor and a reporter GFP are (over)expressed upon excision of the LoxP flanked stop codon (43). We first established a protocol for tamoxifen administration to induce *GFP* mRNA expression. Previously shown specificity will be verified by GFP positivity on sorted cells using MACS and/or FACS. Efficiency can then be calculated by evaluating the percentage of Epcam positive cells that co-express GFP.

Once this mouse model is fully validated, it can be used to further evaluate and characterise the effect of biliary Notch1 overexpression in liver disease and cancer. We have performed a preliminary study to assess the effect of increased Notch1 signalling in LPCs and cholangiocytes in cholestatic liver disease, using the DDC diet. While we did not observe altered histological features after 3 weeks, we did observe decreased expression of stem cell marker *Prom1* and pro- inflammatory marker *Vcam1*, which was previously found upregulated in the pathogenesis of DDC-mediated liver injury (44). To assess the effect of this altered gene expression on the pathogenesis of cholestatic liver disease, we suggest evaluating the effect of increased biliary Notch1 signalling at later timepoints in the DDC feeding regimen, and after a period of recovery.

As inhibition of Notch signalling in LPCs is crucial for differentiation to hepatocytes, we expect decreased potential for hepatocytic differentiation; we will therefore use our mouse model to investigate the role of LPCs in hepatocyte-mediated injury by evaluating the effect of increased Notch1 signalling in the biliary compartment on CDE diet-induced liver injury.

These experiments have been set up in cooperation with the VUB LIVR lab and results are currently being analysed.

Lastly, as it has been proposed that increased HIFα stabilisation affects tumour cells through activation of the Notch signalling pathway, we should evaluate the effect of biliary Notch1 overexpression in the pathogenesis of hepatocellular carcinoma. Increased Notch1 expression promoted by an Afp or Albpromoter (thus hepatocytes and cholangiocytes, or hepatocytes alone respectively) resulted in spontaneous formation of HCC tumours with biliary/LPC features (18, 29). Adversely, antibody-mediated Notch1 inhibition resulted in an increased iCC load in a different study (12). Investigating the effect of increased Notch1 signalling in LPCs and cholangiocytes in the pathogenesis of HCC, and comparing it to the previously described effects of Notch1 overexpression in hepatocytes will allow us to determine if there is a cell specific effect of Notch1 signalling in the liver.

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Chapter 5

Summary

1. SUMMARY

Liver cancer is the 5th most common cancer and the second most common cause of cancer related death worldwide. Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, which is derived from hepatocytes. Liver tumours often arise in a background of chronic liver disease and when tumours outgrow their vascular supply, the newly formed vasculature is often structurally and functionally anomalous resulting in decreased oxygenation or hypoxia. In addition, current therapeutic options also aim at decreasing oxygen and nutrient supply to inhibit tumour growth, which aggravates the reduced tumour oxygenation and might result in an aberrant activation of adaptive responses.

Indeed, in conditions of reduced oxygen tension, the prolyl hydroxylase domains (PHDs) can no longer hydroxylate the hypoxia inducing factor alpha (HIFα), causing HIFα stabilisation and migration to the nucleus were it transactivates several pro-survival genes. This is called the hypoxic adaptive response. Not surprisingly, expression of key markers of the hypoxic adaptive response is related to increased therapy resistance, higher potential for invasion and metastasis and increased expression of liver progenitor cell (LPC) characteristics, all related to poor prognosis in HCC.

Since therapy for HCC inhibits the tumoural oxygen supply, our first aim was to elucidate the timepoints at which activation of the hypoxic response pathway has detrimental effects with respect to tumour outcome, which may allow us to anticipate and adapt current therapeutic strategies. In chapter 3.1, we used a pan-PHD inhibitor, to induce the hypoxic adaptive response at different timepoints in diethylnitrosamine (DEN)-induced hepatocarcinogenesis in mice.

We observed that increased HIFα stabilisation upon pan-PHD inhibition in advanced stage HCC resulted in increased expression of LPC characteristics, which has been shown associated with poor prognosis.

However, pan-PHD inhibition at intermediate stages did not result in increased expression of these LPC characteristics and decreased the HCC tumour burden. This implies a possible safe window for hypoxia inducing treatments for early- and intermediate stage HCC, highlighting the importance of early detection.

Interestingly, increased HIFα stabilisation at the onset of tumour development had detrimental effects on tumour progression, with the development of the mixed HCC-cholangiocellular (HCC-CC) phenotype and increased expression of LPC characteristics, Notch related genes and markers for metastasis. This might imply that increased HIFα stabilisation during tumour initiation, like in tumours arising in a fibrotic liver, can prime future tumour cells to react more aggressively at later stages.

As PHD2 is the main oxygen sensor in the liver, we were interested in the role of PHD2 in the observed effects of early pan-PHD inhibition. Indeed, previous studies in our lab have shown that DEN induction in PHD2 haplodeficient mice also results in the development of the more aggressive mixed HCC-CC phenotype. In **chapter 3.2**, we therefore aimed to further evaluate the effects of PHD2 haplodeficiency in early DEN-induced hepatocarcinogenesis to characterise the events leading to this more aggressive phenotype.

We found that the hypoxic adaptive response was not activated until tumours began to form, at which time mRNA expression of HIF downstream markers, LPC characteristics and Notch3 was increased, however without effect of PHD2 haplodeficiency. We concluded that the PHD2-mediated effects on tumour phenotype we previously observed at later timepoints, are not preceded by an early LPC signature.

Hypoxia-mediated effects can be potentiated by interactions between HIF and Notch signalling.

As we observed increased expression of Notch markers, coinciding with increased HIF signalling in our first 2 studies, we next aimed to investigate a potential therapeutic role for Notch inhibition to counter the effects of increased hypoxic signalling in HCC. In chapter 3.3, we evaluated tumour growth and expression of LPC characteristics in HCC xenograft mice, housed in hypoxic conditions and treated with a Notch inhibitor (gamma secretase inhibitor, GSI).

We found that Notch pathway activation and mRNA expression of LPC markers were higher in tumours of mice housed in hypoxic conditions and that this was associated with increased tumour growth. Importantly, these hypoxia-induced effects were reduced upon GSI treatment. Moreover, GSI also decreased mRNA expression of HIF target genes and LPC characteristics in normoxic conditions. These pre-clinical results are promising for the use of GSI's as an adjuvant in patients who have developed a tumour in a background of fibrosis (characterised by hypoxia) and/or receiving oxygen depriving treatment.

Lastly, to gain further insight in the role of Notch signalling in liver disease and cancer, we initiated the validation of a mouse model for inducible, biliary specific Notch 1 over-expression, in chapter 3.4, which will allow us to further define the cell -and receptor specific effects of Notch signalling in liver disease and cancer.

In our study, we have shown that hypoxia and Notch signalling are involved in the expression of LPC characteristics in mouse models for HCC. We show that activation of the hypoxic response is related to increased expression of LPC markers, and increased tumour growth which are linked to poor prognosis.

Inhibiting the Notch signalling pathway showed promising therapeutic potential to inhibit hypoxia-induced effects in a xenograft mouse model for HCC. However, the roles of different Notch receptors and their potentially differential effects on various cell-types in liver disease and cancer should be further investigated.

2. SAMENVATTING

Primaire leverkanker is de 5e meest prevalente kanker en de 2e meest dodelijke kanker wereldwijd. Kanker van de hepatocyten of hepatocellulair carcinoma (HCC) ontstaat meestal in patiënten met chronische leverziekte veroorzaakt door, onder andere hepatitis B, hepatitis C, chronisch alcoholisme en leververvetting. Deze gaan allen gepaard met verhoogde bindweefselafzetting en dus de vorming van fibrose, waardoor de doorbloeding van de lever bemoeilijkt wordt, en de zuurstoftoevoer belemmerd wordt. Bovendien steunt de behandeling van HCC voornamelijk op het verminderen van de toevoer van nutriënten en zuurstof naar de tumor, om zo tumorgroei te vertragen en overleving te verlengen.

Wanneer cellen verlaagde zuurstofspanning of hypoxie ondervinden, kunnen de prolyl hydroxylase domeinen (PHD) de hypoxie induceerbare factor alpha (HIFα) niet langer hydroxyleren waardoor deze gestabiliseerd wordt en in de nucleus de transcriptie van genen betrokken bij cel overleving stimuleert. Dit proces wordt de hypoxische adaptieve respons genoemd en de expressie van merkers voor dit proces is gerelateerd aan slechte prognose door verhoogde kans op metastase, therapieresistentie en expressie van lever progenitor cel (LPC) kenmerken in HCC.

Aangezien behandeling bij HCC steunt op het induceren van hypoxische omstandigheden, gingen we na of het tijdstip waarop verhoogde HIFα stabilisatie optreedt effect heeft op tumor progressie en karakteristieken. In hoofdstuk 3.1 gebruikten we een PHD inhibitor om verhoogde HIFα stabilisatie te induceren op 3 tijdstippen: vroeg, intermediair en laat in de pathogenese van HCC.

Hier observeerden we dat PHD inhibitie op een laat tijdstip, na het ontwikkelen van tumor noduli, een verhoogde expressie van LPC merkers uitlokt, terwijl HIFα stabilisatie op een intermediair tijdstip, tijdens de vorming van tumor noduli, geen effect had op de expressie van LPC kenmerken en leidde tot minder HCC ontwikkeling in muizen.

Deze bevindingen ondersteunen de hypothese dat er mogelijks een “veilig” tijdskader voor hypoxie inducerende behandelingen bestaat wanneer het ontstaan van neoplastische laesies tijdig gedetecteerd wordt.

Daarnaast resulteerde activatie van de hypoxische adaptieve respons tijdens tumorinitiatie expressie van tot verhoogde LPC karakteristieken, Notch merkers en een verhoogde HCC-tumorlast na het ontwikkelen van tumoren. Het is dus mogelijk dat verhoogde HIFα stabilisatie tijdens tumorinitiatie, zoals kan verwacht worden voor tumoren die ontstaan in een fibrotische lever, toekomstige tumorcellen kan voorbereiden om later een meer agressief fenotype te ontwikkelen.

Omdat PHD2 de belangrijkste zuurstofsensor is, wilden we vervolgens nagaan of de effecten van PHD- inhibitie voornamelijk PHD2- gemedieerd waren. Inderdaad, eerdere studies in ons labo toonden al aan dat HCC inductie in PHD2 haplodeficiente muizen ook leidt tot verhoogde tumorlast en expressie van LPC kenmerken.

Om meer inzicht te krijgen in de mechanismen die bij de ontwikkeling van dit meer agressieve fenotype betrokken zijn, gingen we vervolgens de expressie van HIF, LPC en Notch merkers in de vroege tumor- ontwikkeling van deze PHD2 haplodeficiente muizen na in hoofdstuk 3.2. Hierbij werd geen vroegtijdige verhoogde expressie van LPC of hypoxie- gerelateerde kenmerken vastgesteld op vroege tijdstippen. Op het laatst bestudeerde tijdspunt, net voor de vorming van HCC noduli, observeerden we een sterk verhoogde expressie van HIF, LPC en Notch3 merkers. Op dit tijdspunt was er geen verschil in de expressie van LPC kenmerken en Notch3 tussen PHD2 haplodeficiente en wild type muizen. We concluderen dat de effecten van verminderde PHD expressie waarschijnlijk plaatsvinden tijdens vorming van tumornoduli en niet voorafgegaan worden door een veranderde expressie van LPC of Notch merkers.

Activatie van de Notch signalisatieweg werd al uitvoerig beschreven in HCC, en werd vooropgesteld als de effector van HIF gemedieerde effecten op de prognose van verschillende kankers. In onze PHD- inhibitie studies observeerden we inderdaad een verhoogde mRNA expressie van Notch merkers waardoor we vervolgens het therapeutisch potentieel van Notch inhibitie met behulp van een gamma secretase inhibitor (GSI) wilden nagaan **in hoofdstuk 3.3**. Om een hypoxische adaptieve respons te induceren werden HCC-xenograft muizen in een hypoxische kamer geplaatst. Hier toonden we aan dat tumoren van hypoxische muizen groter waren en gekenmerkt waren door verhoogde Notch signalisatie en expressie van LPC merkers.

Belangrijk was dat GSI behandeling deze hypoxie gemedieerde effecten op tumoren kon onderdrukken. Bovendien was de behandeling niet toxisch en kon gamma secretase inhibitie ook bij normoxie de mRNA expressie van HIF en LPC merkers verminderen. Deze preklinische resultaten zijn een belangrijke stap in de richting van het gebruik van Notch inhibitoren als bijkomende therapie in hypoxische tumoren en/of in combinatie met huidige behandelingen, om nadelige effecten van zuurstoftekort in de behandeling van HCC tegen te gaan.

Om eventuele Notch – inhibitie therapieën verder te optimaliseren moet verder inzicht verworven worden in de precieze invloed van verschillende Notch receptoren op verschillende celtypes in de evolutie van leverziekte en kanker. Daarvoor werd in **hoofdstuk 3.4** een muismodel voor induceerbare verhoogde Notch1 signalisatie in de biliaire lijn ontwikkeld, waarbij de eerste stappen naar de validatie gezet zijn.

In dit onderzoek werd aangetoond dat hypoxie- en Notch signalisatie betrokken zijn bij de expressie van LPC kenmerken in muismodellen voor HCC. We tonen aan dat activatie van de hypoxische adaptieve respons gepaard gaat met verhoogde expressie van LPC merkers en tumorgroei, beide gerelateerd aan een slechtere prognose.

Bovendien tonen we aan dat inhibitie van de Notch signalisatieweg veelbelovend therapeutisch potentieel biedt om de hypoxie geïnduceerde effecten tegen te gaan in een xenograft model voor HCC. Echter, de specifieke rol van verschillende Notch receptoren in leverziekte en kanker dient nog verder onderzocht te worden.

Curriculum Vitae

Personalia

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Master thesis:The role of glucosetransporters and hexokinases in livercirrosis

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2007- 2010 Bachelor in biomedical sciences, Ghent University

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Postgraduate Courses

2016 Statistical thinking and smart experimental design (VIB)

2015 qPCR course (Biogazelle, Bram De Craene)

2015 Clinical studies: study design, implementation and reporting

2014 Authentic Networking (UGent)

2013 Advanced Academic English Conference Skills (UGent):

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Awards and Grants

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UegW Travel grant

Granted by United European Gastroenterology for submitted abstract and participation to the UegWeek in Barcelona (2015)

BWGE Research grant

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EASL Young investigator bursary

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VVGE aanmoedigingsbeurs

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Emmanuel van der Schueren grant

Granted by the flemmish league against cancer (VLK) for research proposal (2012)

Research Communication

Eliene Bogaerts, Femke Wulgaert, Yves-Paul Vandewynckel, Petra Vanwassenhove, Xavier Verhelst, Anja Geerts, Peter Carmeliet, Lindsey Devisscher and Hans Van Vlierberghe; The effect of prolyl hydroxylase domain-2 haplodeficiency on liver progenitor cell markers in early hepatocarcinogenesis. POSTER presentation and ORAL poster champ session at United European Gastroenterology week, Barcelona, Spain, 2015

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Eliene Bogaerts, Femke Heindryckx, Anja Van den Bussche, Anja Geerts and Hans Van Vlierberghe; Hypoxia induces an increased number of progenitor cells in late but not in early stages of hepatocellular carcinoma. ORAL presentation presented at the Belgian week of gastroenterology, La Hulpe, Belgium, 2014

Eliene Bogaerts, Aurelie Comhaire , Femke Heindryckx, Annelies Paridaens , Yves-Paul Vandewynckel, Peter Carmeliet, Anja Geerts, and Hans Van Vlierberghe; The role of hypoxia on liver progenitor cell activation in a mouse model for hepatocellular carcinoma. ORAL presentation presented at the Belgian week of gastroenterology, La Hulpe, Belgium, 2014

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Student supervision and training

Aurélie Comhaire:	Influence of hypoxia on differentiation of liver progenitor cells in the liver -Master in biomedical sciences (2013) Promotor: Prof. Dr. Hans Van Vlierberghe
Femke Wulgaert:	The role of hypoxia on expression of liver progenitor cells at different timepoints during hepatocarcinogenesis - Master in biomedical sciences (2015) Promotor: Prof. Dr. Hans Van Vlierberghe
Sander Lefere:	Biliary Notch1 signalisation in the pathogenesis of fatty liver disease - Master of Medicine in Medicine (2015) Promotor: Prof. Dr. Anja Geerts
Joyca De Temmerman:	The role of correct activation and differentiation of liver progenitor cells in different mouse models for liver damage – Master in biomedical sciences (2016) Promotor: Prof. Dr. Hans Van Vlierberghe

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