INTERPLAY BETWEEN OXYGEN SENSING MECHANISMS AND HEPATITIS B VIRUS REPLICATION

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ABSTRACT

Hepatitis B virus (HBV) preferentially replicates in the liver, a naturally hypoxic organ with an oxygen gradient from 8-4% in the periportal and pericentral regions, respectively. The hypoxia inducible factor (HIF) pathway is the most well studied mammalian oxygen sensing mechanism and is frequently perturbed by pathological states including infection and inflammation. However, the majority of *in vitro* studies have investigated HBV at atmospheric oxygen tensions (18%) where low oxygen sensing mechanisms are inactive and consequently their role in viral replication may be overlooked. Therefore, this thesis studied the interplay between HBV replication and oxygen sensing mechanisms.

Chronic hepatitis B (CHB) is associated with an increased expression of HIF gene transcripts. Further interrogation of a cohort of patients with CHB shows an association between inflammatory responses and HIF transcription. Importantly, this observation is not recapitulated in state-of-art *in vitro*, *in vivo*, and *de novo* HBV infection model systems.

HIF regulates target genes via HIF responsive elements (HREs), and the discovery of two evolutionarily conserved HREs in the HBV genome prompted experiments to understand the role of HIFs in viral replication. HIF siRNA silencing experiments *in vitro* and *in vivo* demonstrate a positive role for HIFs in regulating HBV replication.

As other oxygen sensing mechanisms have been identified in addition to the HIF pathway, further experiments assessed the role of hypoxia and HIF independent mechanisms in regulating HBV transcription. Data suggests that hypoxia positively regulates HBV transcription through both HIF dependent and independent mechanisms. Further investigations reveal that low oxygen

downregulates the HBV host restriction factor Smc6 through HIF independent mechanisms. In addition, the cysteamine (2-aminoethanethol) dioxygenase oxygen sensing pathway regulated interleukin-32 may have anti-viral activities that are repressed under hypoxia.

Collectively, this thesis presents evidences demonstrating key regulatory interactions between HBV and hypoxia, revealing novel therapeutic directions.

DEDICATION

This thesis is dedicated to my grandfather, parents and Lucy.

In memory of my grandfather, Wenzheng Liu, for inspiring me to live life with kindness, humility, and social responsibility.

To my parents, Chunsheng Liu and Shuqin Wang, for their unwavering nurture and support to help me pursue knowledge and passion, as well as being excellent role models teaching me how to live a fulfilling and meaningful life.

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LIST OF COMMON ABBREVIATIONS

ADO	Cysteamine (2-aminoethanethol) dioxygenase
ALT	Alanine aminotransferase
BCP	Basal core promoter
CAIX	Carbonic anhydrase IX
cccDNA	Covalently closed circular DNA
CHB	Chronic hepatitis B
DMSO	Dimethyl sulfoxide
FIH	Factor inhibiting hypoxia inducible factor
HbcAg	Hepatitis B virus core protein
HbeAg	Hepatitis B virus e antigen
HbsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HBx	Hepatitis B virus x protein
HCC	Hepatocellular carcinoma
HIF	Hypoxia inducible factor
HNF	Hepatocyte nuclear factor
HRE	Hypoxia inducible factor responsive element
IFN	Interferon
IL	Interleukin
NTCP	Sodium taurocholate co-transportation polypeptide
ORF	Open reading frame
PCR	Polymerase chain reaction
pCRNA	PreCore ribose nucleic acid
pgRNA	Pre-genomic ribose nucleic acid
PHD	Prolyl hydroxylase domain
PHH	Primary human hepatocyte
rcDNA	Relaxed circular deoxyribose nucleic acid
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau protein

1. INTRODUCTION

1.1 HBV Epidemiology

Hepatitis B virus (HBV) infection is a major global health problem with over 250 million people chronically infected and an estimated 2 billion people showing serological evidence for past or present infections worldwide[1]. There are at least 880,000 mortalities annually from HBV-related liver diseases including hepatocellular carcinoma (HCC) and cirrhosis. HBV can cause acute or chronic infection in the liver. In general, 90% of infected healthy adults recover within the first year but 5% of patients will develop chronic infection with 20-30% progressing to develop cirrhosis and HCC[2]. However, spontaneous viral clearance varies greatly depending on the age of exposure, with neonates and young children at a higher risk of developing chronic infection. Figure 1.1 illustrates the global prevalence of HBV infection[2].

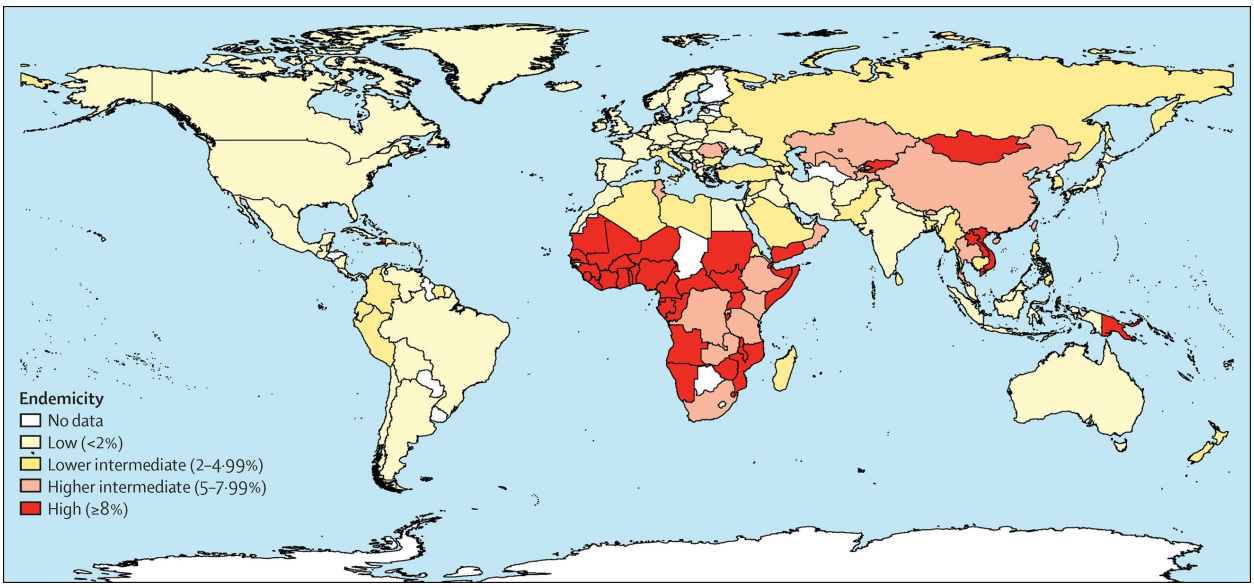


Figure 1.1. Global prevalence of Hepatitis B virus. Adapted from Schweitzer *et al.*[2].

Chronic hepatitis B (CHB) is currently incurable and prophylactic vaccine has no effect on existing infections. CHB is associated with a blunted host innate immune response, high viral antigen and DNA expression along with an exhausted anti-viral response which fails to control HBV replication[3,4]. Existing treatments may be life-long to suppress viral replication. Even previously infected patients who have been cleared of the virus may develop liver cancer[5]. As a result, there is an urgent need to better understand HBV tropism and replication to unravel novel therapeutic targets.

At least ten HBV genotypes, A-J, have been identified as defined by a >8% divergence in nucleotide composition in the viral genome[6]. Each genotype has different patterns of geographical distribution[7]. For example, genotype A is predominantly found in Africa, Europe, and India, genotype B and C in Asia-pacific regions, Northern Canada, and Greenland, genotype D in Africa, the Mediterranean region, and India, and genotypes E-J are more region specific. Table 1.1 summarises the geographical distribution of HBV genotypes A-J[7]. Given this diversity of HBV genotypes, co-infection by more than one genotype is not unusual. Studies in Taiwan have found that 16-33% of HBV positive intravenous drug users have mixed genotype infections[8,9]. As a result, recombination events between genotypes have occurred and resulted in new HBV quasispecies. For instance, recombination of genotypes B and C led to genotype B2 whilst recombination between A, C, and G led to genotype I[10,11]. Overall, HBV genotype is an important factor in determining disease progression and clinical outcomes. For example, patients infected by genotype C might have more severe liver damage compared to genotype B, whilst genotype D might be more associated with cirrhosis development compared to genotype A[12,13].

Table 1.1. HBV genotype and subtype geographic distribution. Adapted from Lin *et al.*[7].

Genotype	Subtype	Geographic distribution
A	A1	Sub-Saharan Africa, India
	A2	Northern Europe, India
	A3	Western Africa
	A4–A7	Gambia, Nigeria
B	B1	Japan
	B2–B5	East Asia, Taiwan, China, Indonesia, Vietnam, Philippines
	B6	Alaska, Northern Canada, Greenland
C	C1-C3	Taiwan, China, Korea, Japan and Southeast Asia.
	C4	Australia
	C5	Philippines, Vietnam
	C6-C11	Indonesia
D	D1–D7	Africa, Europe, Mediterranean countries, India, Indonesia, Australia
E		West and central African, Saudi Arabia
F	F1–F4	Central and South America
G		France, Germany and the United States
H		Central America
I		Vietnam and Laos
J		Japan

There are three main modes of HBV transmission: perinatal, sexual, and blood transmission[7]. The prevalence of each mode differs between geographical locations, and hence HBV genotypes[7]. Perinatal transmission in which HBV is vertically transmitted from mother-to-infant commonly occurs in Asia where genotypes B and C are prevalent. Other HBV genotypes are usually found in areas where sexual and blood transmission commonly occur between adults. It is important to note that risks associated with developing CHB decrease with age of initial infection, possibly due to less developed immune systems in infants and children[2]. Around 90% of perinatal infections and 20-60% of infections between 6 months to 5 years of age result in CHB. This highlights the importance of understanding the interaction between HBV, host factors, and immune response.

1.2 HBV Genome, Viral Transcripts, and Proteins

HBV is a DNA virus in the *Hepadnaviridae* family. It has a partially double-stranded 3.2kb DNA genome, which contains a complete minus strand DNA and an incomplete plus strand DNA at the 3'-end, forming a relaxed circular DNA (rcDNA) as an intermediate form of viral genome. There are four overlapping open reading frames (ORFs), which are called Core (C), Surface (S), Polymerase (P), and Hepatitis B X protein (X) ORFs[14]. These ORFs produce five major mRNA transcripts that encode seven types of proteins[15]. Four promoters are present in the HBV genome, including the basal core promoter (BCP), Sp1 promoter, Sp2 promoter, and X promoter[16]. Figure 1.2 is an illustration of HBV DNA, its overlapping reading frames, RNA transcripts, and proteins.

The C ORF produces preCore RNA (pCRNA) and pre-genomic RNA (pgRNA), which encode e-antigen (HBeAg) and core protein (HBcAg) that form the HBV nucleocapsid, respectively. The S ORF contains the S, preS1, and preS2 regions encoding viral surface proteins (HBsAg). It can produce preS1 and preS2 RNAs which can be translated to three HBsAg proteins, large (L), middle (M), and small (S). The preS1 N-terminal domain is present only in L-HBsAg, preS2 is present in both L-HBsAg and M-HBsAg, and S C-terminal domain is present in all three proteins. Therefore, preS1 RNA produces L-HBsAg while preS2 RNA produces M-HBsAg and S-HBsAg. The P ORF encodes HBV polymerase which is covalently bound to the 5'-end of HBV's minus strand DNA. The X ORF produces HBx RNA which encodes the multifunctional HBV X protein (HBx). DR1 and DR2 are two direct repeats in the 5'-end of the HBV plus strand that enable strand specific DNA synthesis. Two enhancers elements, EnhI and EnhII, are located near the BCP region[14]. EnhI partially overlaps with the X promoter to regulate BCP activity and pC/pgRNA transcription[17]. EnhII overlaps with the BCP region and regulates BCP, Sp1, Sp2, and X promoter activity[18].

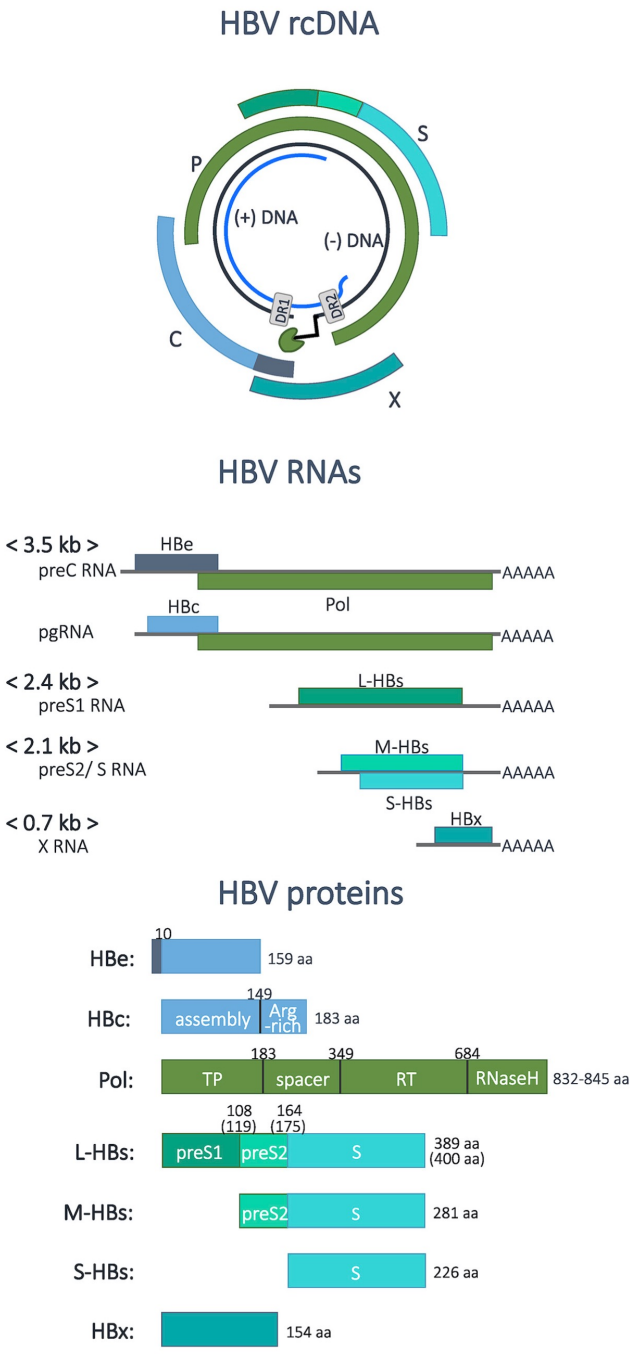


Figure 1.2. Illustration of Hepatitis B virus rcDNA, transcripts, and proteins. HBV relaxed circular DNA (rcDNA) encodes the open reading frames (ORFs) C, S, P, and X, along with direct repeats 1 and 2 (DR1, DR2). HBV RNAs include precore (preC), pregenomic (pg), preS2/S and X RNAs. HBV proteins include hepatitis B e protein (HBe), core protein (HBc), polymerase (Pol), large, medium, and small surface proteins (L-HBs, M-HBs, S-HBs), and HBx protein. Adapted from Tsukuda and Watashi[19].

1.3 HBV Life Cycle

HBV life cycle can be broadly divided into the following stages: cell attachment and entry, nuclear import, rcDNA repair into covalently closed circular DNA (cccDNA), transcription, translation, particle assembly and release[20]. Figure 1.3 provides a schematic illustration of the HBV life cycle.

HBV begins its interaction with host cells by binding to heparin sulphate proteoglycans (HSPGs) on the cell surface[21]. The antigenic loop in the S domain of HBsAg binds hepatocyte specific HSPGs such as glypican 5 (GPC5) via R122 and K141 residues. HBV then binds the sodium taurocholate co-transportation polypeptide (NTCP) receptor with higher affinity[22]. The preS1 domain of l-HBsAg binds NTCP extracellular loops and enables cell entry. HBV is internalised via endocytosis, with clathrin-, caveolae-, and micropinocytosis-dependent endocytosis reported to mediate this process[23].

HBV envelope fusion with endosomal membranes allows the release of HBV genome containing capsid into the cytoplasm[24]. HBV capsid is transported towards the nucleus by microtubule networks. To translocate into the nucleus, HBV interacts with the nuclear pore complex (NPC), large protein complexes in the nuclear envelope. NPC is composed of ~30 nucleoporin (Nup) proteins forming an octagonal complex with a pore across the nuclear envelope. HBV capsid binds importin-α and importin-β on the NPC and is transported into the nucleus through the NPC pore where it binds Nup153 protein at the nuclear basket, an NPC structure inside the nucleus. HBV capsid then undergoes disassembly to release viral genome into the nucleus[25].

Inside the nucleus, the positive strand of the partially double-stranded rcDNA is repaired to form cccDNA, a stable form of HBV genome acting as a reservoir for viral replication[26]. In fact, elimination of the cccDNA reservoir has been a key focus to achieve a sterilising cure for HBV infections. Although it is speculated that host cellular DNA repair enzymes catalyse steps in cccDNA formation, the precise mechanism remains unclear[27]. Recent studies suggest that the minus-strand of rcDNA requires only flap endonuclease 1 (FEN-1) and DNA ligase (LIG1) for repair into cccDNA, whilst plus-strand rcDNA requires proliferating cell nuclear antigen (PCNA), replication factor C (RFC) complex, DNA polymerase delta (POLδ), FEN-1, and LIG1[28,29]. These observations suggest that rcDNA minus- and plus-strands may undergo distinct processes for repair into cccDNA.

HBV cccDNA assembles with host histone proteins and can be regulated through post-translational modification of such histone proteins[30]. Host RNA polymerase II is used to transcribe HBV mRNA from cccDNA[31]. These viral transcripts are then translated into proteins by host ribosomes in the cytoplasm[32]. Host transcription factors are critical for inducing HBV RNA transcription and a number of them have been identified to bind promoter and enhancer regions of the HBV genome. HBV generates new HBV genome through reverse transcription of pgRNA into rcDNA. This process occurs when pgRNA is encapsulated into the nucleocapsid where HBV polymerase is responsible for reverse transcription.

HBV polymerase contains four domains: the terminal protein (TP) domain, spacer domain, reverse transcriptase domain, and RNAseH[33]. Viral polymerase TP domain first binds to a stemloop structure called epsilon signal found at the 5'-end of pgRNA. DNA polymerisation is initiated by addition of 2 deoxy-adenosine monophosphate (dAMP) to deoxy-guanosine monophosphate

(dGMP) on the viral polymerase. Synthesis of the minus strand DNA occurs when the HBV polymerase complex attaches to DR1 domain at the 3'-end of pgRNA. As minus strand DNA is synthesised, pgRNA is degraded by the RNAseH domain of HBV polymerase. The positive DNA strand is synthesised in an opposing direction, where the RNAseH degradation resistant 5'-end of pgRNA is used for protein priming. The HBV DNA produced will serve three functions: 1) viral budding to form new viral particles for release; 2) integration into the host genome where it serves as a transcription template for HBsAg; 3) maintains a stable reservoir of cccDNA in the host nucleus[34]. For viral budding, HBV capsids are enveloped by a host cell membrane bilayer generated from multivesicular bodies (MVB)[35]. HBsAg proteins are present on the MVB membrane. This inward budding process incorporates the viral capsid into an HBsAg containing membrane. These HBV particle are then secreted through endosomal sorting complexes required for transport from the host cell[20].

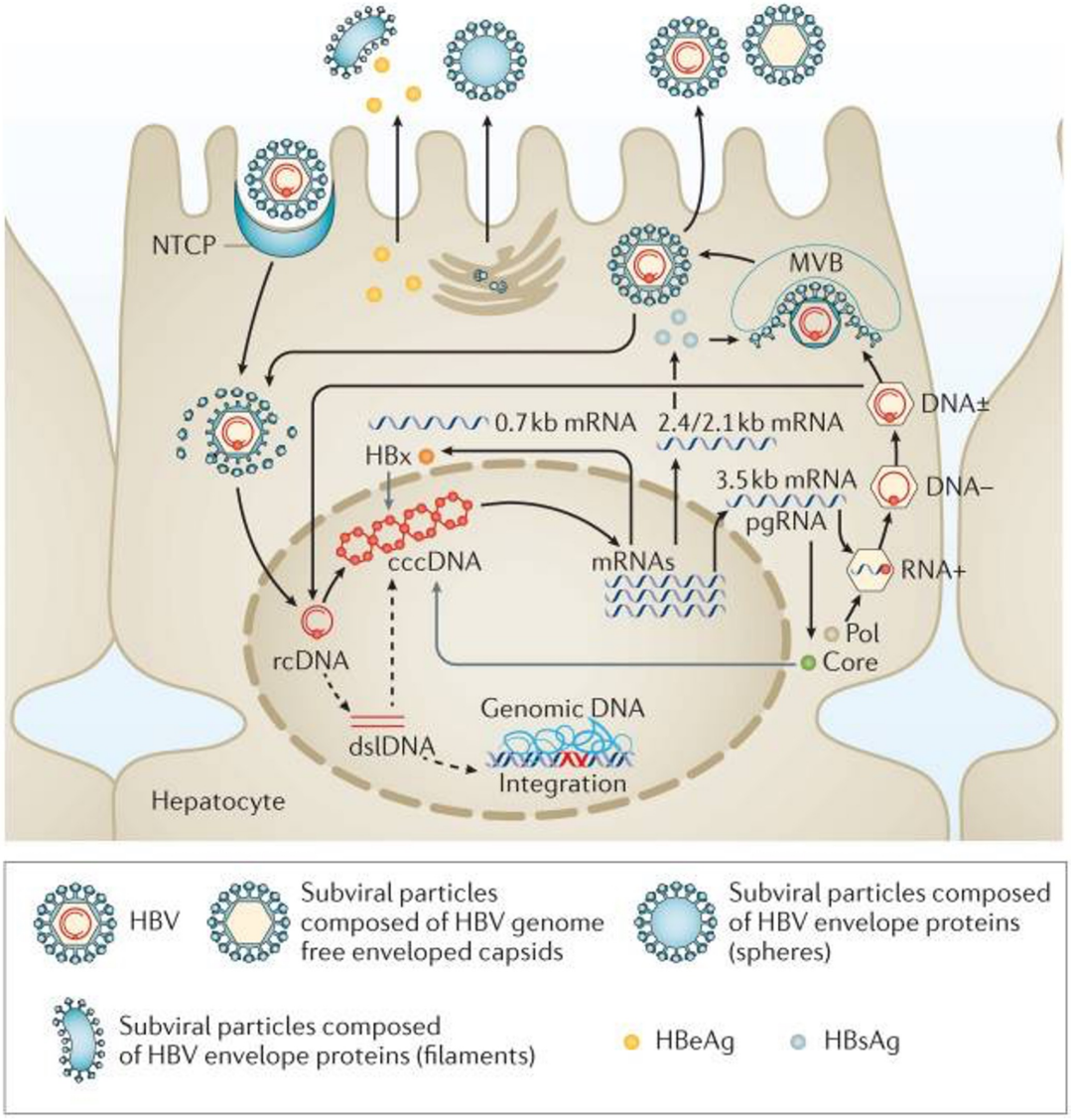


Figure 1.3. Schematic illustration of Hepatitis B viral life cycle. HBV attaches to sodium taurocholate co-transportation polypeptide (NTCP) to enter the host. Viral relaxed circular DNA (rcDNA) is released into the nucleus and repaired into covalently closed circular DNA (cccDNA) where viral transcripts are produced, including pregenomic RNA (pgRNA). HBV pgRNA can produce polymerase (Pol) and core (Core) proteins. Double-stranded linear DNA (dslDNA) may also be generated which can integrate into the host genome. Newly synthesised HBV genome contained in viral capsids can be exported via multivesicular bodies (MVB). Adapted from Thomas and Liang[36].

1.4 HBV Transcription Factors

HBV is dependent on host factors to enable viral transcription, usually through interaction with promoter, EnhI, and EnhII regions of cccDNA[37]. Figure 1.4 provides an overview of host transcription activators and repressors reported to interact with and regulate the HBV genome. A number of ubiquitously expressed host transcription factors have been observed to regulate HBV transcription. For example, CCAAT enhancer binding proteins (C/EBP) are transcription factors known to regulate metabolism, homeostasis, and hepatocyte growth[38]. C/EBP has been reported to bind multiple sites at the BCP and S promoter regions on cccDNA to upregulate HBV transcription[39,40]. Host regulatory mechanisms such as microRNA-155 have been reported to downregulate C/EBP-β and to repress HBV transcription[41].

Another well studied HBV host transcription factor is specificity protein 1 (Sp1), which plays a role in early development, angiogenesis, and cell cycle control[42]. Sp1 has been reported to bind near the BCP, preS1/S2 promoter, and EnhII regions of cccDNA[17,37]. Sp1 binding near the BCP downregulates transcription of the X and S genes while binding to EnhII globally upregulates all HBV transcriptions, highlighting the complex mechanism underlying host factor regulation of HBV transcription.

In contrast, regulatory factor box 1 (RFX1) protein, with a role in regulating host inflammation and cell proliferation, has been reported to repress HBV transcription through interaction with EnhI and BCP regions of cccDNA[43,44]. Interestingly, despite being a ubiquitous transcription factor, RFX1 appears to not bind sequences similar to the HBV EnhI region in non-hepatocytes, suggesting a role for liver specific factors in this interaction[45]. Another example of a transcription repressor is nuclear factor kappa B (NF-κB), an important innate and adaptative immunity

regulator[46]. NF-κB has been reported to compete with Sp1 for binding near the BCP region and inhibit all HBV transcripts. This observation illustrates the dynamic interaction between host transcription activators and repressors in regulating HBV gene expression[47].

The structure maintenance of chromosome 5/6 (Smc5/6) complex was recently identified to restrict HBV transcription[48]. Smc5/6 is a member of the Smc complex family, a highly conserved cellular machinery in bacteria and eukaryotes[49]. The Smc5/6 complex composes of Smc5 and Smc6 proteins tethered by non-structural maintenance of chromosome element (Nse) 4, and other Nse proteins. Depletion of Smc5, Smc6, or Nse components with the exception of Nse2 leads to the destabilisation of the entire Smc5/6 complex[48,50]. Smc5/6 is indispensable for genome stability by resolving stalled replication forks, repairing double-stranded DNA damage, and enabling alternative lengthening of telomeres[51-53]. Decorsière *et al.* showed that the Smc5/6 complex binds to HBV cccDNA to restrict viral transcription[48]. To evade this process, HBV encoded regulatory protein HBx binds to DNA-damage binding protein1 (DDB1) and Cullin4 (Cul4) E3 ubiquitin ligase complex to target Smc5/6 for proteasomal degradation and thereby promote viral transcription. This illustrates the bilateral interaction between viral and host factors to regulate HBV transcription.

In addition to ubiquitously expressed transcription factors, a number of liver specific factors have been reported to play a role in regulating HBV transcription, which could contribute to viral tropism in the liver[37]. The hepatocyte nuclear factor (HNF) family of proteins were initially discovered based on their roles in the liver, and recent studies have found that they also regulate the development of the embryo, kidney, and pancreas[54]. HNF1 has been observed to bind the preS1 promoter and EnhII regions on cccDNA to increase HBV transcription[55,56]. HNF1 appears to

interact with the ubiquitous octamer transcription factor (Oct) to regulate preS1 promoter activity and work with HBx to induce core promoter transcription[55,57]. HNF3 proteins have been observed to bind EnhI, EnhII, and preS1 promoter regions to upregulate HBV transcription[58-60]. As HNF3 has been observed to open compacted chromatin, it has been hypothesised that the host factor may be able to facilitate access and recruitment of other transcription factors to the HBV mini chromosome and to induce transcription[46,61]. HNF4α is another member of the HNF family with a role in regulating lipid metabolism, inflammation, and embryogenesis[62]. HNF4α has been reported to bind EnhI, EnhII, and preS1 regions of the HBV genome to positively regulate viral transcription[63-65]. Host estrogen receptor α (ERα) has been observed to bind HNF4α and inhibit its interaction with EnhI, therefore repressing HBV transcription in female mice[66]. These reports highlight the complex interactions between viral and host factors to regulate HBV transcription and tropism.

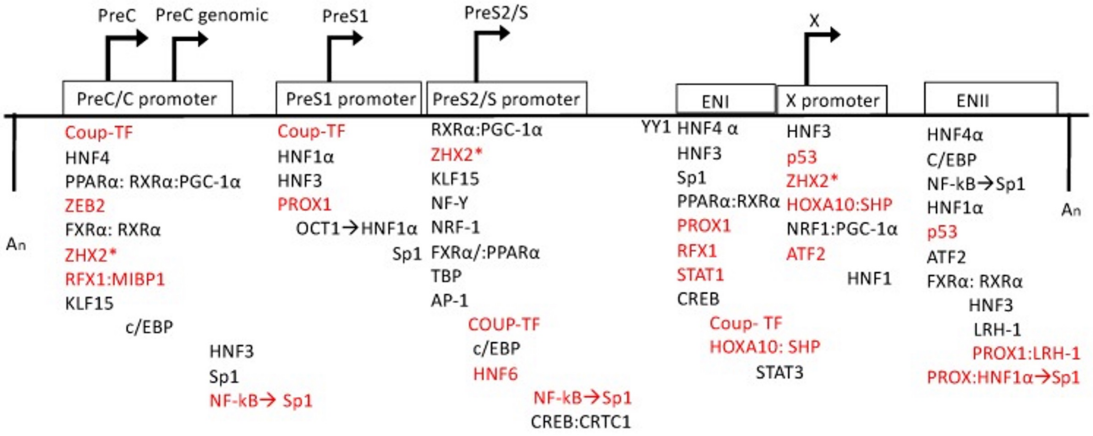


Figure 1.4. Summary of proposed HBV host transcription activators, repressors, and binding sites in the HBV genomes. Black represents transcription activators and red represents repressors. Arrow between transcription factors represents interaction between the two. Asterisk indicates that transcription position is unknown. Adapted from Turton *et al.*[27].

1.5 Natural History of HBV

CHB disease progression can be broadly categorised into five stages according to European Association for the Study of the Liver (EASL) guidelines: HBeAg positive chronic infection, HBeAg positive chronic hepatitis, HBeAg negative chronic infection, HBeAg negative chronic hepatitis, and HBsAg negative phase[67]. CHB could develop in a sequential manner but patients may not go through all stages and often have different duration at each stage[68]. Patients may also progress between stages in a non-sequential and dynamic manner during CHB. It is important to note that current guidelines rely on peripheral measurements of HBV infection and may not fully demonstrate intrahepatic replication in the liver. Figure 1.5 illustrates the natural history of HBV infection[69].

The HBeAg positive chronic infection stage is characterised by high circulating HBV DNA and normal or raised serum ALT. Inflammation and signs of fibrosis in the liver are unusual at this stage. Patients infected perinatally or at a young age commonly go through this phase with prolonged duration. During the HBeAg positive chronic hepatitis stage, HBeAg positive status and high HBV DNA are maintained. However, ALT will be high, which corresponds to moderate to severe liver inflammation and the initiation of fibrosis[67]. Transition from HBeAg positive chronic infection to hepatitis can be rapid in patients infected by HBV as adults or may require many years in others. Some patients might undergo HBeAg seroconversion that associates with reduced levels of HBV DNA. However, other patients could sustain high HBV DNA levels despite the lack of HBeAg and progress to HBeAg negative chronic hepatitis.

The HBeAg negative chronic infection stage is characterised by low to undetectable levels of HBV DNA (<2,000 IU/mL)[67]. Patients usually have normal ALT levels and low levels of inflammation

and fibrosis in the liver. Some patients could have HBV DNA levels above 2,000 IU/mL but their ALT level remains normal. Around 1-3% of patients per year in this stage could progress to HBsAg seroconversion spontaneously. Patients who remain at this stage are at lower risks to developing cirrhosis and HCC but could still progress to HBeAg negative chronic hepatitis. Patients with HBeAg negative chronic hepatitis usually have detectable HBeAg antibodies but fluctuating or persistent moderate to high HBV DNA and ALT levels[67]. This stage has active inflammation and fibrosis in the liver and disease remission is rare.

Finally the HBsAg negative stage is associated with the presence of HBcAg antibodies and undetectable peripheral HBsAg[67]. ALT levels are normal with low to undetectable levels of serum HBV DNA. If a patient progresses to this stage without cirrhosis, cirrhosis and HCC are less likely to develop. It is important to note that HBV cccDNA persists throughout all stages of disease, acting as a potent viral reservoir[67,70].

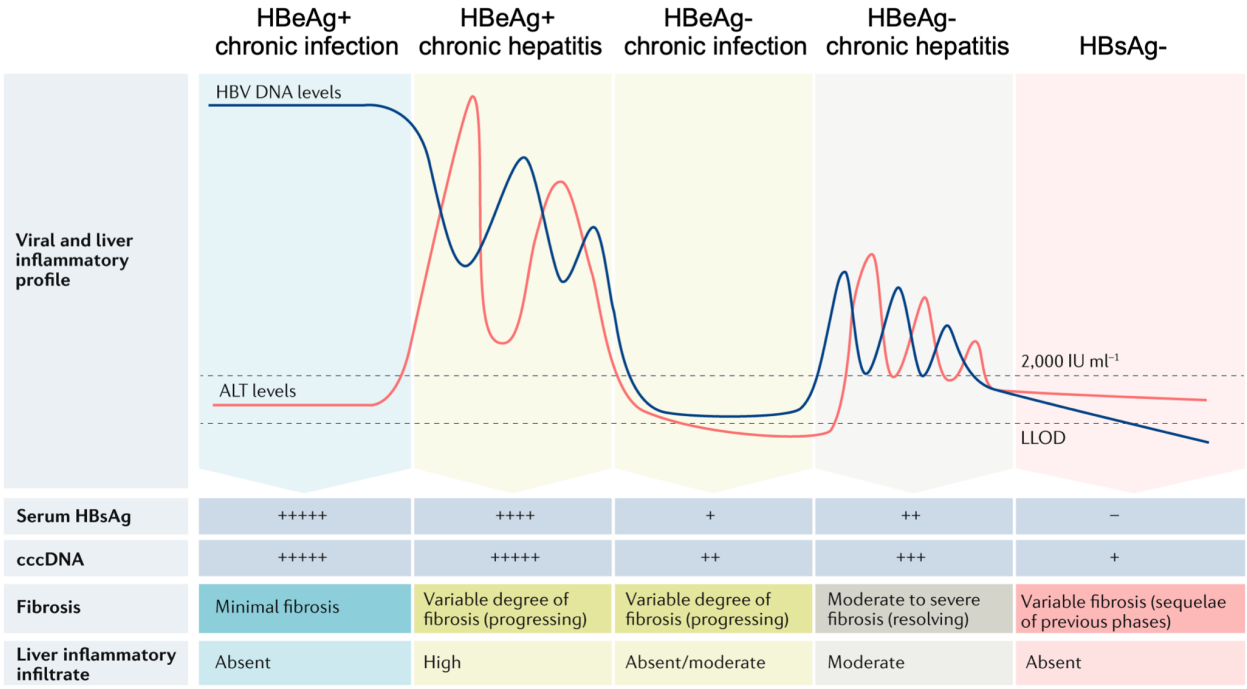


Figure 1.5. Illustration of the natural history of HBV infection. Adapted from Fanning *et al.*[69].

1.6 Model Systems for Studying HBV

1.6.1 *In vitro* models for studying HBV

Four main types of cell lines have been used for studying HBV *in vitro*: hepatoma cells, hepatoma cells overexpressing the entry receptor NTCP, the bipotent progenitor cell HepaRG, and primary human hepatocytes (PHH)[71]. Hepatoma cell lines have been used as the predominant *in vitro* model system for studying HBV over the past three decades[21,71]. Cloned HBV DNA constructs with overlength viral genome can be delivered into hepatoma cells such as HepG2 through transient transfection and produce viral particles. However transfection efficiency and viral replication are limited in such model systems. More recently hepatoma cell lines that have a stable reservoir of episomal HBV cccDNA have been applied as an *in vitro* model for rapid viral parameter analysis[71]. Applying chromatin immunoprecipitation in these model systems have enabled a better understanding of histone and epigenetic regulation of HBV[72].

Hepatoma cells lines with overlength HBV DNA integrated into the host genome such as HepG2.2.15 cells have also been applied, commonly used for therapeutic compound screening[73]. These model systems are strategically applied for purifying HBV particles and studying the impact of HBV integration into the host genome[71]. However, both transient transfection and host genome integration-based model systems do not recapitulate the complete HBV life cycle, limiting the ability to study critical processes such as viral entry and capsid trafficking to the nucleus.

Discovery of NTCP as a hepatocyte-specific HBV entry receptor in 2012 enabled the development of more effective *in vitro* model systems that establish *de novo* infection[74]. Hepatoma cells such as HepG2 that constitutively express NTCP enable *de novo* HBV infection establishment and the study of early steps of the viral life cycle[71]. In fact, hepatoma cell lines expressing NTCP with *de novo* HBV infection can model the entire viral life cycle and allow the identification of previously unknown factors and processes in HBV infection.

The bipotent liver progenitor cell line HepaRG was established in 2002, initially isolated from hepatoma with chronic hepatitis C virus infection[75]. Addition of dimethyl sulfoxide (DMSO), hydrocortisol, and polyethylene glycol is required for differentiation into biliary-like and hepato-like cells to support HBV infection[71]. Differentiated HepaRG cells support HBV infection for over 100 days but infection efficiency and viral spread are limited[76].

HBV naturally infects differentiated hepatocytes in the liver[71]. PHH isolated from the liver de-differentiate *in vitro* and supplementation with DMSO, hydrocortisol, and polyethylene glycol is required to maintain differentiation. Although viral infection in PHH is among the most physiologically relevant *in vitro* model system for studying HBV, chemical supplementation, de-differentiation, and rapid changes in gene expression observed when PHH is cultured *in vitro* could limit HBV susceptibility. Furthermore, PHH is a costly model system with limited availability[71].

1.6.2 Mouse model systems for studying HBV

A number of *in vivo* model systems have been developed to study HBV replication and pathogenesis, including mouse, chimpanzee, and tupaia[77]. Chimpanzees and tupaia are immunocompetent and susceptible to human HBV infection[78,79]. However, these model systems are not easily accessible, costly, and usually result in only transient infections. Ethical considerations further limit the use of chimpanzee as a model system for studying HBV. A number of accessible mouse model systems have been applied to study HBV, with the three main types being transgenic mouse, vector transfected mouse, and human liver-chimeric mouse[77].

First developed in the 1980s and 1990s, HBV transgenic mice have 1.3-fold overlength HBV genome integrated in the host genome with animals born with replicating virus[80,81]. This model system can be used to study middle to late stages of the HBV life cycle, from rcDNA synthesis and RNA transcription, to viral particle assembly and release. These animals have been deployed to study host factor and viral life cycle interaction as well as their impact on viral transcription and antigen expression[82]. Despite having a high level of viral replication, this model system has minimal immunological response and viral gene expression is transcribed from integrated HBV genome rather than cccDNA.

Vector transfected mouse model systems rely on hydrodynamic injection or adenovirus vector methods to transiently deliver HBV genomes into hosts[77,83]. Animals are able to mount an immune response against the viral replication. Viral replication can persist for over 80 days in immunodeficient animals but are rapidly cleared within 15 days in immunocompetent animals that develop HBV-specific T-cells and antibodies[83].

The human liver-chimeric mouse model uses immunocompromised animals to allow integration of human hepatocytes. This system can model the complete HBV life cycle and is particularly useful in assessing promising HBV treatments. However, these animals are immunodeficient and prevent assessment of host immune response to viral infection[77]. Recent studies have created mice engrafted with both human hepatocytes and human immune system via injection of hematopoietic stem cells[84]. Furthermore, chimeric human liver and immune system engraftment can be stably maintained in animals for over five months. These model systems require specific technical expertise and time to establish, but are promising for studying the interaction between HBV infection and immune response.

1.7 Host Immune Response to HBV

Immune response to HBV infection can be broadly categorised as innate and adaptative immune response. Pattern recognition receptors (PRRs) have important roles in innate immunity induction but whether PRRs can detect early HBV infections are controversial[85,86]. Results from *in vitro* monolayer model systems using HepaRG cells and PHH suggest that HBV capsid may be a ligand for toll-like receptor 2 (TLR2) and induce proinflammatory cytokine secretion[87-89]. However, results from 3D microfluidic PHH model systems which better recapitulate the endogenous liver environment suggest that interferon (IFN) is not induced in PHH and Kupffer cells infected by HBV[90]. Regardless of potentially conflicting *in vitro* results in the literature, HBV is generally considered a 'stealth' virus, because studies in humans, chimpanzees, and mice suggest that there is minimal IFN and immune activation during early stages of HBV infection[91-93].

A number of studies suggest that HBV could inhibit innate immune signaling[94-96]. HBV component proteins HBsAg and HBeAg may suppress TLR2, IL-12, and tumour necrosis factor-α (TNF-α) induction in macrophages and monocytes[94]. In CHB patients, increased HBsAg levels may associate with the inhibition of TLR2 and TLR4 regulated cytokines[94]. The HBV encoded protein HBx has also been observed to downregulate IFN production through inhibition of mitochondrial antiviral-signaling protein (MAVS) and stimulation of proteasome degradation[95,96]. These observations align with results suggesting that early HBV infection results in minimal induction of IFN and the innate immune response.

The adaptive immune response has a critical role in regulating HBV infection and chronicity. $CD4^+$ T-cell can facilitate $CD8^+$ T-cell mediated HBV clearance through cytokine signaling[97]. HBcAg-specific $CD4^+$ T-cell responses are detectable during acute viral infection but are depleted during

CHB[98]. Similar to $CD4^+$ T-cell response, HBV-specific $CD8^+$ T-cell proliferative response is significantly lower in patients with persistent infection compared to acute infection[99]. Low HBV-specific $CD8^+$ T-cell response is a strong predictor for persistent HBV infection[100-102]. In CHB, circulating viral-specific $CD8^+$ T-cell is detectable only in patients with lower viral load ($<10^7$ copies/mL)[100]. A number of possible mechanisms leading to $CD4^+$ and $CD8^+$ T-cell suppression in CHB have been identified including inhibition of T-cell entry into the liver, T-cell depletion from long term viral antigen exposure, and activation of anti-inflammatory signaling such as programmed death 1 (PD-1) and regulatory T-cell pathways[103-105]. Past studies have shown that peripheral HBeAg facilitates T-cell tolerance by depleting HBcAg and HBeAg-specific T helper type 1 (Th1) cells[106,107]. In line with this observation, HBeAg positive patients have suppressed HBeAg-specific cytotoxic T-cell response compared to HBeAg negative patients[108]. As T-cell adaptive immune response could control and potentially eradicate HBV infected cells, it has been an active area of research[109].

1.8 The Liver

HBV preferentially replicates in the liver. The liver is a multifunctional organ with a critical role in managing nutrients, maintaining homeostasis, and regulating metabolism[110]. Hepatocytes and cholangiocytes are the two main types of epithelial cells that make up the liver parenchyma[111]. Hepatocytes are terminally differentiated polarised cells accounting for ~60% of the liver. They are responsible for substrate and nutrient absorption, exchange, and processing from the blood as well as metabolite secretion into the bile. Cholangiocytes comprise ~3-5% of the liver and line the intrahepatic bile duct. Other cells in the liver include endothelial cells, hepatic stellate cells, Kupffer cells, and a variety of immune cells[112]. The diversity of cells in the liver represents a complex architecture of labour division with heterogenous distribution of metabolic processes and microenvironment conditions.

The liver is divided into left, right, caudate, and quadrate lobes, made of repeating functional units called lobules that are hexagonal in shape[112]. Afferent blood perfuses hexagonal lobules at their corners and drain into the central vein in the middle. The liver has a double afferent blood perfusion system composed of the portal vein and hepatic artery[113]. The portal vein supplies deoxygenated blood to the liver, accounting for the majority (75-80%) of blood received. The hepatic artery's oxygenated blood accounts for only 25% of total blood supply in the liver. These afferent blood supplies further branch into sinusoidal blood vessels in the lobules[114]. As a result, the liver harbours a naturally low oxygen environment. The liver's oxygen tension is highest at 8% near the periportal region where the portal vein and hepatic artery reside and gradually decreases to 4% at the pericentral region where the central vein resides. This low oxygen gradient can be perturbed by

pathological stress including infection, inflammation and cirrhosis[115]. Figure 1.6 provides a schematic illustration of liver lobule structure and zonation pattern.

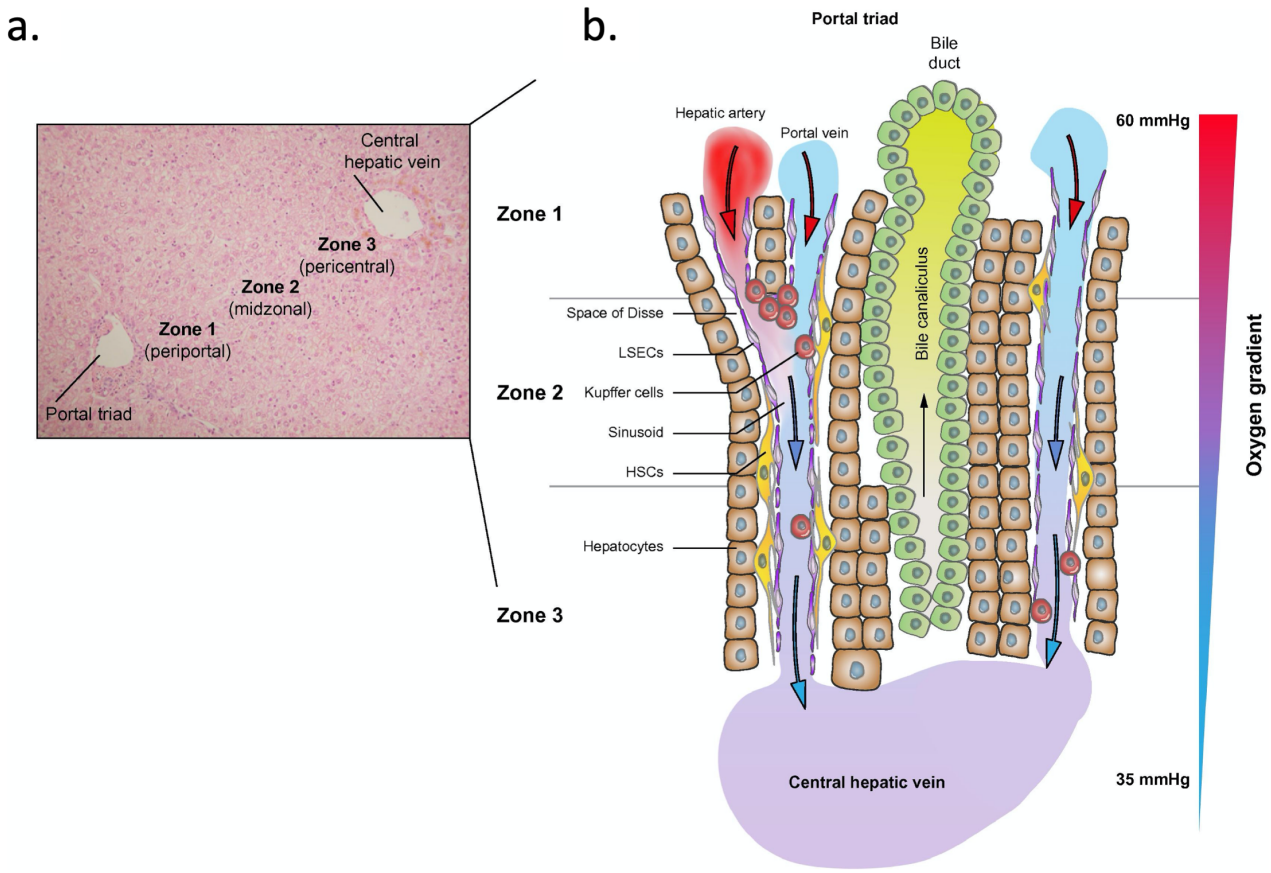


Figure 1.6. Schematic illustration of liver lobule structurer and zonation. Adapted from Wilson *et al.*[115]. (a) Image of liver tissue with haematoxylin and eosin staining depicting zonation pattern. (b) Illustration of the oxygen tension gradient in the liver.

Liver zonation arising as a result of differential oxygen, nutrient, and hormone levels from the periportal to the pericentral region is associated with differential gene regulation at the mRNA, miRNA, and protein levels[116-118]. These different regulations result in a division of tasks across different zones in liver lobules. In a single cell RNA-seq study in mouse, 3,496 or almost 50% of genes out of 7,277 found to be expressed in the liver were spatially zonated across liver lobules[116]. There is a graded rather than binary spatial change in the expression of these genes, in line with a gradual change in oxygen tension, nutrients, and hormones across this gradient. A number of genes associated with oxygen sensing response, metabolism, and molecular signaling displayed a zonation pattern. Ninety-five genes known to be induced by chronic hypoxia were significantly upregulated near the pericentral region, where oxygen tension is lowest in the healthy liver. The Wnt signal transduction pathway is another major mechanism differentially regulated by liver zonation. Genes associated with Wnt signaling were upregulated near the pericentral region and downregulated near the periportal region. Additionally, genes regulated by the Ras signaling pathway were activated near the periportal region. Interestingly, genes associated with bile acid synthesis followed a spatial zonation pattern in the liver, with intermediate pathways spatially activated in a pattern that aligns with their stepwise position in the biosynthesis cascade. A more recent study confirmed that mRNA zonation is consistent with protein expression patterns in the liver[117]. Posttranscriptional regulator microRNAs were differentially expressed dependent on liver zonation. These studies highlight the importance of oxygen tension and hepatocellular microenvironment in liver gene expression, metabolism, and function.

1.9 Oxygen Sensing Mechanisms

Oxygen is essential for human survival. Humans and other organisms have developed a number of oxygen sensing mechanisms to detect and respond to variations in oxygen tension[119]. For example, cysteamine (2-aminoethanethol) dioxygenase (ADO) is an oxygen sensor that directly regulates protein targets including regulator of G protein signaling 4 (RGS4) and interleukin-32 (IL-32) and the lysine-specific demethylase (KDM) 5A and KDM6A activity are dependent on oxygen tension[120-122] (Figure 1.7).

The prolyl hydroxylase domain (PHD)/ factor inhibiting HIF (FIH) – hypoxia inducible factor (HIF) pathway is the most well studied oxygen sensing mechanism (Figure 1.8). HIFs are heterodimeric transcription factors with alpha and beta subunits and bind to a consensus RCGTG(C) motif known as hypoxia responsive element (HRE) located in the promoter and enhancer regions of target genes[119]. There are three oxygen sensitive HIF-α isomers, HIF-1α, HIF-2α, and HIF-3α, which dimerise with the HIF-1β subunit[123]. All HIF subunits are transcription factors in the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family. The bHLH superfamily consists mainly of transcription factors with broad regulatory roles, from development and cell-fate determination to immune response and homeostasis maintenance[124]. Members of the bHLH family form homodimeric and heterodimeric complexes that bind specific DNA sequences to regulate gene transcription. HIF-1α, HIF-2α, HIF-3α, and HIF-1β all possess bHLH and PAS domains responsible for heterodimerisation and DNA binding. When oxygen is abundant, PHD hydroxylates HIF-1α on proline (Pro) -402 and Pro-564, HIF-2α on Pro-405 and Pro-531, and HIF-3α on Pro-492 in the NH_2 terminal transactivation domains (N-TAD) in the oxygen-dependent degradation (ODD) regions [123,125,126]. HIF-α prolyl hydroxylation enables von Hippel Lindau

(VHL) E3 ubiquitin ligase complex binding, poly-ubiquitination and subsequent proteasomal degradation. As an additional regulatory mechanism, a COOH terminal TAD (C-TAD) domain is present in HIF-1α and HIF-2α, where factor inhibiting HIF (FIH) can hydroxylate asparagine (Asn) -803 or Asn-826 respectively under hypoxia[127,128]. Asparagine hydroxylation prevents HIF1 and HIF2 dimer binding to CH1 domain of the p300 transcription coactivator to inhibit HIF target gene transcription. The liver expresses all three isomers of HIF-α, which regulate critical pathways for liver function and pathogenesis, including inflammation, angiogenesis, regeneration, metabolism, fibrosis, and tumorigenesis[129]. Table 1.2. summarises select HIF target genes in the liver and their respective functions (adapted from Nath and Szabo[129]).

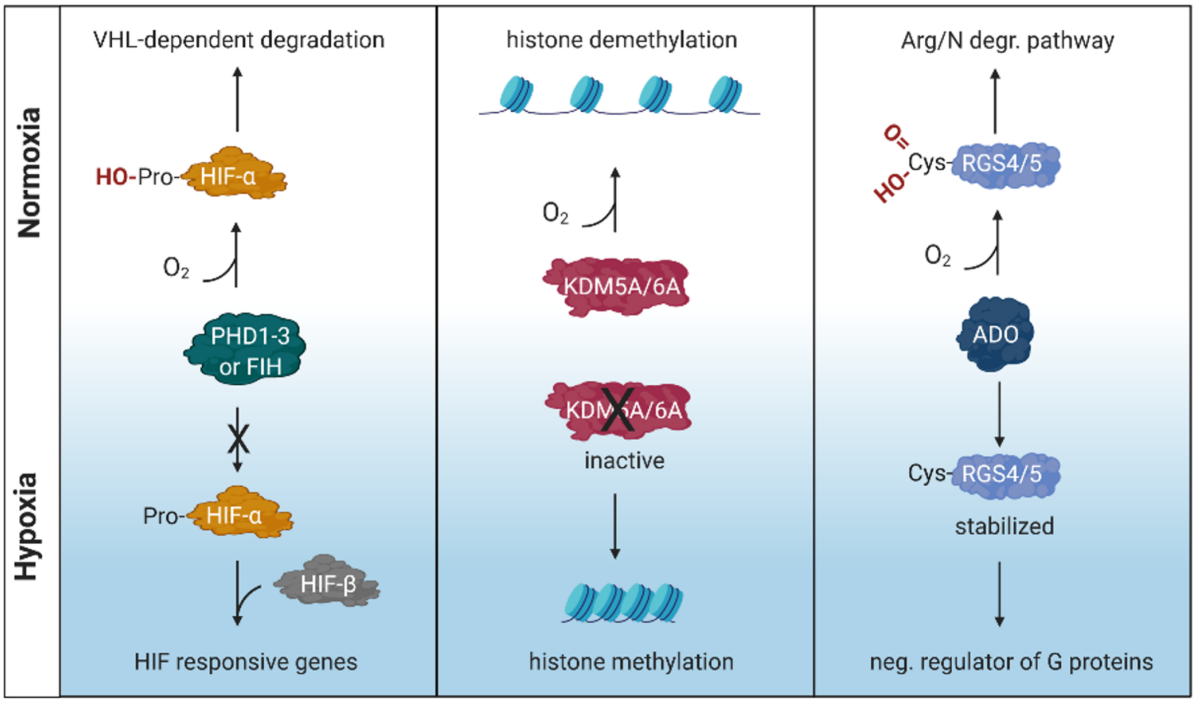


Figure 1.7. Schematic illustration of oxygen sensing mechanisms. Published in Liu *et al.*[75]. These include the prolyl hydroxylase domain (PHD) /factor inhibiting HIF (FIH)-hypoxia inducible factor (HIF) pathway, lysine-specific demethylase (KDM) 5A and KDM6A pathways and the cysteamine (2-aminoethanethiol) dioxygenase (ADO) pathway. RGS, Regulator of G protein signaling; VHL, von Hippel-Lindau tumour suppressor gene.

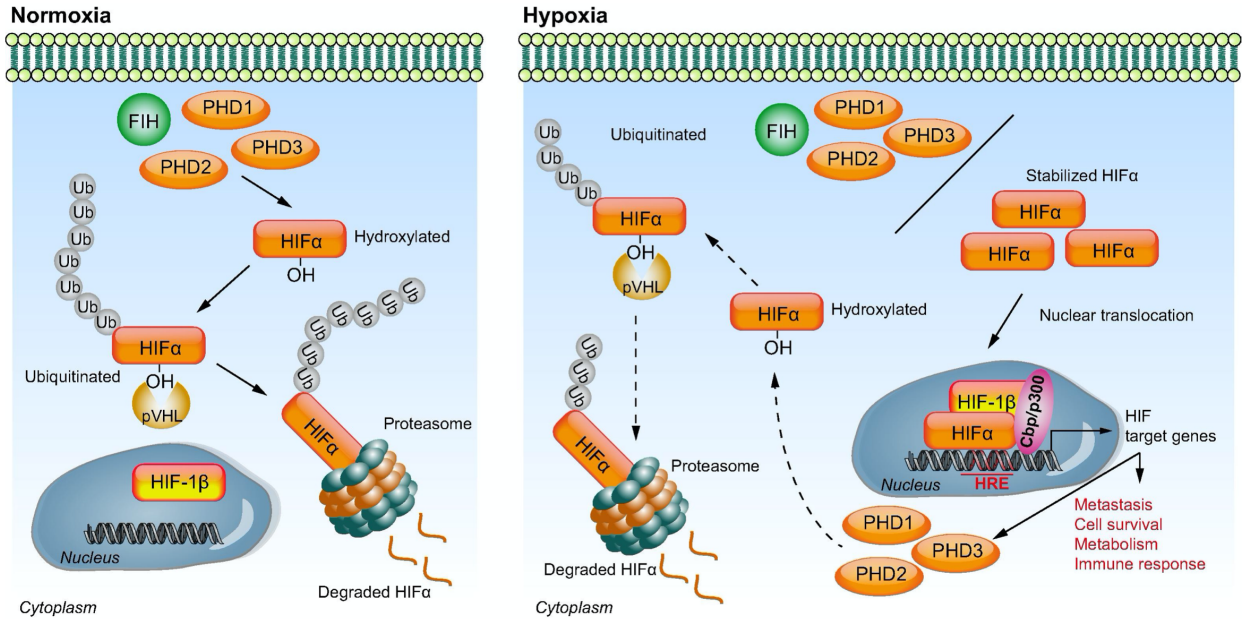


Figure 1.8. Schematic illustration of the VHL-PHD-HIF oxygen sensing mechanism. Adapted from Wilson *et al.*[115].

Table 1.2. Selected HIF target genes and their functions in the liver. Adapted from Nath and Szabo[129].

Gene	Function
Plasminogen activator Inihibitor-1 (PAI-1)	Inflammation, thrombosis, metastasis[130,131]
Prolyl-4 hydroxylase alpha-2 (P4HA2)	Fibrosis, collagen stabilization[132]
Heme Oxygenase 1 (HO1)	Oxidative stress, inflammation[133]
Vascular endothelial growth factor (VEGF)	Angiogenesis, tumour vascularization, ischemia-reperfusion[134-137]
Erythropoietin (EP)	Erythrocyte proliferation, liver regeneration[138]
Ceruloplasmin (CP)	Copper metabolism[139]
Glucokinase (GCK)	Metabolism[140]
Bone Morphogenetic Protein (BMP)	Tumorigenesis[141]

1.9.1 HIF-1α

HIF-1α was the first HIF isoform discovered and isolated by affinity purification from 3'-flanking region of the EPO gene using DNA oligonucleotides[125,142,143]. At least eight HIF-1α splice variants have been reported with different activity levels and tissue specific expression profiles[144-150]. These splice variants can be translated into functional HIF-1α proteins and dimerise with the HIF-1β subunit. HIF-1 is responsible for regulating over 100 target genes with wide ranging functions including glycolysis, proliferation, cell migration, and angiogenesis[125]. HIF-1α, but not HIF-2α is distinctly responsible for regulating glycolytic genes including phosphoglycerate kinase 1 (PGK-1), lactate dehydrogenase A (LDHA), phosphoglucomutase-1 (PGM-1), and pyruvate kinase (PKM)[151]. The liver has an important role in glucose metabolism and glycolysis, and a number of HIF-1 target genes facilitate these functions[152]. For example, glucose transporter 1 (GLUT1) is responsible for glucose transport; LDHA facilitates enzymatic conversion of pyruvate into lactate; PDK1 facilitates the conversion of pyruvate to acetyl-CoA in the Krebs cycle[153].

There is a clear association between HIF-1 and HCC. VEGF is a well-known target of HIF-1 responsible for angiogenesis. Evaluation of nine clinical studies suggest that vascular endothelial growth factor (VEGF) overexpression is prognostic of poor overall survival and progression-free survival in HCC patients[154]. The VEGF inhibitor sorafenib is the current standard of care for patients with advanced unresectable HCC[155].

HIF-1α can induce genes with roles in both proliferation and apoptosis. However, whether HIF-1α signaling results in a proliferative or apoptotic effect appears to be cell-type and microenvironment dependent[143,152]. In the context of the liver, HIF-1α appears to serve a proliferative and antiapoptotic role. Forkhead box M1 (FoxM1) is a transcription factor regulating

proliferation and cancer cell growth[156]. HIF-1α binds FoxM1 promoter to enable its transcription. Co-expression of HIF-1α and FoxM1 in HCC patient tumours results in expedited recurrence and reduced survivorship. HIF-1α can also induce cyclin D1 and cyclin A in hepatoma cell lines to accelerate cell cycle progression and facilitate cell proliferation[157]. In HCC, HIF-1α serves an antiapoptotic role by inducing Bcl-2 to inhibit the proapoptotic mitochondrial serine protease Omi/HtrA2[158]. Taken together, these evidences suggest that HIF-1α has a critical role in liver function and pathology.

1.9.2 HIF-2α

HIF-2α contains the same domains as HIF-1α including bHLH/PAS, ODD, N-TAD, and C-TAD and the two proteins share 48% amino acid homology[159]. HIF-1 and HIF-2 regulate both mutually inclusive and exclusive target genes. The erythropoietin gene (EPO) is a HIF-2 specific target gene expressed in the liver and kidney while VEGF and GLUT-1 are induced by both HIF-1 and HIF-2[138]. Compared to HIF-1α, HIF-2α has different tissue and developmental expression profiles. In fact, HIF-2α binding of HRE is much more cell type specific compared to the more ubiquitous HIF-1α[160]. For example, HIF-2α is highly expressed in adult heart, placenta, lung, and vascular endothelium as well as during embryonic development in fetuses[159]. HIF-1α and HIF-2α also have discrete binding preferences[160]. HIF-2α prefers to bind HRE at enhancer and promoter-distant regions while HIF-1α at promoter regions[160].

HIF-2α has a particularly important role in iron absorption and metabolism. HIF-2α but not HIF-1α facilitates iron absorption in the intestinal epithelium[161]. HIF-2α deletion in mice intestine decreases liver and serum iron concentration and downregulates the master regulator of iron homeostasis, hepcidin. In the liver, constitutive expression of HIF-2α in mice results in aberrant lipid metabolism and hepatic steatosis[162]. Fatty liver disease can progress to cirrhosis and HCC. As a result, HIF-2α could play a major role in liver carcinogenesis[163].

A number of studies demonstrate that HIF-2α has a broadly oncogenic role. HIF-2α promotes proliferation of carcinoma cells lines of diverse origins, including glioblastoma, colorectal cancer, and lung cancer[164]. This is mediated by HIF-2α target genes including epidermal growth factor receptor (EGFR) and insulin like growth factor 1 receptor (IGF1R) tyrosine kinases. In clear cell renal cell carcinoma (ccRCC) associated with the loss of VHL, there is a distinct upregulation in

favour of HIF-2α rather than HIF-1α[143,165,166]. These evidences suggest an important role for HIF-2α in mediating oncogenesis.

1.9.3 HIF-3α

HIF-3α is the least well understood isomer of HIF-α. It shares conserved bHLH/PAS, PAC, ODD, and N-TAD domains with HIF-1α and HIF-2α but lacks the C-TAD domain[167]. Instead HIF-3α has a unique leucine zipper (LZIP) domain at its COOH terminal. HIF-3α protein is endogenously expressed in the human liver, kidney, and lung but its mRNA is transcribed at low levels in these organs[168]. HIF-3α has traditionally been recognised as a negative regulator of HIF-1α and HIF-2α target genes. It is a target of HIF-1 and HIF-2 with its mRNA upregulated under hypoxic conditions[169]. Early studies show that HIF-3α competitively binds HIF-1β to repress HIF-1α and HIF-2α functional activity[170]. However, more recent studies suggest that there could be at least ten alternatively spliced HIF-3α mRNA variants with eight that can be translated into proteins[167]. Each of these variants could have variable sensitivity and responses under hypoxia in a tissue-specific manner. Some short-length variants of HIF-3α were shown to competitively bind HIF-1β to repress HIF-1α and HIF-2α[171,172]. In contrast, a number of HIF-3α full-length protein induced target genes have been identified, such as sex determining region Y-box 2 (Sox2), retinoid acid receptor beta (Rarβ), and forkhead box p2 (Foxp2)[173]. HIF-1α and HIF-3α target genes have been observed to regulate both overlapping and distinct pathways. Both isomers induce genes responsible for biological processes including glucose and amino acid metabolism as well as p53 signaling[174]. However, while HIF-3α induces Janus kinase-signal transducer and activators of transcription (Jak-STAT) and nucleotide-binding oligomerization domain (NOD) -like receptor signaling, HIF-1α regulates VEGF, insulin, and mitogen-activated protein kinase (MAPK) signaling.

1.10 Oxygen Sensing Mechanisms and Viruses

The following literature review on oxygen sensing mechanisms and viruses has been published in *Viruses*[175].

As obligatory intracellular parasites, viruses depend on the host cellular machinery to replicate. Important factors that shape the cellular microenvironment include temperature, pH and oxygen tension. As variable oxygen tension regulates host gene expression, protein modification, metabolism and epigenetic regulation, it is not surprising that oxygen availability can influence multiple steps in the viral life cycle[121,176]. A wide range of oxygen tensions are found within different tissues and organs, ranging from <1% in the skin to 14.5% in arterial blood vessels (reviewed in [177]). However, the majority of *in vitro* studies on viral replication have been conducted at comparatively high atmospheric oxygen tension (18%), where HIFs are not active and consequently their role in viral replication may have been overlooked[178]. Viral infection, inflammatory responses and tissue damage induced reactive oxygen species (ROS) can all stabilise HIF expression, highlighting the complex interplay between oxygen sensing pathways and viral replication[179,180]. Viruses have either DNA or RNA genomes[181] and this can define whether HIFs regulate viral transcription by direct binding to the viral genome or via indirect regulation of host genes. Screening DNA genomes representing the major viral families shows an approximate 50-fold variation in the frequency of HREs relative to the number of ORFs, ranging from 0.3 for HIV-1 (3 HRE/10 ORFs) to 14.7 for Molluscum contagiosum (2,395 HRE/163 ORFs) (Figure 1.9). These data are consistent with variable sensitivity of viruses to low oxygen environments. These data prompted a review of the current knowledge on how oxygen tension impacts viral replication and how viruses manipulate the PHD-HIF pathway (Figure 1.10). Previous reviews have generally

focused on the role of HIFs in viral carcinogenesis and host immunity[182-185]. Using a systems-based approach, the literature in the context of viruses infecting and replicating in different tissue sites was discussed. Table 1.3 summarises mechanisms in which viruses interact with HIFs.

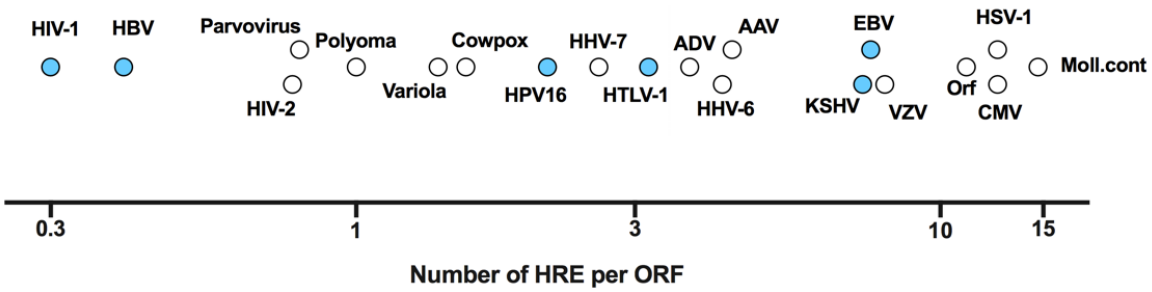


Figure 1.9. Frequency of HIF response elements (HRE) in viral DNA genomes. Published in Liu *et al.*[75]. The frequency of HRE elements (RCGTG) is plotted against the number of open reading frames (ORF) for a range of DNA viruses with those marked in blue discussed in this review. Reference sequences presented were selected from Genbank as follows (accession numbers in brackets); adeno-associated virus (AAV, NC_001401.2), adenovirus (ADV, AC_000007.1), cytomegalovirus (CMV, KU317610.1), cowpox virus (CowPox, NC_003663.2), Epstein–Barr virus (EBV, NC_009334.1), hepatitis B virus (HBV, NC_003977.2), human herpesvirus 6 (HHV-6, NC_000898.1), HHV-7 (NC_001716.2), Kaposi's sarcoma-associated herpesvirus (KSHV, NC_009333.1), human immunodeficiency virus type 1 (HIV-1, NC_001802.1), HIV-2 (NC_001722.1), human papillomavirus 16 (HPV16, NC_001526.4), herpes simplex virus type 1 (HSV-1, NC_001806.2), human T-lymphotropic virus 1 (HTLV-1, NC_001436.1), Molluscum Contagiosum (Moll.cont, NC_001731.1), Orf virus (Orf, NC_005336.1), parvovirus (NC_000883.2), polyomavirus (NC_031757.1), varicella-zoster virus (VZV, NC_001348.1) and variola virus (NC_001611.1).

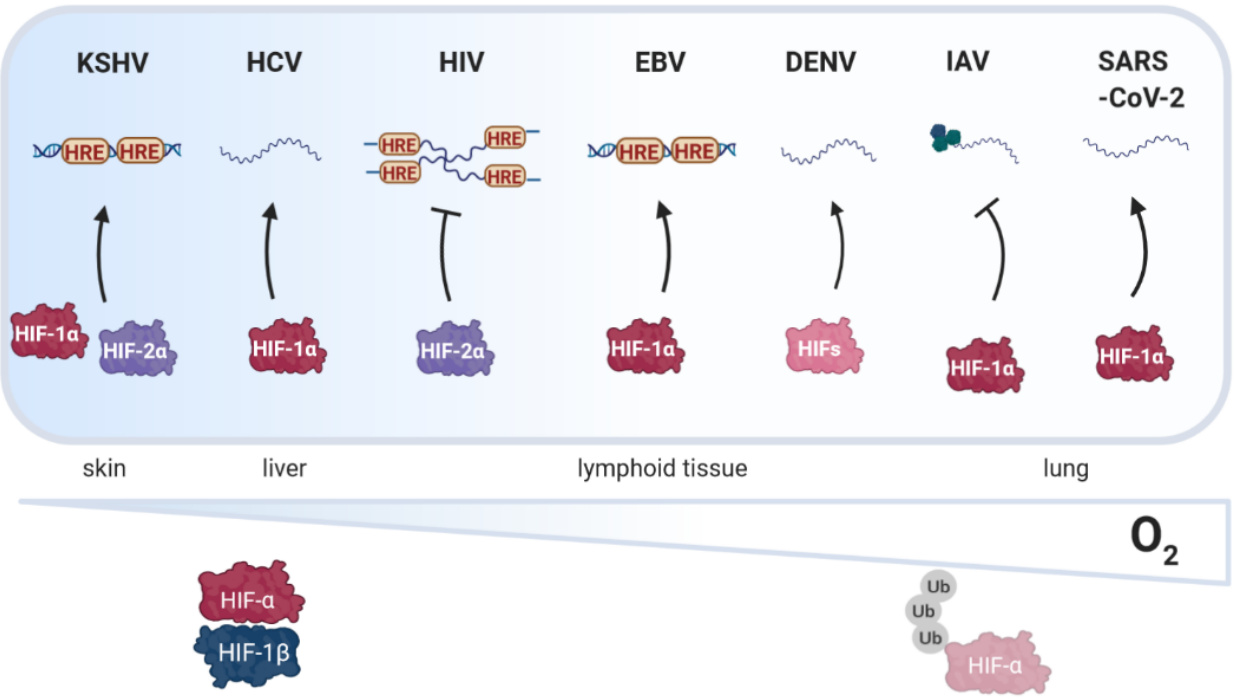


Figure 1.10. Schematic illustration depicting how oxygen tension in different tissues affects viral replication. Published in Liu *et al.*[75]. Hypoxia inducible factor (HIF) signaling enhances (black arrow) the replication of Kaposi's sarcoma associated herpesvirus (KSHV), hepatitis C virus (HCV), Epstein Barr virus (EBV) and dengue virus (DENV). In contrast, human immunodeficiency virus type I (HIV-1), influenza A virus (IAV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) replication is dampened by HIF signaling. HRE, HIF responsive element; Ub, ubiquitin.

1.10.1 The skin and epidermis

The skin plays an essential role in responding to environmental stimuli, where specific deletion of HIF-1α from the epidermis in mice inhibits renal EPO synthesis in response to hypoxia [186]. Furthermore, mice with an epidermal deletion of the VHL factor, a negative regulator of HIF, show increased EPO expression and polycythemia.

Kaposi's sarcoma associated herpesvirus (KSHV)

KSHV is a member of the *Herpesviridae* double-stranded DNA viruses that infects epithelial cells and keratinocytes, where the local oxygen tension is in the range of 1-2.5%[177,187,188]. KSHV causes Kaposi's sarcoma, the most common acquired immunodeficiency syndrome related neoplasm and viral lytic gene activation is associated with oncogenesis[189,190]. In 2003, Haque *et al.* identified functional HREs in the promoter regions of the ORF34 and Rta genes, which are involved in lytic switch, late stage gene expression and viral particle production[191,192]. Hypoxia activated both promoters and while ORF34 promoter was regulated by both HIF-1α and HIF-2α, the Rta promoter was preferentially regulated by HIF-2α. In 2006, Haque *et al.* went on to identify an additional HRE in the ORF35-37 promoter region that was co-regulated by both HIF-1α and HIF-2α[193]. These active HREs in the KSHV lytic gene promoters support an essential role for HIFs in regulating viral latency. KSHV-associated tumours are highly vascularised, a process which is partly mediated by virus-induced HIF signaling. Firstly, KSHV encoded G protein-coupled receptor (vGPCR) induces VEGF expression and associated angiogenesis[189]. The vGPCR induces HIF-1α regulatory domain phosphorylation via p38/MAPK and mTOR pathways[194]. Secondly, the KSHV-

encoded viral IFN regulatory factor 3 (vIFR3) binds to HIF-1α and prevents its degradation[195]. Finally, the KSHV encoded latency-associated nuclear antigen acts as a transcriptional coactivator interacting with HIF-1α protein and enhances HIF-1α mRNA transcription[196,197]. These studies are consistent with the reported 34% overlap between KSHV infection and hypoxia gene expression profiles, suggesting that KSHV exploits the HIF signaling pathway and multiple redundant pathways to ensure HIF expression[198]. Furthermore, inhibiting HIF in immunodeficient athymic mice reduced VEGF expression and tumour growth in a syngeneic mouse model[194]. KSHV-associated angiogenesis was potentiated by the hypoxia mimetic, deferoxamine mesylate, demonstrating an essential role for HIFs in KSHV pathogenesis[199]. KSHV can also cause non-skin related cancers, however, the role of HIFs in these non-skin related cancers have not been studied.

Human papillomavirus (HPV)

HPV are a family of small double-stranded DNA viruses that infect basal epithelia and specific strains have been associated with a risk of oncogenesis [200,201]. The link between HPV strains 16 and 18 and cervical cancer has led to their classification as high risk and integration of HPV16 associates with HIF-1α overexpression [202]. HPV16 and 18 encoded E6 and E7 proteins stabilise HIF-1α and induce VEGF and IL-8 expression that is associated with increased angiogenesis [203-205]. Three studies have suggested alternative non-exclusive mechanisms for HPV to stabilise HIFs: (1) E6 prevents HIF-1α association with VHL [206]; (2) E6 and E7 induce HIF-2α protein via liver kinase B1 (LKB1) modulation [207] and (3) E2 binding to mitochondrial membrane components of the respiratory chain induce ROS[208]. There are limited clinical studies addressing the translational impact of HIFs in HPV associated cancers. However, in a cohort of patients with oropharyngeal

squamous cell carcinoma there was some evidence for HIF-1α expression in the tumour associating with clinical outcome [209]. Jo *et al.* reported that elevated VEGF levels in a cohort of patients diagnosed with HPV16-associated oropharynx squamous cell carcinoma was independent of HIF-1α expression[210]. Given the technical limitations in staining for HIF-1α in tissue and the oxygen rich environment of the oropharynx, *ex vivo* patient sample processing methods may require optimisation before interpreting these studies[211]. Further clinical studies are required to fully examine the relationship between HPV, HIFs and patient outcomes.

1.10.2 The liver

The liver is a naturally low oxygen environment with the highest oxygen tension near its periportal region at 8%, gradually decreasing to 4% in the pericentral area. A recent single cell sequencing study of the mouse liver reported this oxygen gradient to associate with liver zonation, a phenomenon where hepatocytes show distinct functional and structural heterogeneity across the liver[116,212]. These infections are the leading cause for HCC worldwide and associated with significant mortality, accounting for more than 1.3 million deaths per year [213]. Hepatitis B and C viruses are a global health problem causing acute and chronic infections that can lead to liver cirrhosis and HCC.

Hepatitis C virus (HCV)

HCV is a positive-sense single-stranded RNA virus in the *Flaviviridae* family that infects the liver. Low oxygen has been reported to increase HCV RNA levels in human hepatoma Huh-7 and HepG2 model systems [214,215]. These studies reported differences in the HIF-dependency of the low oxygen-increase in HCV RNA that most likely reflects the different oxygen tensions used in the experimental models: Vassilaki *et al.* cultured the infected cells at 3% oxygen and showed a minimal role for HIFs in regulating HCV replication, whereas Wilson *et al.* reported a positive role for HIFs in regulating HCV replication at 1% oxygen[214,215]. Since the HIF-PHD and FIH degradation pathways may still be functionally active at 3% oxygen there may be limited HIF transcriptional activity, highlighting the importance of the % oxygen applied in *in vitro* model systems and how this impacts on HIF signaling. The study by Vassilaki *et al.* highlights the role

for hydroxylases within the 2-oxoglutorate (2-OG) oxygenase family in regulating HCV replication. A recent report showed the importance of N6-methyladenosine (m^6A) modification in the replication of HCV and other *Flaviviridae* RNAs and viral particle genesis that was mediated by fat mass and obesity-associated protein (FTO), a member of the 2-OG oxygenases[216].

The phospholipase autotaxin is hypoxic regulated and pharmacological inhibition or siRNA silencing of autotaxin-lysophosphatidic acid signaling reduced HCV replication, suggesting a role for HIFs to promote HCV replication via this pathway [217]. Autotaxin generates the biologically active lipid lysophosphatidic acid that plays a pro-tumorigenic role in a wide number of cancers. Farquhar *et al.* reported a positive association between hypoxic gene expression in HCV associated HCC tissue and autotaxin transcript levels[217]. Furthermore, the HCV encoded core and E1/E2 glycoproteins stabilised HIF expression by inducing an unfolded protein-stress response[215,218-221]. Further reports showing that HCV induction of NF-kB, STAT-3, PI3-K-aKT, and p42/44 MAPK signaling stabilised HIF-1α, highlight the multiple pathways for HCV induced HIF signaling[217,222-224]. Nassimuzzaman *et al.* reported HIF dependent VEGF expression in HCV infected cells and suggested a role for HCV in activating angiogenesis[224]. This observation was extended by Wilson *et al.* who showed that HCV-induced VEGF reduced hepatoma cell polarity and potentiated viral transmission and increased epithelial-mesenchymal transition, suggesting a role for HCV stabilised HIFs in promoting fibrosis and liver injury[225].

Hepatitis B virus (HBV)

In contrast to our understanding of HIF's role in regulating HCV replication and pathogenesis, there has been limited studies exploring the impact of HIFs on HBV life cycle. Several reports have studied the role of the HBV HBx protein in HIF stabilisation. HBx is known to be oncogenic and interacts with a myriad of host factors. A number of studies have argued for a role of the HBV protein HBx to regulate HIF. Yoo *et al.* argued that HBx induced HIF-1α protein and mRNA, and HIF-1α nuclear translocation via two distinct mechanisms: a) through modulation of the MTA1 and HDAC1/2 pathways to prevent HIF-1α deacetylation and 2) inhibition of HIF-1α and VHL binding. Moon *et al.* also demonstrated that HBx binds HIF-1α bHLH/PAS domain, but not VHL, to induce HIF-1α stability[226]. In 2014, Liu *et al.* demonstrated that HBx mutations K130M and V131 further enhanced HIF-1α upregulation, and HBx C-terminal truncation or deletions decreased its effects on HIF-1α stability induction[227]. A significant limitation of these studies is their dependence on HBx overexpression systems. Transient plasmid transfection systems with hypoxia reporter constructs were often used in past studies to assess HBx stabilization of HIFs. Given recent knowledge that HBx degrades Smc6 and silences episomal DNA, interpretations from earlier studies using *in vitro* plasmid systems need to be reassessed[228].

Several clinical studies have reported an association between increased HIF expression and HBV-related disease outcome. Xie *et al.* showed that HIF-1α expression in HCC associated with shorter survival [229]. Similarly, Osman *et al.* demonstrated that HIF-1α expression in HCC associated with larger tumour size, multifocal malignancies and more advanced disease[230]. Genetic variation in HIF-1α has also been linked with HBV-HCC risk, where the CG haplotype associated with increased risk relative to the CA haplotype[231]. Furthermore, a HIF-2α single nucleotide

polymorphism rs13419896 associated with an increased risk of liver cirrhosis[232]. Similarly, high KDM5B expression showed a negative association with HCC prognosis [233]. These clinical studies support translational studies to explore the application of HIF inhibitors to HBV related pathologies.

1.10.3 The immune system

Lymphoid organs operate at oxygen levels in the range of 0.5–4.5% O_2 [234-236], which are sufficient to activate the HIF pathway[237].

Human immunodeficiency virus type I (HIV-1)

Human immunodeficiency virus type I (HIV-1) is a retrovirus with an RNA genome that is reverse transcribed into cDNA and integrated into the host genome where it serves as a transcriptional template. If the integrated provirus is methylated and transcriptionally silenced, the viral genome becomes latent. Reactivation of HIV from latent reservoirs is a crucial step of 'shock-and-kill' treatment approaches. HIV-1 primarily replicates in CD4+ T cells and the major virus reservoirs are thought to be in lymphoid tissues: hence this virus has evolved to replicate in cells that may experience widely differing oxygen tensions. Accumulating evidence shows that a hypoxic environment inhibits HIV replication and reactivation[238,239]. HIF-2α can repress HIV transcription under low oxygen conditions (1% oxygen) via a direct interaction with a conserved HRE in the U3 region of the long terminal repeat (LTR)[238]. In addition, hypoxic reduction in cyclin T1 activity was reported to reduce Tat mediated transcription[239]. Duette *et al.* reported the hypoxia mimetic cobalt chloride induced a modest 1.5 fold increase in HIV-1 replication, however, since these mimetics activate additional signaling pathways further investigations are required to explore these differences[240,241,242].

Chronic immune activation, metabolic changes and inflammation during HIV-1 replication induce mitochondrial ROS[240]. The HIV-1 accessory protein Vpr was reported to increase HIF-1α

expression via induction of ROS and is consistent with an independent report showing that expression of Vpr in the monocytic cell line U937 increased HIF dependent glycolysis[243,244]. In line with these findings, clinical studies reported HIF-1α expression in the brain of AIDS patients diagnosed with dementia and increased HIF-2α and VEGF protein in kidney biopsies from patients with HIV-associated nephropathy[243,245]. Taken together, these data suggest that the hypoxic environment may be relevant to any successful shock-and-kill antiviral strategy.

Human T-lymphotropic virus type 1 (HTLV-1)

The human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that integrates into the host genome. An estimated 5-10 million people worldwide are infected by HTLV-1 and in 5-10% of cases this leads to a CD4-T cell malignancy, known as adult T-cell leukaemia or a progressive inflammatory disease of the spinal cord. In contrast to HIV-1, hypoxia enhanced HTLV-1 reactivation from latency via a HIF independent process that involved the 2-OG metabolite, suggesting a role for oxygenases in reactivating the genome[246]. Furthermore, there is evidence that the HLTV-1 encoded transactivator protein, Tax, induces HIF-1α protein expression and suppresses expression of the cellular proapoptotic BH3-only proteins Bim and Bid[247]. Since HTLV-1 may benefit from a hypoxic environment through several different mechanisms, a better understanding of these interactions could identify therapeutic targets.

Epstein Barr virus (EBV)

Epstein Barr virus (EBV) is a double-stranded DNA virus in the family of *Herpesviridae* that replicates in $CD20^+$ B cells and epithelial cells of the oropharynx and is associated with a number of malignancies, including Hodgkin's lymphoma and Burkett's Lymphoma[248]. As described for KSHV, HIF-1α directly binds the primary latent-lytic switch BZLF1 gene promoter, Zp, and activates transcription[249,250]. The EBV latent membrane protein 1 (LMP1), an oncogenic protein capable of B-cell immortalisation, increases HIF-1α protein expression by upregulating the Siah1 E3 ubiquitin ligase that degrades PHD1 and PHD3[248,251-253]. In addition, the loss of VHL/HIF-1α complexes stabilises HIF-1α and associated HIF signaling, including pyruvate dehydrogenase kinase 1 (PDK1) and the pyruvate kinase M2 (PKM2) isoform, resulting in an increase in lactate production and glucose consumption[254].

There is limited evidence of an association between the survival of patients with EBV^+ nasopharyngeal cancers and HIF-1α or VEGF expression[255]. Although LMP1 expression was noted in all EBV+ nasopharyngeal carcinomas, there was limited evidence of LMP1 co-localisation with HIF-1α. Despite the lack of a direct correlation between HIF expression and nasopharyngeal cancer clinical outcome, Yang *et al.* reported that LMP1 blockade increased the sensitivity of nasopharyngeal carcinoma to radiotherapy by downregulating HIF-1α and VEGF activity and decreasing phosphorylated JNKs/c-Jun signaling[256]. This observation was further validated through an *in vivo* experiment showing a significant reduction in tumour growth.

Dengue virus (DENV)

Dengue virus (DENV) is a member of the *Flavivirus* genus that includes yellow fever, Zika, West Nile and Japanese encephalitis viruses. DENV is the most prevalent arbovirus disease in the world, with a spectrum of symptoms ranging from asymptomatic to severe haemorrhagic fever. DENV is transmitted by *Aedes aegypti* mosquito with potentially 3.9 billion people currently at risk of contracting infection[257]. Severe dengue fever most often occurs in secondary/tertiary infections and is thought to be mediated via antibody-dependent enhanced (ADE) infection along with a skewed T cell response. DENV primarily replicates in macrophages and infection is associated with an oxidative stress response that plays a role in the immunopathology of the disease[258,259,260]. Gan *et al.* showed that hypoxia enhanced antibody-dependent DENV infection of THP-1 cells and primary human monocytes by two independent mechanisms[261]. Firstly, HIF-1α upregulated fragment crystallisable gamma receptor IIA (FcγRIIA). Secondly, membranous lipid concentrations were increased under hypoxia independently of HIF-1α. These processes increased antibody-opsonised DENV infection of monocytes. HIFs were also reported to promote DENV RNA replication and translation through a HIF-1α dependent mechanism in Huh-7 hepatoma cells[262]. DENV induces an oxidative stress response that may lead to HIF expression and hypoxic reprogramming[263]. Frakolaki *et al.* reported that DENV infection activated a HRE-luciferase reporter plasmid in Huh7 cells under atmospheric oxygen tension[262]. However, no further experiments were conducted to directly examine HIF protein levels or transcription of HIF target genes, limiting the conclusions of the study. Further studies are required to dissect the influence of physiological oxygen tension and tissue specific mechanisms on DENV regulation as well as the influence of DENV replication on local oxygen tension and metabolism.

1.10.4 The respiratory tract

Oxygen levels in the lung can vary in different locations within adults but also during development. Le *et al.* reported a median oxygen tension of 5.6% for adult lung tissue[264]. HIFs play an important role in fetal lung development and when the lung is hypoxic, loss of HIF expression can lead to impaired lung development or even death[265]. At the same time HIF upregulation due to lung damage, pulmonary hypertension or in lung cancer can negatively correlate with disease outcome.

Influenza A virus (IAV)

Influenza A virus (IAV) is a negative-sense single-stranded RNA virus associated with respiratory infections. IAV strains differ in their preference to infect the upper or lower respiratory tract[266]. Zhao *et al.* reported that IAV H1N1 (PR8) replicated to a higher titre in mice with targeted HIF-1α knockout in type II alveolar epithelial cells (AEC2), accompanied by increased lung inflammation and mortality[267]. Although the underlying mechanisms require further exploration, these data support an indirect role for HIF-1α in regulating IAV replication by inhibiting autophagy as a result of decelerated glycolysis. IAV has been reported to increase glycolysis by enhancing glucose uptake, lactate production and oxygen consumption rates[268,269]. Oral treatment with BEZ235 (a putative PI3 K/mTOR inhibitor) decreased glycolysis and reduced virus replication and mortality in a mouse model[270]. This observation contrasts with data from Zhao *et al.* that may reflect cell-type dependent metabolic responses in the lung[267]. It is likely that the role of HIF in influenza infection is more complicated as Zhang *et al.* recently showed that RIG-I like receptor (RLR) activation, a key sensor of IAV infection, suppresses glycolysis by inhibiting hexokinase[271].

HIF-1α is an important regulator of metabolic processes and is potentially regulated by immune responses against the virus. Indeed, HIF-1α induces proinflammatory cytokines and plays a key role in AEC2 differentiation and alveolar repair following IAV infection through activation of Notch signaling[272-274].

Supplemental oxygen (hyperoxia) given to premature infants associates with an increased risk of lung dysfunction and susceptibility to respiratory infections in adult life[275-277]. In a mouse model hyperoxia caused a significant reduction of pro-surfactant protein C-positive alveolar epithelial Type II cells by 8 weeks of age[278]. This finding is supported by a report identifying a protective role of superoxide dismutase to hyperoxia in a transgenic mouse model. Hence, oxygen tension and metabolic changes can impact the alveolar epithelial balance and response to injury. Further studies would elucidate the metabolic effects of HIF on different cell types in the lung, IAV replication, and whether oxygen tension affects IAV tissue tropism.

Infection with IAV leads to acute lung injury which activates hypoxia signaling and HIF induction. Several groups reported that IAV infection stabilises HIF-1α, although their proposed mechanisms vary. Zhao *et al.* showed an increase in HIF-1α expression in H1N1 (PR8) infected A549 cells with a modest increase in mRNA levels [267]. Ren *et al.* observed HIF-1α induction during H1N1 (PR8) infection as a result of reduced FIH expression[279]. Huo *et al.* reported no increase in HIF mRNA or protein levels, but an increase in nuclear translocation. Since IAV infection can result in a caspase-dependent enlargement of nuclear pores and non-specific protein uptake by the nucleus, this finding requires further validation[280]. Infection of the murine mastocytoma cell line P815 with different IAV strains (human H1N1, avian H5N1 and H7N2) showed that HIF-1α was activated by H7N2 but not by H1N1 or H5N1[281], suggesting differing abilities of IAV strains to

activate HIF-signaling pathways. It was interesting to note that H7N2 infection which activated HIF-signaling replicated to lower titre. Given the known effect of HIF-1α on degranulation and inflammatory factor production in immune cells, these differences could have wide-ranging implications[282]. HIF signaling is likely to be an important determinant for the outcome and recovery from IAV infections.

SARS-Coronavirus-2 (SARS-CoV-2)

SARS-CoV-2 is a positive-sense single-stranded RNA virus in the *Coronaviridae* family and the causal agent of coronavirus disease 2019 (COVID-19)[283]. As of March 2021, over 120 million people have been infected by SARS-CoV-2 with 2.7 million fatalities[284]. SARS-CoV-2 primarily targets the respiratory tract and can result in pneumonia and severe acute respiratory distress syndrome especially in the elderly and in individuals with comorbidities[285,286,287]. Epithelial cell death and lung inflammation are major hallmarks of SARS-CoV-2 induced tissue damage[288]. Although the clinical presentation is heterogeneous, with many mild cases, a defining feature of severe COVID-19 is a marked hypoxaemia. Recent studies show multi-organ involvement in severe COVID-19 disease[289], including the gastrointestinal tract[290] and central nervous system[291-294]. SARS-CoV-2 encoded Spike protein binds human angiotensin-converting enzyme (ACE2) and the transmembrane proteases, serine 2 (TMPRSS2) and furin, to trigger the fusion of viral and cell membranes[295,296]. Hypoxia has been reported to reduce ACE2 expression in lung pulmonary arterial smooth muscle cells and haematopoietic stem cell precursors via regulating ACE1[297,298]. This is consistent with a recent report showing a gradient of ACE2 expression in proximal (high) versus distal (low) pulmonary epithelial cells that associates with SARS-CoV-2 infection[285,287]. In

contrast, neonatal hyperoxia increases ACE2 and TMPRSS2 expression in an age-dependent fashion via a loss of type II alveolar epithelial cells[299]. Given reports that neonatal hyperoxia associates with risk of more severe IAV infection in adults via a loss of type II alveolar epithelial cells, this provides an explanation for the increased severity of COVID-19 in elderly and people with pre-existing co-morbidities[272-274].

HIF-1α protein was detected in monocytes isolated from bronchoalveolar lavage and circulating neutrophils in COVID-19 patients[300,301]. These observations were validated *in vitro* and showed that SARS-CoV-2 infection of monocytes induced mitochondrial ROS and stabilised HIF-1α expression and associated gene transcription[300]. SARS-CoV-2 infection of human brain organoids also showed evidence of virus-dependent HIF expression[302]. In contrast, Appelberg *et al.* reported that SARS-CoV-2 infection of human hepatoma Huh-7 cells repressed HIF-1α expression, suggesting cell-type specific effects of viral infection on this pathway[303]. Codo *et al.* demonstrated that pharmacological inhibition of mitochondrial ROS and associated HIF-1α activity in monocytes reduced SARS-CoV-2 infection and interleukin-1β expression in monocytes[300]. Importantly, inhibition of HIF-1α restored T cell proliferation and rescued apoptosis in co-cultured A549 cells, suggesting that targeting the HIF oxygen sensing pathway could offer a novel therapeutic modality for COVID-19. However, the diverse role of HIFs and other hallmarks of a low oxygen environment in the inflamed lung are likely to be complex and require further investigation.

Table 2.3. Mechanisms in which viruses interact with HIFs. Published in Liu *et al.*[304].

Virus	Viral component	Mechanism categorisation	Mechanism description	Citation
KSHV	G protein-coupled receptor (vGPCR)	Direct interaction	- Triggers HIF-1a phosphorylation via p38 and MAPK pathways - Upregulates HIF-1α and HIF-2α proteins via mTOR pathway modulation	194
	Latency-associated nuclear antigen (LANA)	Direct interaction	- Directly interacts with HIF-1α to enhance binding to HIF-1α promoter and upregulate HIF-1α mRNA	196,197
	Viral IFN regulatory factor 3 (vIFR3)	Direct interaction	- Stimulates HIF-1a transcription and protein stability by blocking HIF-1α degradation	195
HPV	HPV16 E6 and E7 proteins	Direct interaction	- Regulate HIF-2α protein via LKB1 modulation	207
	HPV16 E6	Direct interaction	- Prevents HIF-1α and VHL binding and reduces HIF-1α ubiquitination to upregulate HIF-1α protein	206
	HPV18 E2	Indirect interaction	- Upregulates ROS by binding to inner mitochondrial membrane components of the respiratory chain to stabilise HIF-1α	208
HCV	HCV virus	Indirect interaction	- Oxidative stress stabilises HIF-1α protein via NF-kB, STAT-3, PI3-K-aKT, and p42/44 MAPK pathway dependent mechanisms	217,222-224
	Core protein	Indirect interaction	- Regulates HIF-1α protein but not HIF-2α by unfolded protein-stress response	218-221
	E1E2 Glycoproteins	Indirect interaction	- Regulates HIF-1α protein by unfolded protein-stress response	215

HBV	HBx protein	Direct interaction	- HIF-1α protein and mRNA upregulation through: (1) upregulation of MTA1 and HDAC1/2 which perturbed HIF-1α deacetylation and (2) prevention of HIF-1α association with VHL and down-stream degradation	305-307
	HBV virus	Indirect interaction	- Inflammation and oxidative stress induced by viral infection can upregulate transcriptional activity of HIF	304
HIV	HIV virus, Viral protein R (Vpr)	Indirect interaction	- ROS induction increases HIF-1α mRNA and protein	240,243,244, 308
HTLV-1	Transactivator protein (Tax)	Direct Interaction	- induces HIF-1α protein expression	247
EBV	Latent membrane protein 1 (LMP1)	Indirect interaction	- HIF-1α protein degradation is inhibited via proteasomal degradation of PHD1 and PHD3 through Siah1 E3 ubiquitin ligase upregulation - additionally, the loss of VHL/HIF-1α complexes stabilises HIF-1α and associated HIF signaling	248,251-254
DENV	DENV	Indirect interaction	- induces an oxidative stress response that might lead to HIF stabilisation and hypoxic reprogramming	263
IAV (PR8)	IAV	Direct interaction	- FIH-1 inhibition leads to a reduction in HIF-1a degradation	279
		Indirect interaction	- Inflammatory responses (including acute lung injury) activate hypoxia signaling and HIF induction - Increased HIF-1α nuclear translocation	281
SARS-Cov-2	SARS-Cov-2 virus	Indirect interaction	- Induces mitochondrial ROS and upregulates HIF-1α protein	300-302

1.11 Aims and objectives

The systems-based literature review on the interplay between oxygen sensing mechanisms, viral replication and pathogenesis reveals that understanding of the topic varies widely between viruses. Knowledge on the role of hypoxia and oxygen sensing pathways in regulating HBV replication is particularly limited. Low oxygen tension and the PHD-HIF pathway have an important role in the liver, HBV's preferred organ of replication. However, the majority of past studies on the HBV life cycle have been conducted at relatively high atmospheric oxygen tension (18%). To this end, this thesis investigated the interplay between oxygen sensing mechanisms and HBV replication. This thesis will address three aims by answering the following questions:

1. Does HBV infection perturb HIF signaling?
2. Does the HIF signaling pathway regulate HBV replication?
3. Are there any HIF independent oxygen sensing mechanisms that can regulate HBV replication?

Work from this thesis has led to the discovery that oxygen sensing mechanisms have critical roles in regulating HBV replication and pathogenesis. Conversely, this work also identified that chronic HBV infection perturbs HIF signaling in the liver. Knowledge generated from this thesis has important implications for targeting the PHD-HIF pathway as an exciting therapeutic modality for HBV pathogenesis and hypoxia culture systems as novel approaches for studying viral replication.

2. MATERIALS AND METHODS

Portions of this chapter have been published in *Scientific Reports* and *Journal of Hepatology*[72,304].

2.1 Tissue Culture

HepG2, HepG2-NTCP, HepG2-Core, HepG2-LMS, HepG2.2.15, HepG2-HBV-pEpi, HEK293T, U2OS and HepAD38 cell lines were cultured with Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX with 10% fetal bovine serum (FBS), 1 mM Sodium Pyruvate, 1% non-essential amino acids, and 50 IU penicillin/streptomycin mL^{-1} (Life Technologies, UK). 786O-VA and 786O-VHL cell lines were cultured in the same medium with additional 0.5mg/mL geneticin (Sigma, UK). HepG2 cells were cultured on calf skin collagen (Sigma, UK) coated surfaces. Tissue culture surface was coated with calf skin collagen via 1-hour long incubation in 1:50 dilution calf skin collagen solution. HepaRG cells expressing HBx under the control of a Tetracycline inducible promoter (HepaRG-HBx_{WT}) were cultured in Williams E medium supplemented with 10% FBS, 50 U penicillin/streptomycin mL^{-1}, 5 μg human insulin mL^{-1} and 5×10^{-7} M hydrocortisone hemisuccinate (Sigma, UK). HBx protein induction by Tetracycline was confirmed with Western blot. As a control HepaRG cells expressing an inactive HBx null mutant (HepaRG-HBx_{STOP}) were generated, where three point nonsense mutations (relative to EcoRI site: C to A, 1393nt; C to A, 1396nt and C to T, 1397nt) were introduced to generate three stop codons (respectively, TGA, 1393nt; TGA, 1396nt; TAA, 1397nt) in HBV genotype D[309]. All cell lines were cultured at 37°C and 5% CO2 humidified Galaxy 170S Incubator (Eppendorf, UK). HepG2 is an epithelial-like adherent hepatocellular carcinoma cell line derived from a 15 year-old Caucasian male[310].

HEK293T is an epithelial adherent embryonic kidney cell line[311]. U2OS is an epithelial adherent osteosarcoma cell line derived from a 15 year-old Caucasian female[312]. 786O is an adherent renal cell adenocarcinoma cell line derived from a 58 year-old Caucasian male[313]. HepAD38 is an adherent hepatoblastoma cell line derived from a 15 year-old Caucasian male[314]. HepaRG is an adherent liver cell line derived from a female with HCV infection and cholangiocarcinoma[75].

Table 2.1. List of cell lines used in this thesis study.

Name	Tissue of origin	Source
HepG2	Human hepatoma	American Type Culture Collection (ATCC)
HepG2-NTCP	HepG2 with NTCP overexpression	Gift from Prof. Stephan Urban, University of Heidelberg
HepG2-Core	HepG2 with HBcAg overexpression	Gift from Prof. Ulrike Protzer, Technical University of Munich
HepG2-LMS	HepG2 with HBsAg overexpression	Gift from Prof. Ulrike Protzer, Technical University of Munich
HepG2.2.15	HepG2 with integrated 1.3 overlength HBV genome	Gift from Prof. Ulrike Protzer, Technical University of Munich
HepG2-HBV-pEpi	HepG2 with epichromosomal replicating copies of HBV	Gift from Prof. Ulrike Protzer, Technical University of Munich
HEK293T	Embryonic kidney	Gift from Dr. Ya-Min Tian, University of Oxford
U2OS	Osteosarcoma	Gift from Dr. Ya-Min Tian, University of Oxford
786O-VA	Renal cell adenocarcinoma	Gift from Dr. Véronique Lafleur, University of Oxford
786O-VHL	786O cells overexpressing functional wildtype VHL	Gift from Dr. Véronique Lafleur, University of Oxford
HepAD38	Hepatoblastoma	ATCC
HepaRG-HBx$_{WT}$	Human hepatoma HepaRG cell line overexpressing wildtype HBx	Gift from Prof. David Durantel, University of Paris
HepaRG-HBx$_{STOP}$	Human hepatoma cell inactive HBx null mutant	Gift from Prof. David Durantel, University of Paris

2.2 Adenovirus Transduction

Ad-HBx and Ad-Ova adenovirus vectors express the HBV genotype D HBx gene and chicken ovalbumin gene under control of the Transthyretin (TTR) promoter. Promoter and insert were inserted into the E1 region of adenovirus (Ad5ΔE1/E3) backbone plasmid pAd/PL-DEST through Gateway recombination following the manufacturer's instructions (Gateway System; Invitrogen, Karlsruhe, Germany)[304]. Adeno virus stocks were titrated using the cytopathic effect in HepG2 cells as previously described[315]. Cells were transduced with 25 multiplicity of infection adenovirus vector expressing HBx or ovalbumin for 1 hour, rinsed with phosphate saline buffer (Gibco, UK) (PBS), and incubated at 18% or 1% oxygen tension for 4 hours, 8 hours, 24 hours or 48 hours to examine the role of HBx in regulating HIF signaling.

2.3 Plasmid Transfection

Target cells were cultured in 6-well plates with 10% FBS supplemented growth media overnight. Media were replaced with penicillin/streptomycin free DMEM media prior to transfection and cells were transfected with Fugene-6 (Promega) complexed with appropriate plasmids per manufacturer's protocol. Cells were allowed to incubate overnight, subject to corresponding treatments, and harvested for analysis as described in each chapter. Plasmids used for this thesis are listed in Table 2.2.

2.4 HBV Promoter Assay

HepG2-NTCP cells were transfected using Fugene-6 (Promega) with pGL3-HBV-BCP-Luciferase (provided by Wang-Shick Ryu, Yonsei University, South Korea) or pGL3-HRE-Luciferase promoter constructs with triple repeat of the inducible nitric oxide synthase (iNOS) gene HRE binding sequence (provided by Margret Ashcroft, University of Cambridge, UK) and 24 hours later cultured under 18% or 1% oxygen with samples harvested at the stated times[316,317]. Luciferase activity in cell lysates was quantified with a Luciferase assay (Promega) as per manufacturer's protocol. Briefly, samples were lysed with luciferase lysis buffer (Promega) and stored at -20^{o}C. Samples were then thawed, transferred to 96-well luminometer plates, mixed with a luciferase assay substrate solution (Promega), and analysed using a Centro LB960 Microluminometer.

Table 2.2. List of plasmids used in this thesis study.

Name	Vector	Insert	Source
pGL3-HBV-BCP-Luc	pGL3	HBV BCP luciferase	Gift from Dr. Chunkyu Ko, Technical University of Munich
pGL3-HRE	pGL3	HRE luciferase	Gift from Prof. Margaret Ashcroft, University of Cambridge
pcDNA-IL32	pcDNA3.1	IL32	Gift from Dr. Thomas Keeley, University of Oxford
pcDNA-GFP	pcDNA3.1	GFP	Gift from Prof. Margaret Ashcroft, University of Cambridge

2.5 SDS-PAGE and Western Blot

Samples were harvested using RIPA lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, and Roche protease inhibitor cocktail tablets). Lysed samples were mixed with 4× Laemmli reducing buffer (BioRad, UK), and incubated at 95°C for 5 minutes. A 10% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) was used for protein separation in a Mini Protean 3® system (BioRad, UK). Protein samples at 10-20µg were loaded and allowed to separate for 1-2 hours at 100V in a running buffer (25mM Trizma Base, 0.2M Glycine, and 0.1% SDS). Proteins were transferred to PVDF membranes (Amersham) by electroblotting for 90 minutes at 100V in a transfer buffer (25mM Trizma Base, 0.2M Glycine, and 200mL methanol). Membranes were cut at the 70kDa molecular weight mark, blocked in PBST, 5% skimmed milk (Sigma) for at least 1 hour, incubated overnight with respective primary antibodies, and incubated for 1 hour with respective HRP-linked secondary antibodies. Blots were developed using Pierce SuperSignal West Pico chemiluminescent substrate kits and visualised with a ChemiDoc XRS+ imaging system (BioRad, UK). Antibodies used in this thesis study are listed in Table 2.3.

Table 2.3. List of antibodies used in this thesis study.

Antibody	Manufacturer	Catalogue number	Working dilution
Mouse anti-HIF-1α	BD Transduction Labs	610959	1:1,000
Mouse anti-HIF-2α	Novus	NB100-132	1:1,000
Rabbit anti-HIF-1β (ARNT1)	Novus	NB100-110	1:1,000
Rabbit anti-HBx	Abcam	AB39716	1:500
Mouse anti-β-actin	Sigma	A5441	1:10,000
Mouse anti- CAIX	Gift from Prof. Adrian Harris, University of Oxford		1:1,000
Mouse anti-Smc6L1	Abgent	AT3956a	1:1,000
Rabbit anti-Nse4A n-terminal	Abgent	AP9909a	1:1,000
Polyclonal goat anti-mouse immunoglobulins HRP	Dako	P0447	1:3,000
ECL anti-rabbit IgG, HRP linked whole antibody	GE Healthcare Life Sciences	NA934V	1:5,000

2.6 PCR Quantification of RNA Transcripts

Total cellular RNA was extracted using a RNeasy mini kit (Qiagen) following the manufacturer's instructions and samples treated with RNase-Free DNaseI (14 Kunitz units/reaction, Qiagen) for 30 minutes at room temperature as per manufacturer's protocol for on-column DNA digestion. RNA concentration was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific) and cDNA synthesised with 0.25-1µg of RNA in a 20µL total reaction volume using a random hexamer/oligo dT strand synthesis kit in accordance with the manufacturer's instructions (10 minutes at 25°C; 15 minutes at 42°C; 15 minutes at 48°C; SensiFast, Bioline). Polymerase chain reaction (PCR) amplification of HBV RNAs were performed using primers as previously described[318] using a SYBR green real-time PCR protocol (qPCRBIO SyGreen, PCR Biosystems) in a Lightcycler 96™ instrument (Roche). The amplification conditions were: 95°C for 2 minutes (enzyme activation), followed by 45 cycles of amplification (95°C for 5 seconds; 60°C for 30 seconds). HIF target and hypoxic genes were amplified using TaqMan® Gene Expression assays (Thermo Fisher) and amplified using a Taqman real-time PCR protocol (qPCRBIO probe, PCR Biosystems) using the same conditions as listed above. Information on primers used listed in Table 2.4.

Table 2.4. List of primers used in this thesis study. Sequences are listed from the 5' to the 3' direction.

Primer target	**Sequence**	**Manufacturer and catalogue number**
HBV-pgRNA TaqMan® Gene Expression Assay	ND	ThermoFisher AIKAMSS
CAIX TaqMan® Gene Expression Assay	ND	ThermoFisher Hs00154208_m1
VEGFA TaqMan® Gene Expression Assay	ND	ThermoFisher Hs00900055_m1
SMC6 TaqMan® Gene Expression Assay	ND	ThermoFisher Hs00226378_m1
B2M Control Mix	ND	Applied Biosystems 4325797
NDRG1 forward	TTTGATGTCCAGGAGCAGGA	
NDRG1 reverse	ATGCCGATGTCATGGTAGGT	
B-Actin forward	CCAACCGCGAGAAGATGA	
B-Actin reverse	CCAGAGGCGTACAGGGATAG	
4T PCR T1 pC/pg locus forward	GGGGAACTAATGACTCTAGCTACC	
4T PCR T1 pC/pg locus reverse	TTTAGGCCCATATTAGTGTTGACA	
4T PCR T2 pC/pg preS1 loci forward	CAAGGTAGGAGCTGGAGCATTC	

4T PCR T2 pC/pg preS1 loci reverse	GAGGCAGGAGGCGGATTTG	
4T PCR T3 pC/pg preS1 preS2 loci forward	CTCCAGTTCAGGAACAGTAAACCC	
4T PCR T3 pC/pg preS1 preS2 loci reverse	AGGAATCCTGATGTGATGTTCTCC	
4T PCR T4 pC/pg preS1 preS2 HBx loci forward	ACGGGGCGCACCTCTCTTTA	
4T PCR T4 pC/pg preS1 preS2 HBx loci reverse	GTGAAGCGAAGTGCACACGG	
Mouse HIF-1b forward	CTCACGAAGGTCGTTCATCTGC	
Mouse HIF-1b reverse	CCACAAAGTGAGGTTCTCCTTCC	
Mouse VEGFA forward	CTGCTGTAACGATGAAGCCCTG	
Mouse VEGFA reverse	GCTGTAGGAAGCTCATCTCTCC	
Mouse PHD2 forward	GGCGAACGATTGAGGCTTCCTT	
Mouse PHD2 reverse	GCTGGTGACAGCAAAGAGAAGG	
Mouse FMO2 forward	GCATAGGTCTCATCCAGCCTCT	
Mouse FMO2 reverse	TAGTCGTCTCCGAAGGCAAGCT	
Mouse CYP2c38 forward	CCTTGTCCCTAACAACCTACCC	
Mouse CYP2c38 reverse	GGAACTCCTTGCTGTCATGCAG	
Mouse RNF186 forward	GCTTAGCTGTCAGCACACCTTC	
Mouse RNF186 reverse	ACAGCAGTGACCTTTCGGCACA	
Mouse RGS16 forward	ATCCGATCAGCCACCAAACTGG	
Mouse RGS16 reverse	GAAGCAACTGGTAGTGGCAGCT	
Mouse B-Actin forward	CATTGCTGACAGGATGCAGAAGG	
Mouse B-Actin reverse	TGCTGGAAGGTGGACAGTGAGG	

2.7 Gene Set Enrichment Analysis

GSEA was performed with GSEA_4.0.3[319] using publicly available Affymetrix microarray datasets[320,321] from Gene Expression Omnibus (GEO) (accessions: GSE83148, GSE14323). Gene sets were derived from HepG2 RNA-seq data[160] downloaded from GEO (GSE120886) or PHH RNA-seq accessed from the published supplementary material[322]. To determine whether HBV infection induces hypoxia-responsive genes, the mRNA expression patterns of the liver chimeric mice RNA-seq dataset for the top 80 hypoxia-induced genes as identified in HepG2 hepatic cells were interrogated[160]. In-house datasets were compared with RNA-seq[323] from HBV-infected primary human hepatocytes. Data were retrieved from GEO (accessions: GSE120886, GSE93153, GSE118295). For consistency, all datasets were re-analysed with the same Partek Flow bioinformatic pipeline. Microarray analysis was performed with Partek Genomics Suite (v6.6) as previously described[324] and data presented using Graphpad Prism 7.

2.8 HBV Transgenic Mice and HBV or HIF-1β Specific siRNA Delivery

Animal experiments were conducted in accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). HBV transgenic (HBVtg) male and female mice (strain HBV1.3.32)[81,82] carrying a 1.3-fold overlength HBV genome on a C57BL/6J background between 12-15 weeks were used. The HBV specific siRNA (siHBV) was designed to silence all HBV transcripts by targeting the 3´region of the HBV genome and the control siRNA (siCtrl) does not target any viral or known host transcripts. SiRNAs targeting HIF-1β (siHIF-1β),

siHBV, or siCtrl were complexed with Invivofectamine 3.0 reagent (ThermoFisher Scientific) before injecting 1 μg/g body weight into the tail vain. Serum HBsAg, HbeAg, or ALT were analysed using Architect HbsAg quantitative assay (Abbott Laboratories), Architect HbeAg quantitative assay (Abbott Laboratories), or Reflotron GPT/ALT test (Roche) as per manufacturer's protocol. Immediately after sacrificing the mice and preparation of the liver, approximately 1-1.5 cm long piece of liver was placed in 500μL RNAlater. After storage for 24 hours at 4°C the tissue was transferred to -20°C and stored until RNA preparation. Total RNA was prepared using the RNeasy Mini kit as per manufacturer's protocol (Qiagen). For HBcAg liver staining, liver samples were fixed in 4% buffered formalin for 48 hours, dehydrated and embedded in paraffin. Two μm-thin sections were stained with a rabbit anti-HBV core antibody (OriGene technologies Inc.; Rockville, MD, USA; CAT#: AP10430PU-N) on a Leica Bond MAX system (Leica Biosystems, Nussloch). Tissue slides were scanned using a SCN 400 slide scanner (Leica Biosystems).

2.9 HBV Infected Human Chimeric Mice and RNA-Sequencing

Animal experiments conducted in accordance with guidelines of the Imperial College London local genetic manipulation safety committee and the Health and Safety Executive of the United Kingdom. Mock and HBV infected mice were sacrificed and livers harvested for RNA isolation. RNA purity was assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and integrity determined using a 2100 Bioanalyzer Instrument (Agilent Technologies). RNA-sequencing (RNA-seq) was conducted at the Beijing Genomics Institute (BGI, Hong Kong). FASTQ format data was aligned to the human genome, genes were quantified with differential

expression determined. Scatter dot plots of fold change values were plotted with Graphpad Prism version 7. RNA-seq data are deposited in the GEO archive at NCBI, with the accession number SE145835 and entitled: Transcriptional profiling of hepatocytes isolated from chronically HBV-infected human chimeric mice.

2.10 Human and Non-Human HBV Phylogeny

The HBV Database (http://hbvdb.ibcp.fr)[325] was searched for the HRE motif RCGTGC and the number and location of matches enumerated. The neighbor-joining phylogenetic tree is based on that of McNaughton and colleagues[326] and was built from an alignment of complete (~3.2Kb) NCBI referent genomes of human and non-human HBV with the following accession numbers: Anatidae (Geese): Snow goose; NC_005950.1, Duck; NC_001344.1, Sheldgoose; NC_005890.1 and Ross's goose; NC_005888.1. Other birds: Heron; NC_001486.1, Tinamou; NC_035210.1 and Parrot; NC_016561.1. Other species: Tibetan frog; NC_030446.1, White sucker fish; NC_027922.1 and Bluegill fish; NC_030445.1. Bats: Tent-making bat; NC_024445.1, Horseshoe bat; NC_024444.1, Roundleaf bat; NC_024443.1 and Long-fingered bat; NC_020881.1. New world monkeys: Woolly monkey; NC_028129.1 and Capuchin Monkey; KY703886. Other Mammals: Woodchuck; NC_004107.1 and Ground squirrel; NC_001484.1. Old World Primates: Orangutan; F193863, Gorilla; FJ798097 and Chimpanzee; FJ798098. Human: Genotype (Gt) A; EU054331, X02763, Gt B; AB219428, D00331 Gt C; GQ924620, GQ358158, Gt D; AF121240, FJ904433, Gt E; AB106564, AY738145, Gt F; AY090458, X75658, Gt G; AB064313, AF160501 and Gt H; AY090454, FJ356716 (HBV sequences are referents from the HBV database[325]).

2.11 HBV Genesis and Infection

HBV was purified from a HepAD38 producer line as previously reported[327]. Briefly, virus was purified using centrifugal filter devices (Centricon Plus-70 and Biomax 100.000, Millipore Corp., Bedford, MA) and stocks with a titre between $3x10^9$ and $3x10^{10}$ viral genome equivalents (vge) per mL stored at -80°C. HBV-X- virus was purified from a HepG2 based cell line containing a HBV 1.3x overlength integrated viral genome where both 5' and 3' HBx genes were knocked out by a point mutation that changes the eighth amino acid to a stop codon (CAA-to-TAA) as previously described[328]. HepG2-NTCP cells were treated with 2.5% DMSO for 3 days and inoculated with HBV at a MOI of 200 in the presence of 4% polyethylene glycol 8000. After 18-20 hours the inoculum was removed by washing with PBS and the cells cultured in the presence of 2.5% DMSO. Secreted HBe and HBs antigen were quantified by ELISA as per manufacturer's protocol (Autobio, China).

2.12 SiRNA Silencing

HepG2-NTCP cells were trypsinised and mixed with 50nM of either control or HIF-1α/HIF-2α/HIF-1β siRNA complexed with DharmaFECT4 transfection reagent (Dharmacon) according to manufacturer's instructions. Transfected cells were seeded and left to recover for 4 hours after which media was replenished. Cells were infected with HBV at a MOI of 200, 24 hours post siRNA delivery for 6 hours, treated with or without appropriate compounds, then transferred to either 18% or 1% oxygen for 72 hours after which cells were harvested in RLT buffer (Qiagen) for subsequent western blot or PCR analysis. Information on siRNAs used are listed in Table 2.5.

Table 2.5. List of siRNAs used in this thesis study.

Target	Manufacturer	Catalogue number
siRNA HIF-1α	Ambion	4390824, s6539
siRNA HIF-2α (EPAS1)	Ambion	4390824, s4700
siRNA HIF-1b (ARNT1)	Santa Cruz	sc-29733
Scrambled siRNA Control (Negative Control)	Ambion	4390843
siRNA Mouse HIF-1b 1	ThermoFisher	AM16708
siRNA Mouse HIF-1b 2	ThermoFisher	AM16708
Negative control siRNA-1	ThermoFisher	AM4404021

2.13 NTCP Fluorescent Microscopy

HepG2-NTCP cells or Huh7 not expressing NTCP as control were seeded in 96-well plates and incubated overnight. Media was removed, cells were rinsed with PBS, and incubated with media containing a synthetic green fluorescent protein (GFP) tagged PreS1 peptide that binds NTCP (Myrcludex B) for 1 hour. Media was removed, cells were rinsed with PBS, and imaged with fluorescent microscopy.

2.14 Statistical Analysis

Results shown as mean ± standard deviation with statistical analysis performed with Prism7 (GraphPad, USA) or Excel (Microsoft, USA). Statistical tests used are described in figure legends with $P<0.05$ being considered statistically significant.

3. HBV MODULATION OF THE HIF PATHWAY

Portions of this chapter have been published in Scientific Reports[304].

3.1 Introduction

The liver harbours a naturally low oxygen environment, where it receives oxygenated blood from the hepatic artery and oxygen-depleted blood via the hepatic portal vein, resulting in an oxygen gradient of 4-8% across the pericentral and periportal areas, respectively[329]. Importantly, this oxygen gradient is readily perturbed in pathological states such as infection, inflammation and cirrhosis[115]. In addition to hypoxia, inflammation and oxidative stress can promote HIF-transcriptional activity. The host inflammatory mediators NF-κB and TNF-α have been reported to induce HIF-1α transcription[182,330]. ROS produced by immune cells provide a further mechanism for inflammation-driven HIF-signaling[179,180,331]. Current knowledge supports a role for the PHD-HIF pathway to facilitate replication of viruses with a selective tropism for low oxygen environments[175]. As reviewed previously, a number of viruses induce the HIF signaling pathway including hepatitis C virus, human papillomavirus, Kaposi sarcoma-associated herpesvirus and human cytomegalovirus[175,195,215,222,224,332,333]. In particular, several reports have suggested that HBV protein HBx interacts with and stabilises HIFs[226,227,229,305-307,334-336]. However, this proposed HBx-HIF interplay awaits validation in *in vitro* and *in vivo* HBV replication model systems.

Given previous reports on the role of viral infection and inflammation in modulating the PHD-HIF pathway, this chapter explores the impact of HBV infection on HIF signaling. This chapter reports an upregulation of hypoxic gene transcripts in the liver of chronic HBV infected patients[320].

Studies to investigate the underlying mechanism using *in vitro* viral infection models and *in vivo* HBV transgenic mice and human liver chimeric mice models show limited evidence of hypoxic gene expression. *In vitro* studies with cells engineered to express HBV proteins including HBx, HBcAg, and HBsAg show minimal perturbation of HIF signaling consistent with results from full replicating model systems. These studies highlight a role for inflammatory responses in driving HIF signaling in CHB that is not recapitulated by current infection-competent model systems.

3.2 Results

3.2.1 Increased hypoxic gene transcripts in CHB

To determine whether hypoxic regulated gene transcripts are increased in CHB, Gene Set Enrichment Analysis (GSEA) was conducted using published microarray data of liver biopsy samples from chronic HBV infected patients (n=90), that were free of cirrhosis or HCC, and non-viral infected control subjects (healthy, n=6)[320]. This cohort study contained 69% male and 31% female with half of all patients over the age of 40. Ethics approval was obtained from the Fudan University ethics committee in Shanghai, China. A panel of 43 hypoxia gene signatures was obtained from the Molecular Signatures Database (MSigDB v 7.0) that included hypoxia regulated genes from diverse tissues and cancer types. There was an enrichment (false discovery rate (FDR) <0.05) of 19 gene sets in the CHB cohort (Figure 3.1a). Since hypoxic-transcriptional responses can vary between cell and tissue types and none of the MSigDB signatures were liver-derived, analysis was conducted using RNA-sequencing (RNA-seq) data from human hepatoma derived HepG2 cells cultured at 0.5% oxygen for 16 hours (Table 3.1)[160,337]. A total of 80 hypoxic upregulated genes were identified (greater than 2 fold change, FDR<0.05) and GSEA showed an enrichment (FDR=0.077) in CHB patients (Table 3.2, Figure 3.1b). To extend these results, analysis of acute transcriptional response of primary human hepatocytes (PHHs) cultured at 1% oxygen for 4 hours identified 113 upregulated genes (FC>2; FDR<0.05) and GSEA showed an enrichment in CHB (Figure 3.1c)[322]. Since PHHs can de-differentiate and lose hepatocyte-specific function *in vitro*, it was reassuring to observe an overlap of hypoxic regulated genes in HepG2 cells and PHHs (Figure 3.1d)[338].

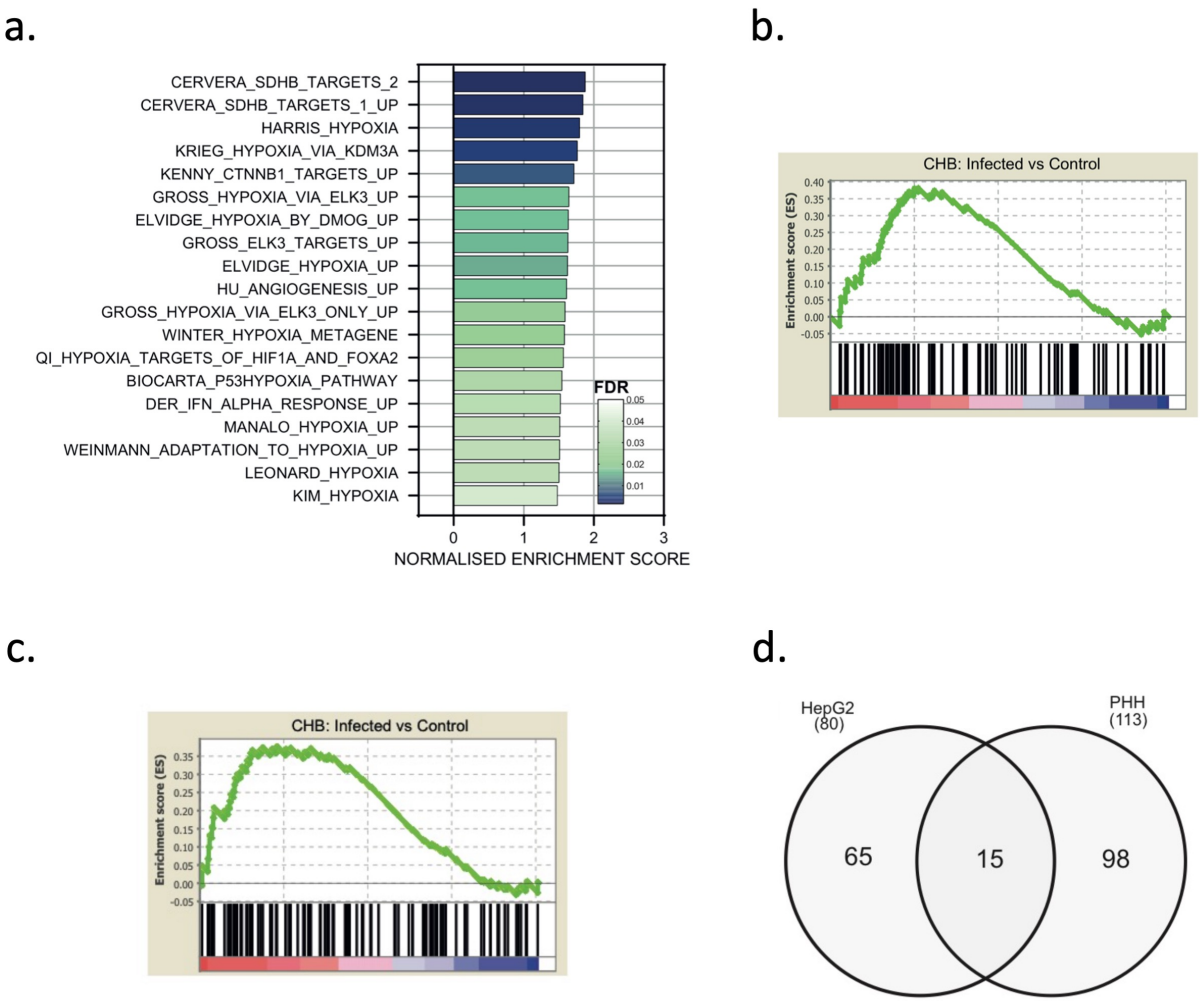


Figure 3.1. Hypoxia gene signatures were significantly upregulated in CHB. (a) Databases were assessed in the CHB cohort and 19 significantly upregulated hypoxia gene signatures identified (FDR<0.05), ranked by Normalised Enrichment Score (NES). Further details on each hypoxia gene set are provided in Table 3.1. (b) Gene set enrichment analysis (GSEA) shows a significant enrichment of HepG2 defined hypoxic genes in CHB cohort (FDR = 0.077). The gene set was based on Fold Change > 2, and FDR < 0.05; 80 genes satisfied these criteria and are listed in Table 3.2. Red horizontal bars on the bottom indicate genes with positive correlations and blue horizontal bars indicate negative correlations, with left being the highest positive correlation and right being the highest negative correlation. Black vertical bars represent genes present in the dataset for analysis. (c) A panel of primary human hepatocyte (PHH) derived hypoxic gene signature was also assessed in CHB patients. GSEA shows an enrichment of PHH derived hypoxic genes in CHB cohort versus healthy controls (FDR=0.351). (d) Differential gene expression in HepG2 cells (0.5% oxygen for 16 hours) and PHHs (1% oxygen for 4 hours), using a cut-off of at least 2 fold change, with an FDR of 0.05, showed 80 differentially expressed genes in HepG2 cells and 113 in PHHs, with 15 common overlapping genes. Analysis conducted by James Harris and Emanuele Marchi.

Table 3.1. Hypoxic gene signatures from MSigDB that are enriched (FDR<0.05) in CHB. Gene signatures are ranked by NES. The table also shows the number of genes in each signature (Size); the number of genes in common with the HepG2 derived signature (Overlap); and the tissue or cell line from which this signature was derived (Origin). Analysis conducted by James Harris and Emanuele Marchi.

HYPOXIA_UPREGULATED_GENE_SIGNATURES				
RANK	**SIGNATURE**	**SIZE**	**OVERLAP**	**ORIGIN**
1	CERVERA SDHB TARGETS 2	111	1	Hep3B and HeLA Cells
2	CERVERA SDHB TARGETS 1 UP	113	2	Hep3B and HeLA Cells
3	HARRIS HYPOXIA	80	12	Pan-cancer
4	KRIEG HYPOXIA VIA KDM3A	50	2	Renal and colon carcinoma
5	KENNY CTNNB1 TARGETS UP	48	1	Breast and colon cancer
6	GROSS HYPOXIA VIA ELK3 UP	197	1	Skin endothelium
7	ELVIDGE HYPOXIA BY DMOG UP	130	26	Breast cancer cell line MCF7
8	GROSS ELK3 TARGETS UP	27	0	Skin endothelium
9	ELVIDGE HYPOXIA UP	164	29	Breast cancer cell line MCF7
10	HU ANGIOGENESIS UP	20	0	nSCLC
11	GROSS HYPOXIA VIA ELK3 ONLY UP	31	2	Skin endothelium
12	WINTER HYPOXIA METAGENE	232	25	Head and neck cancers
13	QI HYPOXIA TARGETS OF HIF1A AND FOXA2	30	3	Adeno- and prostate carcinoma
14	BIOCARTA P53 HYPOXIA PATHWAY	20	1	Cardiovascular endothelium
15	DER IFN ALPHA RESPONSE UP	70	1	Fibroscarcoma
16	MANALO HYPOXIA UP	201	23	Pulmonary artery endothelium
17	WEINMANN ADAPTATION TO HYPOXIA UP	25	0	Hypoxia tolerant lung cancer cells
18	LEONARD HYPOXIA	42	10	Kidney tubule cells
19	KIM HYPOXIA	19	6	Fibroblasts

Table 3.2. Hypoxia upregulated genes in HepG2 cells.

Rank	Gene_Symbol	Gene_Description	Accession_Number(s)
1	LOXL2	Lysyl oxidase like 2	NM_002318
2	SMIM3	Small integral membrane protein 3	NM_032947
3	LOC154761	Family with sequence similarity 115, member c pseudogene	NR_015421
4	WDR54	Wd repeat domain 54	NM_032118 /// XM_005264586 /// XM_006712111
5	DOK3	Docking protein 3	NM_001144875 /// NM_001144876 /// NM_001308235 /// NM_001308236 /// NM_024872 /// XM_00
6	HCAR3	Hydroxycarboxylic acid receptor 3	NM_006018
7	EHD2	Eh domain containing 2	NM_014601
8	TNS1	Tensin 1	NM_001308022 /// NM_001308023 /// NM_022648 /// XM_011511711 /// XM_011511712 /// XM_01
9	HK1	Hexokinase 1	NM_000188 /// NM_033496 /// NM_033497 /// NM_033498 /// NM_033500 /// XM_005269735 ///
10	SLC2A3	Solute carrier family 2 member 3	NM_006931
11	GYS1	Glycogen synthase 1	NM_001161587 /// NM_002103 /// NR_027763
12	PLAC8	Placenta specific 8	NM_001130715 /// NM_001130716 /// NM_016619
13	IGFBP1	Insulin like growth factor binding protein 1	NM_000596 /// NM_001013029
14	FXYD1	Fxyd domain containing ion transport regulator 1	NM_001278717 /// NM_001278718 /// NM_005031 /// NM_021902
15	LSP1	Lymphocyte-specific protein 1	NM_001013253 /// NM_001013254 /// NM_001013255 /// NM_001242932 /// NM_001289005 /// NM
16	SLC6A8	Solute carrier family 6 member 8	NM_001142805 /// NM_001142806 /// NM_005629
17	TFF1	Trefoil factor 1	NM_003225
18	TUBB1	Tubulin beta 1 class vi	NM_030773
19	RIMKLA	Ribosomal modification protein rimk like family member a	NM_173642 /// XM_006710585
20	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	NM_001145443 /// NM_001282630 /// NM_001314063 /// NM_004566 /// XM_005252463 /// XM_00
21	CKB	Creatine kinase b	NM_001823
22	RASSF5	Ras association domain family member 5	NM_031437 /// NM_182663 /// NM_182664 /// NM_182665
23	SLC51A	Solute carrier family 51 alpha subunit	NM_152672
24	SPAG4	Sperm associated antigen 4	NM_003116 /// XM_005260519 /// XM_005260520 /// XM_011529009 /// XM_011529010 /// XM_01
25	HAVCR1	Hepatitis a virus cellular receptor 1	NM_001099414 /// NM_001173393 /// NM_001308156 /// NM_012206 /// XM_006714840 /// XM_01
26	ANGPTL4	Angiopoietin like 4	NM_001039667 /// NM_016109 /// NM_139314 /// NR_104213 /// XM_005272484 /// XM_00527248
27	LOX	Lysyl oxidase	NM_001178102 /// NM_001317073 /// NM_002317
28	CA9	Carbonic anhydrase 9	NM_001216 /// XM_006716869 /// XM_006716870 /// XR_428428
29	IGLON5	Iglon family member 5	NM_001101372
30	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	NM_004567 /// XM_005265230 /// XM_005265231 /// XM_011533829 /// XM_011533830 /// XM_01
31	PLEKHA2	Pleckstrin homology domain containing a2	NM_021623 /// XM_011544605 /// XM_011544606 /// XM_011544607 /// XM_011544608
32	LOXL3	Lysyl oxidase like 3	NM_001289164 /// NM_001289165 /// NM_032603 /// XM_011533134
33	CHST15	Carbohydrate (n-acetylgalactosamine 4-sulfate 6-o) sulfotransferase 15	NM_001270764 /// NM_001270765 /// NM_014863 /// NM_015892 /// XM_005269891 /// XM_00526
34	TMCC1	Transmembrane and coiled-coil domain family 1	NM_001017395 /// NM_001128224 /// NM_015008 /// NR_033361 /// XM_006713542 /// XM_00671
35	BHLHE40	Basic helix-loop-helix family member e40	NM_003670
36	EGFL7	Egf like domain multiple 7	NM_016215 /// NM_201446 /// NR_045110 /// NR_045111 /// NR_046367 /// XM_006717141 ///
37	EGLN3	Egl-9 family hypoxia inducible factor 3	NM_001308103 /// NM_022073 /// XM_006720015
38	KCTD11	Potassium channel tetramerization domain containing 11	NM_001002914
39	FGF11	Fibroblast growth factor 11	NM_001303460 /// NM_004112 /// NR_130156
40	TMEM45A	Transmembrane protein 45a	NM_018004 /// XM_005247569
41	ISM2	Isthmin 2	NM_182509 /// NM_199265 /// NM_199296 /// XM_011536489
42	NDRG1	N-myc downstream regulated 1	NM_001135242 /// NM_001258432 /// NM_001258433 /// NM_006096 /// XM_011516791 /// XM_01
43	SERPINE1	Serpin family e member 1	NM_000602 /// NM_001165413
44	ESPN	Espin	NM_031475 /// XM_005263501 /// XM_011542231 /// XM_011542232 /// XM_011542233 /// XM_01
45	ADM	Adrenomedullin	NM_001124
46	RNASET2	Ribonuclease t2	NM_003730
47	FAM110C	Family with sequence similarity 110 member c	NM_001077710 /// XM_011510372 /// XM_011510373 /// XM_011510374
48	IGFBP3	Insulin like growth factor binding protein 3	NM_000598 /// NM_001013398
49	PDK1	Pyruvate dehydrogenase kinase 1	NM_001278549 /// NM_002610 /// NR_103729 /// NR_103731 /// XM_006712594 /// XM_00671259
50	PDGFB	Platelet derived growth factor subunit b	NM_002608 /// NM_033016
51	UNC93A	Protein unc-93 homolog a	NM_001143947 /// NM_018974 /// XM_011535905 /// XM_011535906 /// XM_011535907 /// XM_01
52	PPP2R2C	Protein phosphatase 2 regulatory subunit bgamma	NM_001206994 /// NM_001206995 /// NM_001206996 /// NM_020416 /// NM_181876 /// XM_00524
53	FAM13A	Family with sequence similarity 13 member a	NM_001015045 /// NM_001265578 /// NM_001265579 /// NM_001265580 /// NM_014883 /// XM_00
54	PLIN2	Perilipin 2	NM_001122 /// NR_038064 /// XM_006716719
55	GBE1	Glycogen banching enzyme	NM_000158
56	LCN15	Lipocalin 15	NM_203347 /// XM_006717105 /// XM_011518672
57	ZNF395	Zinc finger protein 395	NM_018660
58	RAB42	Ras-related protein rab-42	NM_001193532 /// NM_152304
59	PGK1	Phosphoglycerate kinase 1	NM_000291
60	PPP1R3C	Protein phosphatase 2 regulatory subunit 3c	NM_005398
61	BNIP3L	Bcl2/adenovirus e1b 19kda protein-interacting protein 3-like	NM_004331 /// XM_005273617 /// XM_011544630
62	ANKRD37	Ankyrin repeat domain 37	NM_181726 /// XM_005262981
63	ANKZF1	Ankyrin repeat and zinc finger domain containing 1	NM_001042410 /// NM_001282792 /// NM_018089 /// XM_005246663 /// XM_011511392 /// XR_42
64	APOL1	Apolipoprotein l1	NM_001136540 /// NM_001136541 /// NM_003661 /// NM_145343 /// NM_145344 /// XM_00526179
65	DDIT4	Dna damage inducible transcript 4	NM_019058
66	PNPLA7	Patatin-like phospholipase domain-containing protein 7	NM_001098537 /// NM_152286 /// XM_006717102 /// XM_006717104 /// XM_011518664 /// XR_92
67	BNIP3	Bcl2 interacting protein 3	NM_004052
68	RORA	Rar related orphan receptor a	NM_002943 /// NM_134260 /// NM_134261 /// NM_134262 /// XM_005254584 /// XM_011521873 /
69	NDUFA4L2	Ndufa4 mitochondrial complex associated like 2	NM_020142 /// XM_005269033 /// XM_011538573
70	PIGZ	Phophatidylinositol glycan anchor biosynthesis class z	NM_025163 /// XM_006713758 /// XM_011513190 /// XM_011513191 /// XM_011513192
71	ABCA7	Atp binding cassette transporter a7	NM_019112 /// NM_033308 /// XM_006722616 /// XM_006722617 /// XM_006722618 /// XM_01152
72	HR	Protein hairless	NM_005144 /// NM_018411 /// XM_005273569 /// XM_006716367
73	EPO	Erythropoietin	NM_000799
74	TMEM145	Transmembrane protein 145	NM_173633 /// XM_005258781 /// XM_011526791 /// XM_011526792
75	MIR210HG	Mir210 host gene	NR_038262
76	CITED2	Cbp/p300 interacting transactivator with glu/asp rich carboxy-terminal domain 2	NM_001168388 /// NM_001168389 /// NM_006079
77	PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	NM_000935 /// NM_182943 /// XM_005247535 /// XM_005247536
78	INHA	Inhibin alpha	NM_002191
79	BIRC7	Baculoviral iap repeat containing 7	NM_022161 /// NM_139317
80	ALDOC	Aldolase, fructose-bisphosphate c	NM_005165 /// XM_005257949 /// XM_011524556

CHB reflects a dynamic interaction between virus infected hepatocytes and immune cells. Periods of active hepatitis, as measured by the elevated activity of the liver enzyme alanine aminotransferase (ALT), is associated with increased viremia[339]. Grouping the CHB cohort by ALT activity showed an enrichment (FDR=0.110) of the hypoxic HepG2 derived gene set in patients with elevated ALT (ALT >40IU/mL) (n=57) compared to those with normal ALT (n=25) (Figure 3.2a). To evaluate whether hypoxic gene expression is observed in other inflammatory liver diseases, a cohort of hepatitis C virus (HCV) infected patients with cirrhosis (n=41) was analysed. Patients analysed from this published dataset did not develop HCC and were a part of the Adult to Adult Living Donor Liver Transplant Cohort Study. Ethics approval was obtained from Virginia Commonwealth University, University of North Carolina, University of California Los Angeles, University of Pennsylvania, and Northwestern University review boards. Analysing this cohort revealed an enrichment of the hypoxic HepG2 gene signature (FDR=0.006) in the infected group compared to normal controls (n=19) (Figure 3.2b)[321]. Analysing the leading-edge genes in the HBV (n=30) and HCV (n=23) cohorts identified 10 common genes, however, the majority of genes (57%) were unique to their respective cohorts. These data are consistent with a role for inflammatory pathways in the regulation of hypoxic transcriptional responses. To evaluate this model, enriched pathways in CHB liver were examined using the hallmark gene sets from MSigDB. This analysis identified genes associated with allograft rejection as the most significantly upregulated gene set in CHB. Interestingly HIF-1α was one of the leading-edge genes in this subset; contributing to the core enrichment score. There is increased HIF-1α mRNA levels in the CHB patients compared to control subjects (Log2 fold change (FC) = 2.648, p=0.005). Moreover, there is an increase in inflammatory signaling pathways in CHB liver: 'TNF-α signaling via NF-κB', 'Inflammatory Response' and 'Interferon Gamma Response' (Figure 3.2c). In summary these data

support the conclusion that the hypoxic phenotype in the diseased liver is likely driven by local inflammation, rather than a direct result of viral infection.

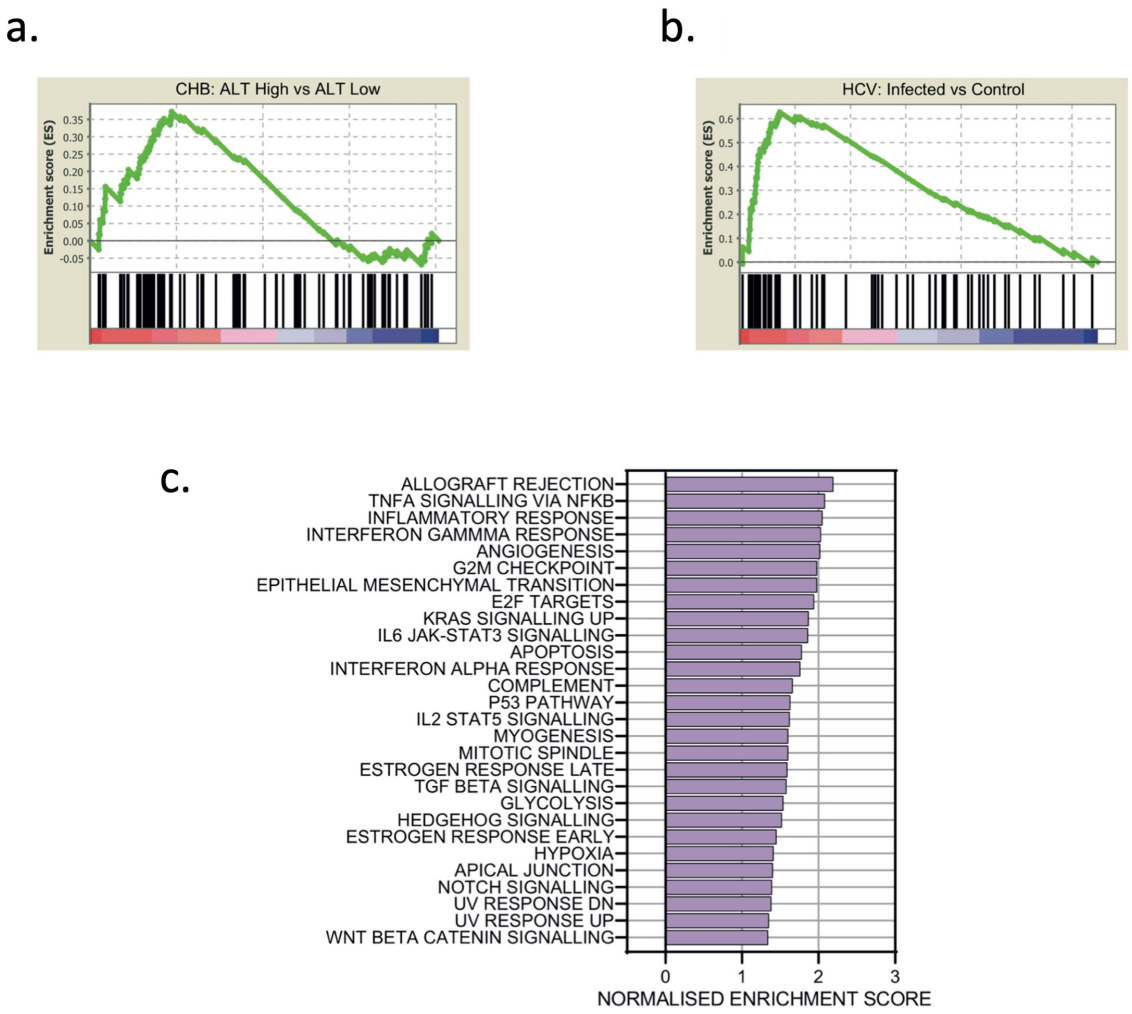


Figure 3.2. A role for inflammatory response in driving HIF signaling in CHB. (a) CHB cohort was grouped by peripheral ALT activity, with subjects > 40IU/L (n=57) or < 40IU/L (n=25). GSEA shows a significant enrichment of HepG2 defined hypoxic genes in patients with elevated ALT (FDR=0.110). Red horizontal bars on the bottom indicate genes with positive correlations and blue horizontal bars indicate negative correlations, with left being the highest positive correlation and right being the highest negative correlation. Black vertical bars represent genes present in the dataset for analysis. (b) HepG2 hypoxic gene set was enriched (FDR=0.006) in HCV infected patients with cirrhosis (n=41) compared to normal liver controls (n=19). (c) MSigDB hallmark gene sets identified the most upregulated pathways in the CHB cohort: 28 gene sets were significantly enriched (FDR<0.05) and are ranked by NES. Analysis conducted by James Harris and Emanuele Marchi.

3.2.2 Silencing viral gene expression in HBV transgenic mouse model has a minimal effect on HIF target gene transcripts

Since HBV can only infect humans and hominoid primates, no immune competent animal models are available that support natural HBV infection. One of the most-widely used murine models for studying HBV replication are transgenic mice expressing HBV from a single integrated genome (HBVtg). HBVtg mice have been reported to develop HCC that show similar chromosomal aberrations and gene expression patterns to human HBV-associated HCC[340]. To study the effect of HBV on HIF transcriptional activity in this model system, HBVtg mice were treated with liver-targeted siRNAs targeting all of the major viral RNAs (siHBV)[341] or with an unspecific control siRNA (siCtrl). PCR measurement of viral transcripts including pC/pgRNA, PreS1/S2 and HBx RNA showed a significant reduction in the siHBV treated mice that was accompanied by a reduction in peripheral HBeAg and HBsAg (Figure 3.3a).

In the siCtrl group, male mice had higher HBV RNA, HBcAg expressing hepatocytes and serum HBeAg and HBsAg levels than females (Figure 3.3b). However, the limited number of animals prevented any meaningful statistical analysis. siHBV silencing showed comparable efficacy to reduce viral RNAs, HBeAg, and HBsAg between males and female (Figure 3.3c). Finally, HBcAg staining in the liver clearly shows successful silencing in siHBV treated mouse compared to siCtrl mouse (Figure 3.3d).

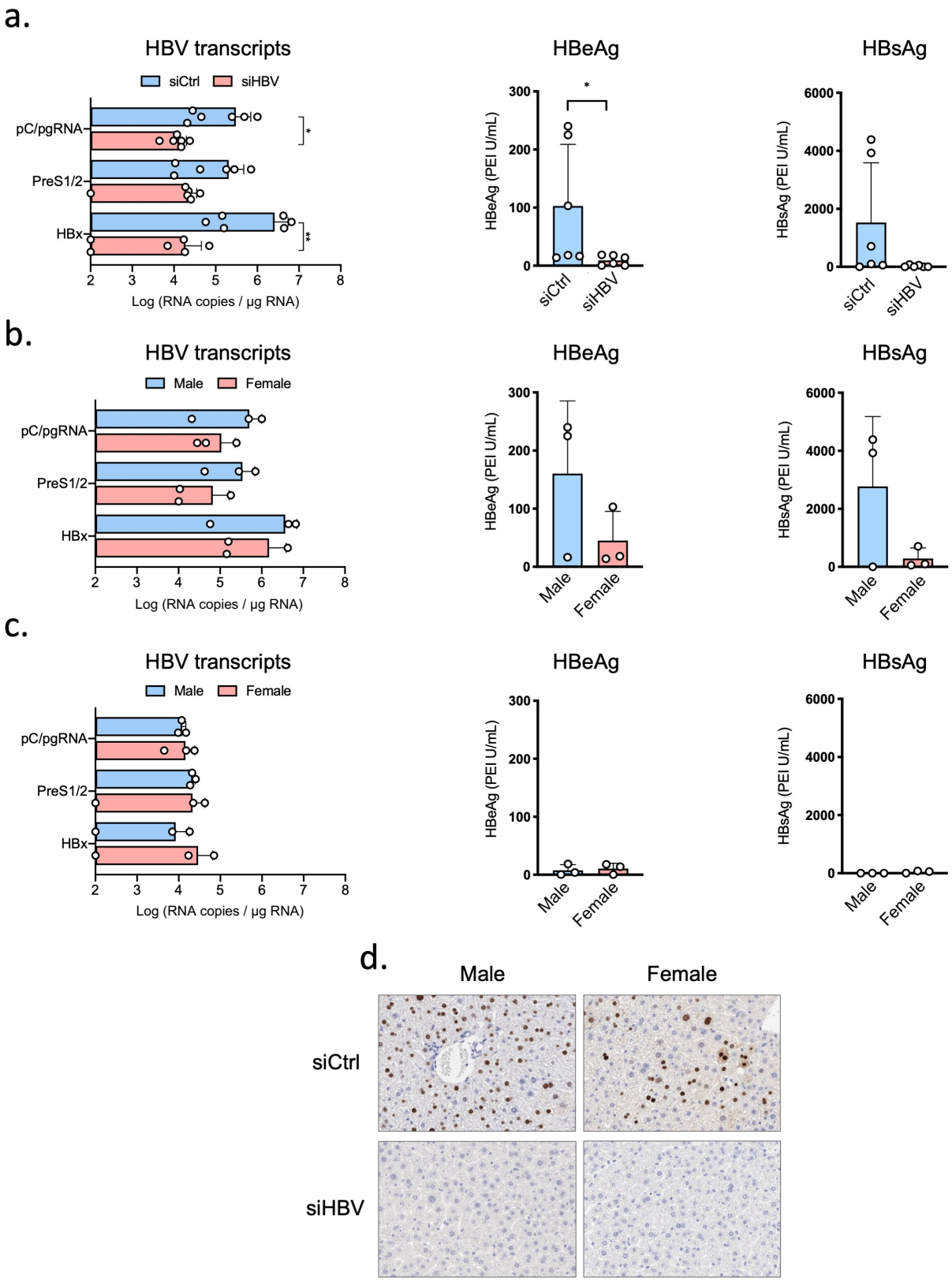


Figure 3.3. Effective silencing of viral transcription in HBV transgenic mice. Figure legend on the following page.

Figure 3.3. Effective silencing of viral transcription in HBV transgenic mice. HBV transgenic mice (n=6 per group, 3=males, 3=females) were injected with liver directed siRNAs targeting the HBx region (siHBV) which is commonly shared by all viral RNAs or with a control siRNA (siCtrl). Seven days later efficacy of siHBV silencing was assessed by quantifying the hepatocyte HBV transcripts, serum HBeAg, or HBsAg presented as a comparison between siCtrl vs siHBV groups (a), between males versus females in the siCtrl group (b), or between males versus females in the siHBV group. Hepatocyte HBcAg expression was assessed via immunostaining and representative results shown (d). Mann Whitney analysis was applied with $p<0.05$ deemed as significant. *In vivo* experiments conducted with support from Thomas Michler.

To understand the impact of siHBV silencing on HIF signaling, the HIF regulated gene transcripts *CAIX, VEGFA, GLUT1*, and *PHD2* were assessed in mouse liver samples. Silencing HBV mRNAs and antigens had a minimal effect on the HIF regulated gene transcripts (Figure 3.4). These studies suggest a minimal role of HBV encoded proteins or RNAs in perturbing HIF transcriptional activity.

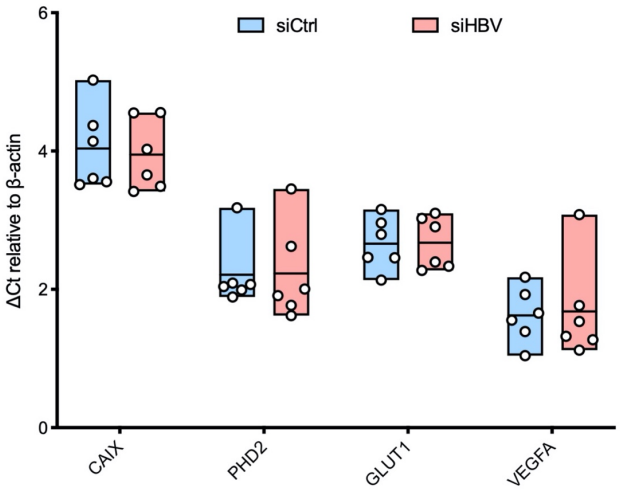


Figure 3.4. Effect of silencing viral transcription in HBV transgenic mice on HIF target gene transcripts. Transcription of HIF target genes (*CAIX*, *PHD2*, *GLUT1*, *VEGFA*) in the liver were assessed in HBV transgenic mice presented in Figure 3.3. Results are presented as a comparison between siCtrl and siHBV groups. Hypoxia target gene values are expressed as ΔCt values by subtracting the Ct value of the housekeeping gene β-actin from Ct value of the gene of interest. 2-way-ANOVA with Bonferroni correction analysis was applied with $p<0.05$ deemed as significant. Each data point represents data from a single animal.

3.2.3 Functionally active HBx protein does not affect HIF-α protein expression or target gene transcripts

As HBx was previously reported to stabilise HIFs, the bipotent HepaRG cell line that expresses HBx (HepaRG-HBx_{WT}) under a tetracycline (Tet) inducible promoter was used to study HBx-HIF interplay[226,229,305-307,334-336,342,318,328]. HBx promotes viral transcription by degrading the Smc5/6 complex. Western blot analysis confirmed that treating the cells with Tet induced HBx expression, which resulted in a loss of Smc6 expression and restored the replication defective HBV mutant lacking HBx (HBV_{X-}) (Figure 3.5a, Figure 3.5b)[48], confirming that HBx in this model system is functionally active.

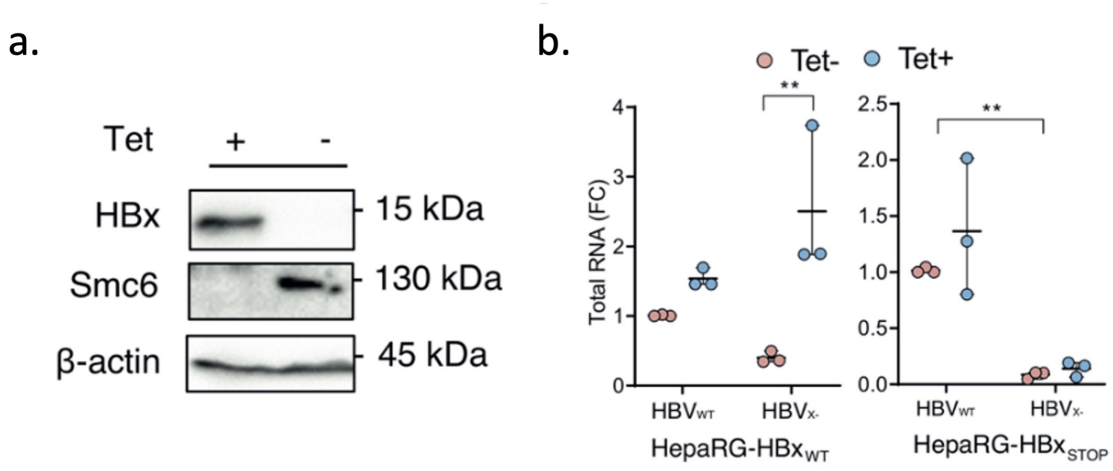


Figure 3.5. Effect of tetracycline induction on HBx expression and function in HepaRG cells. HepaRG cells encoding HBx were incubated with Tet (50 μM) for 24 hours and HBx protein and Smc6 expression detected by western blot (a). Differentiated HepaRG cells encoding HBx_{WT} or mutated HBx_{STOP} were treated or not with Tet and infected with wild type HBV (HBV_{WT}) or HBV lacking HBx (HBV_{X-}) and 13 days later infection was assessed by measuring total viral RNAs (b). The data is normalised for each cell line relative to HBV_{WT} infection without Tet and represents 3 biological repeats; 2-way-ANOVA with Bonferroni correction was applied with $p<0.05$ deemed as significant. Error bar represents standard deviation. HBx transcomplementation experiment conducted with support from Valentina D'Arienzo.

As a control for these experiments, HepaRG cells encoding HBx with three nonsense mutations (HepaRG-HBx$_{STOP}$) was used. To assess whether HBx can promote or stabilise HIF expression, HepaRG-HBx$_{WT}$ or HepaRG-HBx$_{STOP}$ cells were treated with Tet and cultured at 1% oxygen, a typical oxygen concentration used to model hypoxia *ex vivo*, or standard 'normoxic' laboratory conditions of 18% oxygen for 24 hours. HBx had a minimal effect on HIF-1α or HIF-2α protein or mRNA levels (Figure 3.6a, Figure 3.6b) in HepaRG cells cultured at 18% oxygen. Culturing HepaRG-HBx$_{WT}$ or HepaRG-HBx$_{STOP}$ cells under 1% oxygen confirmed that the cells express both HIF-1α and HIF-2α and HBx had a negligible effect on either HIF isoform (Figure 3.6a). To assess whether HBx altered HIF transcriptional activity, the mRNA levels of four HIF-regulated host genes (*CAIX, BNIP3, VEGFA* or *GLUT1*) were quantified and CAIX protein assessed (Figure 3.6b, Figure 3.6a). These studies showed a minimal effect of HBx on HIF expression or transcriptional activity.

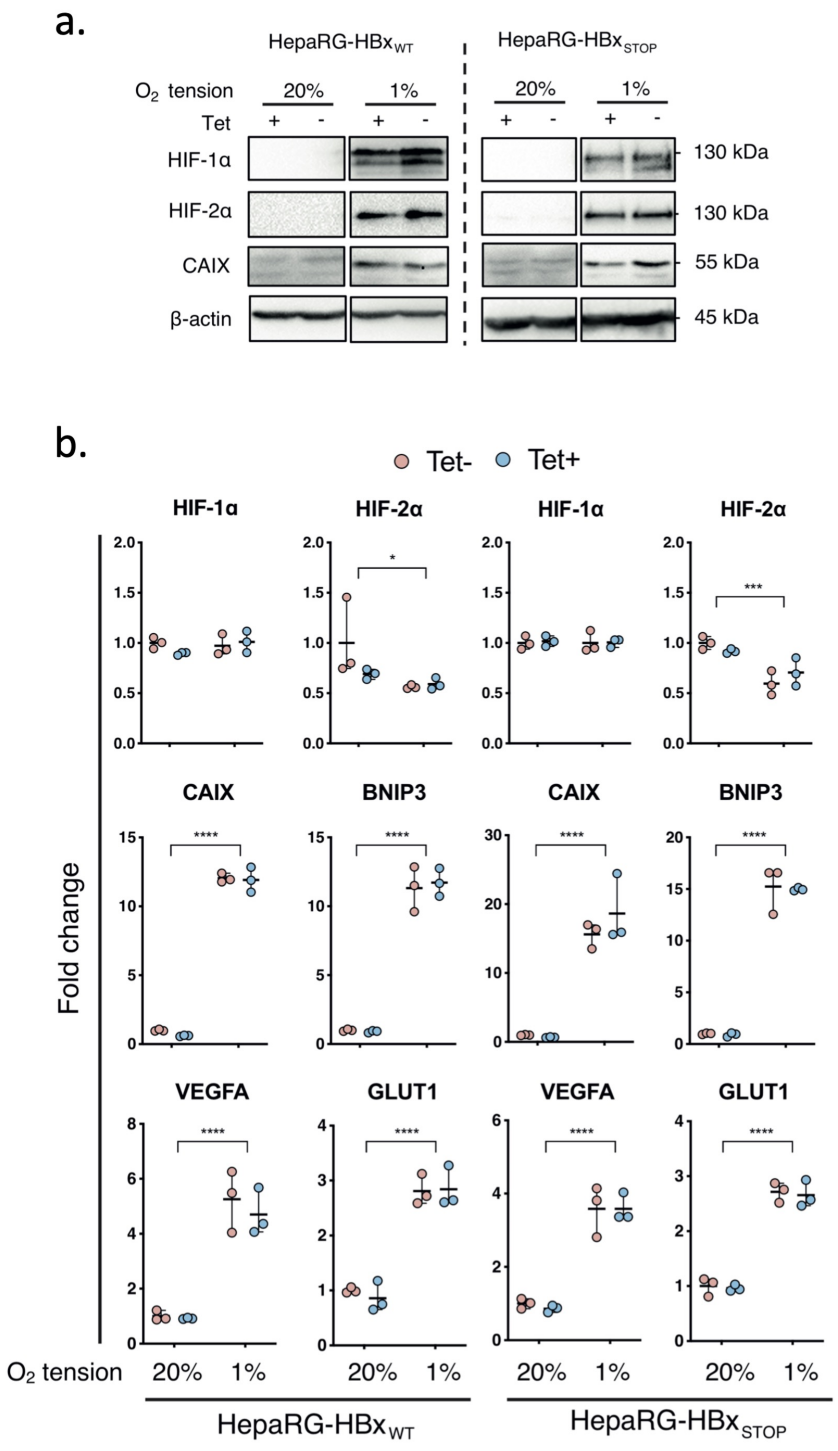


Figure 3.6. Effect of HBx on HIF expression and transcriptional activity in HepaRG cells. Figure legend on the following page.

Figure 3.6. Effect of HBx on HIF expression and transcriptional activity in HepaRG cells. HepaRG cells encoding WT or mutated HBx (STOP) were incubated with or without Tet (50 μM, 24 hours) and cultured under 18% or 1% oxygen conditions for 24 hours. Cells were lysed and expression of HIF-1α, HIF-2α, carbonic anhydrase IX (CAIX) and housekeeping gene β-actin assessed by western blot (a) and mRNA levels of *HIF-1α*, *HIF-2α* and several HIF target genes (*CAIX, BNIP3, VEGFA* and *GLUT1*) quantified by qPCR (b). Normality distribution was assessed by D'Agostino-Pearson test; 2-way-ANOVA with Bonferroni correction was applied with $p<0.05$ deemed as significant. Data is representative of results from 3 biological repeats.

Under normoxic conditions HIFs are hydroxylated by the oxygen-dependent PHDs and targeted for proteosomal degradation. Oxygen reperfusion of hypoxic cells results in a time-dependent loss of HIFs and whether HBx could alter the kinetics of HIF expression was assessed. There was a comparable decrease in HIF-1α and HIF-2α proteins after 10-20 minutes of oxygen reperfusion in both Tet treated and untreated cells (Figure 3.7), demonstrating that HBx has a negligible effect on the kinetics of HIF degradation. In summary, these results demonstrate that HepaRG cells are responsive to low oxygen and show a significant increase in hypoxia-associated gene transcription that is not impacted by HBx.

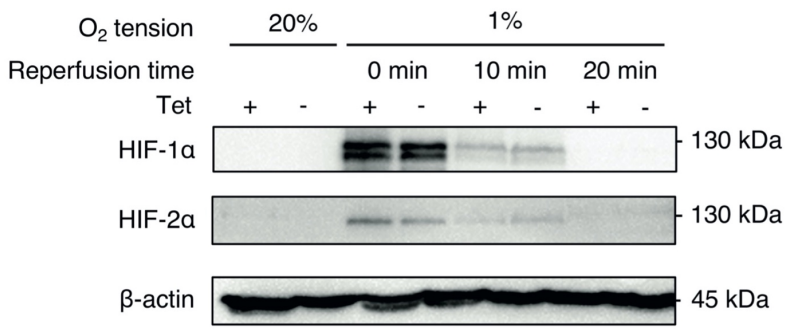


Figure 3.7. Effect of oxygen reperfusion on HIF expression in HepaRG cells. HepaRG cells encoding wild type HBx were incubated with Tet (50 μM, 24 hours) and cultured at 18% or 1% oxygen for 24 hours. The hypoxic cultures were returned to 18% oxygen. After 10 or 20 minutes, cells were lysed and screened for HIF-1α or HIF-2α and housekeeping gene β-actin expression. Data is representative of results from 3 biological repeats.

To extend these results an adenoviral vector encoding HBx (Ad-HBx) was used and demonstrated HBx expression in transduced HepG2-NTCP cells that resulted in a degradation of Smc6. Furthermore, transducing HepG2-NTCP cells with Ad-HBx restored the replication of HBV_{X-} further demonstrating its functional activity (Figure 3.8a). HepG2-NTCP cells transduced with Ad-HBx or adenoviral vector expressing ovalbumin (Ad-OVA) were cultured at 18% or 1% oxygen and cells harvested over a 48 hours period. HBx expression was confirmed 24 hours post-transduction (Figure 3.8b) and HIF-1α expression after 8 hours at 1% oxygen. Comparable expression levels of HIF-1α were noted in both Ad-HBx and Ad-OVA transduced cells, suggesting a negligible effect of HBx on HIF-1α induction. These results further highlight a minimal role of HBx in regulating HIF1α or HIF2α mRNA or protein expression.

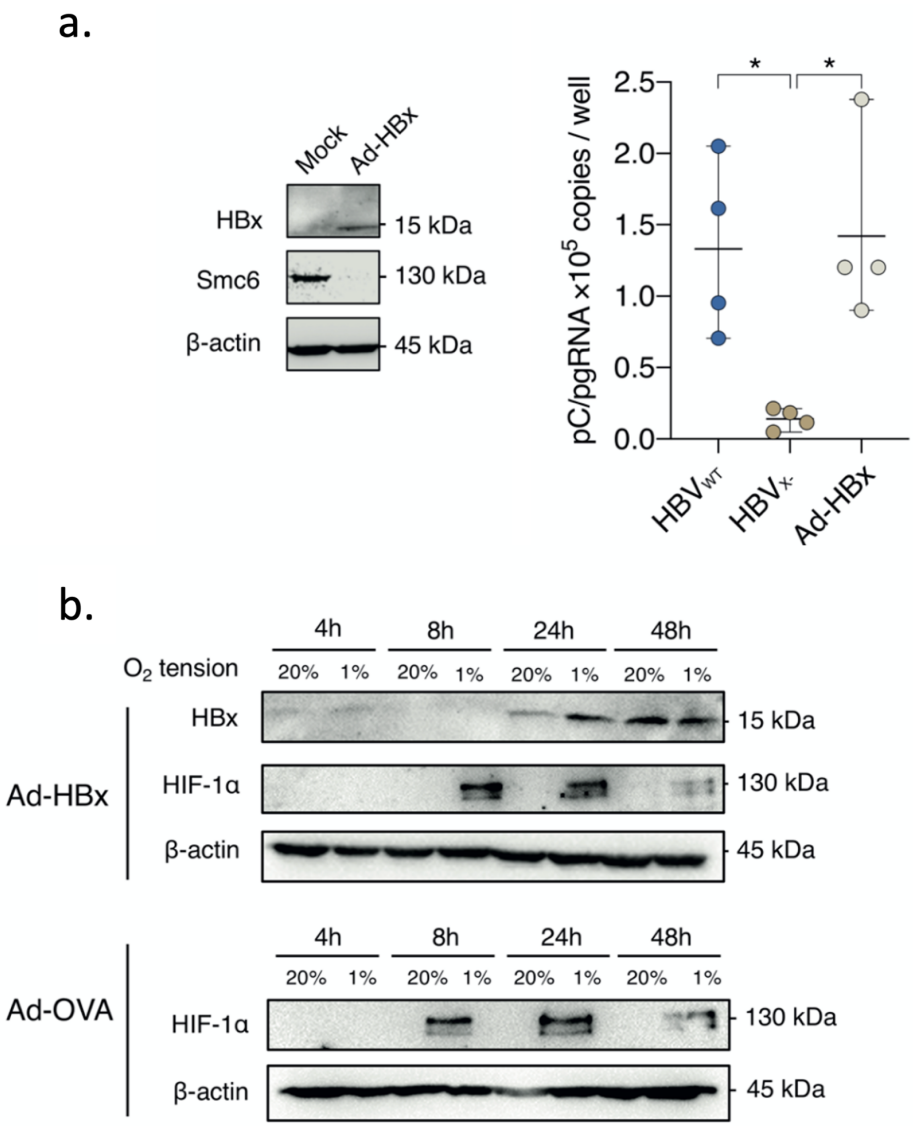


Figure 3.8. Effect of HBx expression on HIF expression and transcriptional activity in HepG2 cells. HepG2-NTCP cells were transduced with Ad-HBx and 24 hours later the cells were probed for HBx and Smc6 expression. In a parallel experiment HepG2-NTCP cells were infected with HBV or a mutated virus lacking HBx (HBV_{X-}) (MOI of 200) in the presence or absence of Ad-HBx and pC/pgRNA measured at 6 days post-infection (a). HepG2-NTCP cells were transduced with Ad-HBx or Ad-OVA and HBx and HIF-1α expression assessed at selected times after culturing at either 18% or 1% oxygen (b). Normality distribution was assessed by D'Agostino-Pearson test; 2-way-ANOVA with Bonferroni correction was applied with $p<0.05$ deemed as significant. Data is representative of results from 3-4 biological repeats.

3.2.4 HBV encoded core and surface antigen proteins have a minimal effect on HIF-α protein expression

The HBV proteins HBcAg and HBsAg have important roles in host factor interaction. HBcAg has been reported to regulate a number of host transcription factors while HBsAg is highly expressed in CHB[343-346]. Therefore, further experiments were conducted to explore whether HBcAg and HBsAg have a role in modulating HIF-α expression. To assess whether HBcAg and HBsAg stabilise HIF-α proteins, HepG2 cells engineered to express HBcAg (HepG2-Core) or L-HBsAg, M-HBsAg, and S-HBsAg (HepG2-LMS) were compared with parental HepG2 cells. HBcAg expression in HepG2-Core cells was validated by western blot and HBsAg expression in HepG2-LMS cells was confirmed with ELISA (Figure 3.9a, Figure 3.9c). Neither HepG2, HepG2-Core, or HepG2-LMS cells expressed HIF-1α, HIF-2α, or CAIX protein when cultured under normoxic conditions (Figure 3.9b, Figure 3.9d). Incubation at 1% oxygen tension stabilised HIF-1α, HIF-2α and CAIX proteins at comparable levels in HepG2, HepG2-Core, and HepG2-LMS cells. These data suggest a minimal role for HBcAg or HBsAg in regulating HIF-1α and HIF-2α expression. Collectively, these results suggest that HBV proteins HBx, HBcAg, and HBsAg have minimal roles in regulating HIFs.

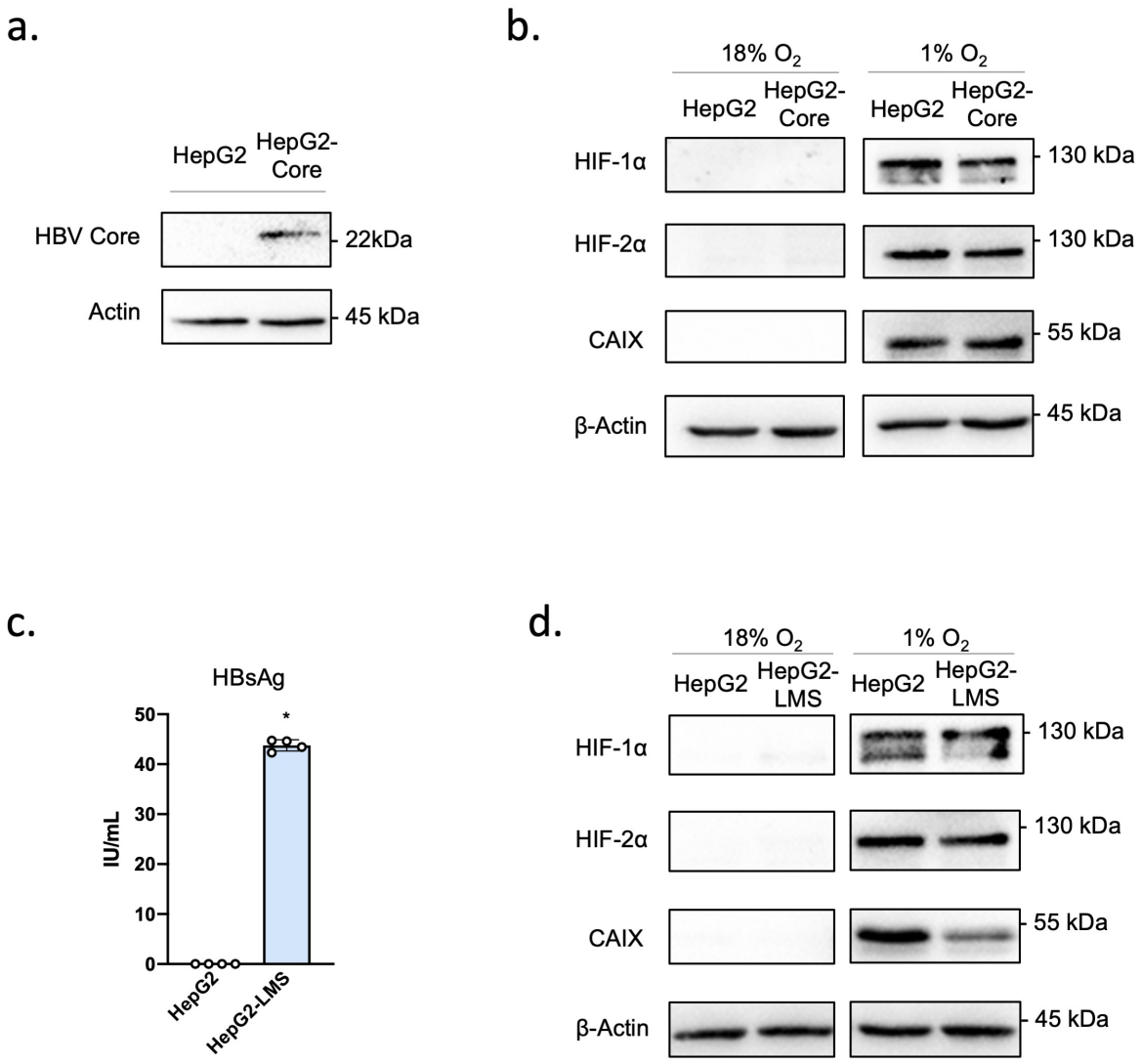


Figure 3.9. HBV core protein (HBcAg) and surface protein (HBsAg) expression in HepG2 cells has a minimal effect on HIF expression or activity. (a) HepG2 cells stably expressing HBcAg (HepG2-Core) and parental HepG2 cells were subject to western blot analysis to confirm HBcAg expression. (b) HepG2 or HepG2-Core cells were incubated at 18% or 1% oxygen for 24 hours and harvested for western blot analysis of HIF-1α, HIF-2α, and CAIX proteins. (c) Supernatants from HepG2-LMS or parental HepG2 cells were assessed for HBsAg by ELISA. Mann Whitney analysis was applied with $p<0.05$ deemed as significant. (d) HIF-1α, HIF-2α, and CAIX proteins were assessed in HepG2 or HepG2-LMS cells under 18% or 1% oxygen. Data is representative of results from 3 biological repeats. Error bar represents standard deviation.

3.2.5 HBV infection of HepG2 cells, primary human hepatocytes, and human liver chimeric mice does not affect HIF target gene expression

To complement the HBx, HBcAg, and HBsAg studies described above, the effect of HBV infection on HIF oxygen sensing pathways was studied in current state-of-art *in vitro* and *in vivo* models. HepG2-NTCP cells were infected with HBV, cultured under normoxic conditions and sampled after 3 and 9 days to assess HIF-1α or HIF-2α expression. HBV replication was confirmed by measuring HBeAg (53.9 ± 2.7 IU/mL) and HBsAg (12.6 ± 4.4 IU/mL), however, no HIF or CAIX expression in normoxic infected or non-infected cells was detected (Figure 3.10a). As a control HepG2-NTCP cells were treated with the PHD inhibitor (FG-4592 at 30 μM) and showed HIF protein expression (Figure 3.10a). Furthermore, analysing a published RNA-seq data from HBV infected primary human hepatocytes showed no evidence of hypoxic gene upregulation (Figure 3.10b)[323]. To further validate these conclusions, chimeric human liver FRGN mice were used to assess whether HBV infection would induce HIF signaling in this model[347]. Female FRGN mice between 8-12 weeks of age were transplanted with 0.5×10^6 cryopreserved adult PHH by intrasplenic injection and monitored for engraftment by measuring serum human albumin levels (at least 0.1mg human albumin per mL in peripheral blood)[347]. Engrafted animals were infected with 0.5 million genome equivalent (GE) copies of HBV per mouse and monitored for HBV replication. Once stable viremia was established (minimum $5x10^7$ GE mL^{-1} of serum) the mice were sacrificed and livers harvested from the infected (n=4) and uninfected (n=3) animals for RNA isolation and RNA-seq. Analysing these RNA-seq data sets showed minimal evidence for an increase in hypoxic transcriptional activity in the HBV infected livers (Figure 3.10b). For comparative purposes, hypoxic genes that were upregulated in the CHB cohort are shown (Figure

3.10b), demonstrating the influence of inflammation on gene regulation and highlighting the limitations of current HBV replication systems to model CHB[320].

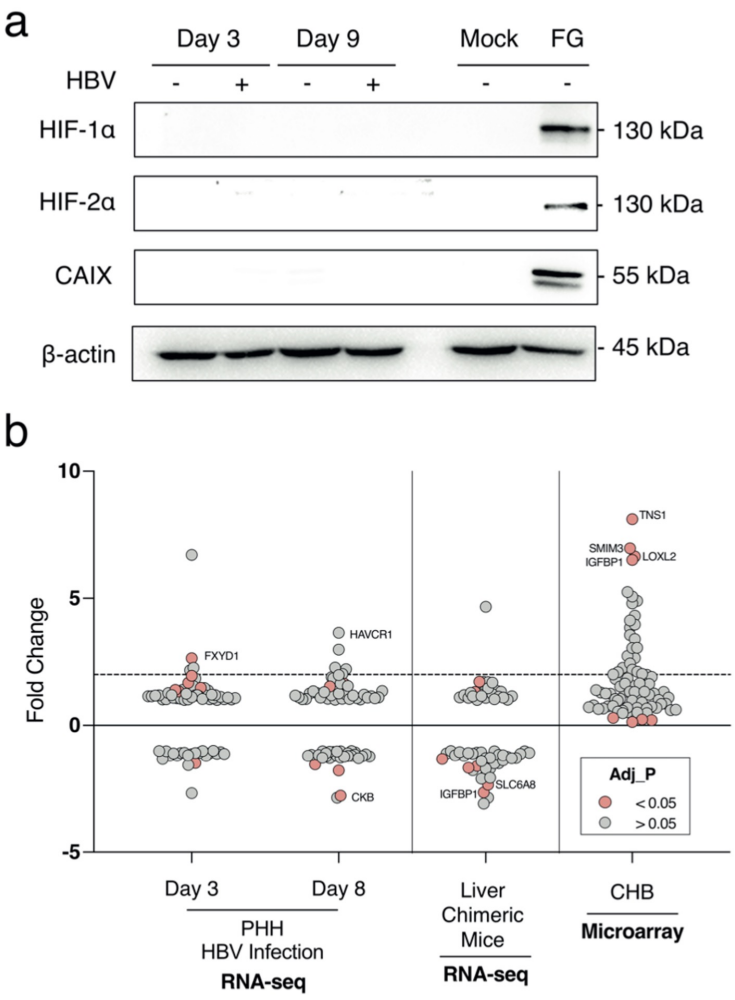


Figure 3.10. Comparing hypoxia gene signatures in HBV infected hepatocytes and humanised liver chimeric mice. Mock or HBV-infected HepG2-NTCP cells (MOI of 200) were harvested after 3 or 9 days, lysed and assessed for HIF-1α, HIF-2α or CAIX expression and the housekeeping gene β-actin by western blotting. As a positive control HepG2-NTCP cells were treated with the HIF PHD inhibitor FG-4592 (FG) at 30μM for 24 hours and protein lysates analysed by western blotting (a). Induction of hypoxic genes in transcriptomic data of HBV infected primary human hepatocytes, HBV infected human liver chimeric mice and a CHB cohort (b). Fold change was calculated for each of the 80 genes in HBV infection against the healthy controls, where the dotted line represents a 2-fold change. For the CHB cohort, fold change was calculated from the raw Affymetrix, differential expression was tested using multiple t-tests and significance determined by p value <0.05. Figure 3.10b analysis conducted with support from Efstathios Giotis, Ana Ortega-Prieto, and Marcus Dorner.

3.3 Discussion

3.3.1 CHB and HBV model systems

Past studies on the interplay between HBV and oxygen sensing mechanisms have almost exclusively focused on the interplay between HBx and HIF. HBV infection is a complex condition which can transition from acute to chronic disease, and whether HIF signaling is upregulated in CHB remains unexplored[7]. Furthermore, it is not known whether full replicating HBV infection can perturb HIF signaling and this chapter examined the role of HBV and viral proteins in regulating HIF.

This chapter identified increased hypoxic gene signatures in a CHB cohort and confirmed hypoxic gene expression using MSigDB derived gene sets and gene signatures from HepG2 and PHH data sets[160,322]. There was an increase in *HIF-1α* mRNA levels in this CHB cohort, consistent with a transcriptional regulation by inflammatory mediators. These results support a model where HBV-associated inflammatory responses induce HIF expression and these complex virus-host interactions are not recapitulated by simple *in vitro* culture systems, HBV transgenic mice or immunodeficient human liver chimeric mouse models.

3.3.2 HBV infection, inflammatory responses, and HIF signaling

As both HBV and HCV infections associate with liver inflammation, this chapter explored hypoxic gene expression in both viral diseases and show clear evidence for increased expression in chronic hepatitis C infection (Figure 3.2b)[348]. Analysing the leading-edge genes from the HBV (n=30) and HCV (n=23) cohorts identified 10 common genes, suggesting some gene specific differences between these chronic infections.

Bioinformatic analysis in this chapter identified 25 hypoxia upregulated gene transcripts in CHB, including lysyl oxidase like 2 (LOXL2), small integral membrane protein (SMIM3), tensin 1 (TNS1), and insulin like growth factor binding protein 1 (IGFBP1). Notably, LOXL2 overexpression in HCC was previously associated with high tumour grade, metastasis, and poor patient overall and disease-free survival[349]. LOXL2 was reported to mediate its pathogenic effects in HCC angiogenesis via vasculogenic mimicry signaling, cytoskeleton reorganization, and bone-marrow derived cell recruitment[349,350]. Hypoxia and HIF-1α signaling were previously reported as key regulators of LOXL2, consistent with observations in this chapter[350,351]. IGFBP1 transcripts were increased in CHB and this gene was recently reported to be regulated by HIF-2α in *in vitro* and *in vivo* model systems[352]. Furthermore, IGFBP1 is a known NF-κB target gene and was induced by HBV infection[353]. These data suggest co-regulation of IGFBP1 by inflammatory pathways including NF-κB and oxygen sensing mechanisms such as HIF signaling, which is consistent with the observation that inflammatory gene enrichment is associated with hypoxia gene signature in CHB.

NF-κB signaling, the NF-κB target TNF-α, and IFN-γ response are among the most upregulated inflammatory pathways in CHB (Figure 3.2c). HBV is known to induce inflammatory mechanisms

that regulate HIF signaling. NF-κB serves an important role in chronic inflammatory response, infection recognition and HIF signaling regulation[354-356]. In NF-κB signaling, the protein IκBα binds NF-κB dimers to inhibit its signaling function while IKKα and IKKβ activate NF-κB through IκBα phosphorylation and catalysis[357]. Host PRRs such as Toll-like receptors have been reported to target pathogens including HBV, induce the NF-κB pathway, and signal for cytokines, chemokines, and immune response related gene expression[358,359]. This understanding aligns with the observation that CHB patients have upregulated inflammatory signaling pathways.

HBV proteins are also known to induce NF-κB. HBx expression can upregulate endogenous host transcription factors, including NF-κB through association with the mitochondrial complex[360,361]. Studies suggest that HBx could disrupt mitochondrial physiology, metabolism, and membrane potential leading to ROS production. Treatment with the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetylcysteine (NAC) abolishes ROS associated NF-κB induction by HBx[360]. HBsAg has also been shown to activate NF-κB activity and induce its targets including TNF-α through interaction with host β2-glycoprotein, phosphorylation of Ser32/36 on IκBα to cause IκBα degradation, and as a result activation of the NF-κB complex[362].

NF-κB is known to induce HIF signaling and promote expression of VEGF and CAIX[356]. In *in vivo* model systems, NF-κB induces *HIF-1α* transcripts in the liver of hypoxic mice[330]. Importantly, NF-κB inactivation due to IKKβ deletion results in reduced HIF-1α protein and VEGF transcripts in mice housed under hypoxia. The presence of a putative NF-κB binding site at 197/188 bp from HIF-1α promoter initiation site provides a potential mechanism for these observations[356]. NF-κB binding to HIF-1α promoter was confirmed through electrophoretic mobility shift assays and chromatin immuno-precipitation (ChIP). NF-κB can also upregulate other HIF protein subunits

including HIF-2α and HIF-1β through the induction of its target gene TNF-α[363]. Genome sequence alignment and *in vitro* studies show that NF-κB modulation of the HIF pathway is evolutionarily conserved across humans, mice, and *Drosophila*[363]. Collectively, studies on the interaction between HBV, NF-κB, and the HIF pathway align with and provide a potential mechanism for the observation that HIF and NF-κB signaling are upregulated in CHB patients.

IFN-γ is another inflammatory signaling pathway that is enriched in CHB patients. Cytokines such as IFN-γ and the NF-κB target TNF-α are important regulators in host response against HBV infection, by inhibiting viral replication and orchestrating immune defense[364,365]. The presence of HBV HBsAg protein induces IFN-γ production by cytotoxic T cells[366]. Treating CHB patients with IFN-γ reduced the severity of liver fibrosis[367,368]. IFN-γ has been shown to activate HIF signaling, by upregulating HIF-1α and the HIF target gene VEGFA in *in vitro* and *ex vivo* experiments[369,370]. IFN-γ induction of ROS which upregulates HIF signaling has been proposed as a mechanism for the observed interactions between these pathways[370].

Infection could induce ROS in the liver which contributes to chronic liver disease and HCC[371,372]. ROS can stabilise HIF under normoxic conditions[373]. Treatment with H_2O_2 and glucose oxidase that generate ROS stabilise HIF-1α and HIF-2α while antioxidants can abolish this effect[373-376]. Three models have been proposed as potential mechanisms for ROS stabilisation of HIF-α: 1) ROS could alter the oxidation state of HIF-α ODDD domain to disrupt VHL-HIF binding and inhibit HIF degradation[377]. 2) ROS could alter the oxidation state of the iron co-factor critical for PHD enzyme function, inhibiting HIF-α hydroxylation and degradation[378]. 3) ROS induction of ERK phosphorylation and the PI3K/AKT pathway leads to upregulation in HIF mRNA transcription[373,377,379,380].

Collectively, the interaction between HBV infection, inflammatory responses and HIF signaling support the observation that the PHD-HIF pathway is induced in CHB. HIF signaling perturbation in CHB is likely mediated by complex and multifaceted mechanisms including NF-kB, IFN-γ, ROS and cellular stress response pathways. Future investigations to unravel precise mechanisms causing HIF functional activity induction in CHB could facilitate discovery of novel targets for the treatment of HBV-associated pathologies.

3.3.3. Transgenic mouse model system for HBV infection

To ascertain whether HBV induced HIF signaling in the transgenic mouse model, the effect of silencing viral RNA and on HIF signaling was investigated *in vivo*. An earlier study showed the efficacy of silencing the integrated HBV genome[82]. Four HIF target genes were selected for analysis in the HBV transgenic mice: CAIX, PHD2, GLUT1, and VEGFA. CAIX is expressed in the liver, specifically regulated by HIF-1 but not HIF-2, and a well documented marker for tumour hypoxia[381-383]; PHD2 is an upstream regulator for HIF degradation but its promoter contains putative HRE for HIF-1 binding[384]. In contrast, GLUT1 and VEGFA are both well documented HIF-1 and HIF-2 target genes[385,386]. Surprisingly, successful silencing of HBV replication had a minimal impact on these gene transcripts, providing evidence that an *in vivo* transgenic mouse HBV model system does not recapitulate upregulated hypoxic gene transcripts observed in CHB patients.

3.3.4 HBx and HIF signaling

Given previous reports that HBx can stabilise HIFs, experiments were conducted to understand whether functionally active HBx could regulate HIF-1α and HIF-2α mRNA, protein and transcriptional activity *in vitro*[226,229,305-307,334-336,342]. There was minimal evidence for HBx regulation of HIFs in three independent model systems: an inducible HepaRG-HBx cell line; an Ad-HBx transduced cell, and in *de novo* infection of HepG2-NTCP or PHHs. These experiments were distinct from earlier reports on at least three counts. Firstly, due to the technical difficulties in visualizing HBx by western blotting or immunofluorescent imaging, many of the earlier studies

did not confirm HBx expression. Secondly, the majority of studies did not validate the functional activity of the expressed HBx protein. Finally, several studies assessed HBx stabilization of HIF-1α using transient plasmid transfection systems with hypoxia reporter constructs, rather than directly measuring HIF expression and target gene activity. Given current knowledge that HBx degrades Smc5/6 which silences episomal DNA transcription, the interpretation of these earlier plasmid based systems is now uncertain[228]. Confirmation of the expression and function of HBx in *in vitro* models and quantification of HIF transcriptional activity under normoxic or hypoxic conditions in this chapter add confidence to the conclusion that HBx does not modulate HIF expression or transcriptional activity in the model systems used.

Guerrieri *et al.* identified and validated a role for HBx in regulating genes involved in endocytosis, predominantly members of the Ras-related in brain (Rab) family[387]. Anti-HBx chromatin immunoprecipitation studies identified HBx binding sites that included RAB1A, RAB2B and RAB5B promoters and none of the validated Rab genes were listed in the hypoxic gene set used in this chapter (Table 3.2). Furthermore, GSEA of the CHB cohort or screening reactome gene sets showed a modest enrichment in the 'Transferrin Endocytosis' pathway (Figure 3.2c), suggesting a minimal overlap between HBx and HIF regulated genes. Furthermore, results from this chapter show that other viral encoded proteins, including HBsAg and HBcAg, had a minimal role in regulating HIF. This is consistent with past clinical observations that HBsAg positivity does not correlate with HIF-1α expression although HIF-1α is associated with tumour size and metastasis of HBV-related HCC[388].

3.3.5 Implications on immunocompetent model systems for studying HBV

In summary, this chapter shows that HIF signaling is activated in CHB patients, but this is not recapitulated in state-of-art *in vitro* or *in vivo* model systems. These differences could be explained by an inability of current systems to model the complexity of CHB. Current *in vitro* and *in vivo* model systems are not able to recapitulate inflammatory signaling and ROS production as potential mechanisms for HIF induction in CHB. Nonetheless, the observation of increased hypoxic gene signature expression in CHB patients offers an important insight on biomarker discovery for early HCC detection. This is in agreement with previous studies that have associated higher HIFα mRNA and protein expression in HCC with worse prognostic outcomes for HCC patients[389-391]. Moreover, as the liver harbours a natural physiological low oxygen environment, future investigations exploring how oxygen sensing pathways regulate HBV replication and pathogenesis may identify novel therapeutic targets.

To better understand the interplay between HBV infection, inflammatory mechanisms, and HIF signaling, future studies could use a syngeneic immunocompetent model system for studying HBV infection[84,392]. A number of potential model systems can be explored including the woodchuck hepatitis virus (WHV), immunocompetent HBV mice, and immune cell co-cultures. WHV is related to HBV in terms of genetic and virological traits and WHV infection in woodchucks results in similar immune response and natural disease history to HBV infection in humans. However, the WHV life cycle also has distinctions compared to HBV. For example, while woodchuck hepatocytes infected with WHV contains 1 to 50 copies of cccDNA per cell, HBV infected hepatocytes from HBV patients have a greater range of cccDNA per cell, from 0.035 to 195 copies with a median value of 1.5 copies per cell[393,394]. In addition, propagating woodchucks as a model

system is a lengthy process that requires significant technical expertise. In contrast, immunocompetent mice models that are HBV tolerant might be more readily established with a more accurate representation of the HBV life cycle in humans, but would require specific technical expertise[395]. However, studies suggest a high level of viral replication might only be maintained for a short period and could be cleared as early as seven to fourteen days in a immunocompetent HBV mouse model[83,396]. Therefore, an immunocompetent HBV mouse model might not accurately recapitulate CHB and immune response in humans. Unlike the woodchuck and immunocompetent mouse model systems, an immune cell co-culture model system could be a more cost-effective method to study the interaction between HBV and specific immune cells of interest. However, such simple *in vitro* model systems may not fully recapitulate complexity underlying the interplay between the immune system and HBV in CHB[109,397].

3.4 Summary

Existing literature has largely focused on the role of the HBV protein HBx in stabilising HIF. A number of clinical investigations suggest that HIF expression could be associated with worse outcomes for HBV associated pathologies such as cirrhosis and HCC[389-391]. Therefore, this chapter explored the role of HBV in regulating HIF signaling.

Highlights of findings from this chapter include the following:

- In a cohort of CHB patients, HIF target genes and various inflammatory pathways including NF-κB, TNF-α, and IFN-γ were significantly upregulated. However, siRNA silencing of HBV replication in HBV transgenic mice had a minimal effect on HIF signaling.
- Further validation with chimeric human liver FRGN mouse model with HBV infection, published RNA-seq data from HBV infected PHH, and HBV *de novo* infection in HepG2-NTCP cells show minimal evidence of HIF signaling induction by HBV infection.
- *In vitro* studies show that the HBV protein components HBx, HBsAg, and HBcAg do not perturb HIF signaling.

This chapter and various clinical studies have explored the impact of HBV infection on HIF signaling and pathogenesis. The physiological hypoxic environment in the liver and evidences suggesting the stabilisation of HIF in CHB from this chapter raise a number of questions on whether HIF has a role in regulating HBV replication. In particular the following three questions have not been addressed and will be explored in remaining chapters:

1) Does HIF have a role in regulating HBV replication?
2) Does low oxygen tension have a role in regulating HBV replication?
3) Do other oxygen sensing mechanisms have a role in regulating HBV replication?

4. A ROLE FOR HIFS IN REGULATING HBV REPLICATION

4.1 Introduction

Work from the previous chapter shows that HIF signaling is induced in chronic HBV infection but this observation cannot be recapitulated in state-of-art HBV *de novo* infection, transgenic mouse, and chimeric liver mouse model systems[304]. Past studies on the interplay between HBV, hypoxia, and the PHD-HIF pathway have largely focused on the effect of HBV on this signaling pathway and related pathologies such as HCC development[175]. However, little is known whether the PHD-HIF pathway regulates the HBV life cycle. Previous studies have reported on the differential expression of genes in the mouse that associate with liver zonation and local oxygen tension[116,117]. In particular single-cell RNA-seq demonstrates that hypoxia response genes are differentially expressed from the perivenous to pericentral region of the liver, where oxygen tension changes from 8% to 4%, respectively[116]. Furthermore, viral infection and inflammatory responses are known to activate HIF signaling[178-180]. Collectively, these observations suggest that oxygen sensing mechanism is active in the inflamed liver and could regulate HBV replication.

This chapter explores the role of PHD-HIF signaling in HBV replication. Discovery of two evolutionarily conserved HREs in the HBV genome prompted *in vitro* experiments to explore the role of HIFs on HBV transcription. Further siRNA HIF silencing experiments confirm a role for HIF-1 in regulating HBV transcription. Since the HIF-1α binding partner HIF-1β can also dimerise with aryl hydrocarbon receptor (AhR) and the AhR binding site xenobiotic-responsive element (XRE) is present in the HBV genome, experiments were conducted to assess the role of AhR-HIF-1β signaling pathway on HBV transcription. Finally, siRNA silencing of HIF-1β in a HBV

transgenic mouse model provides further evidence to support a positive role for HIFs in regulating HBV replication.

4.2 Results

4.2.1 Pharmacological inhibition of HIF prolyl hydroxylase positively regulates HBV transcription *in vitro*

Two HREs were identified in the BCP region of the HBV genome upon screening over 7,000 HBV sequences in a public database: located within EnhI (1236-1240) and in the antisense strand between EnhI and EnhII (1605-1599) (Figure 4.1)[325]. These HREs are also present in HBV sequences dating back to the Bronze age and medieval period, sequenced from samples ranging from 7,100 to 200 years old[398]. Further analysis of closely related *hepadnaviridae* HBV lineages shows that both HRE-1 and HRE-2 are conserved in viruses infecting humans, higher primates, and woodchucks with the exception of HBV genotype H. In contrast, viruses infecting monkey, bat, rodent, and bird lack these HRE motifs. These data suggest an evolutionary conserved role for HIFs in regulating human HBV and prompted investigations to understand the role of PHD in HBV regulation.

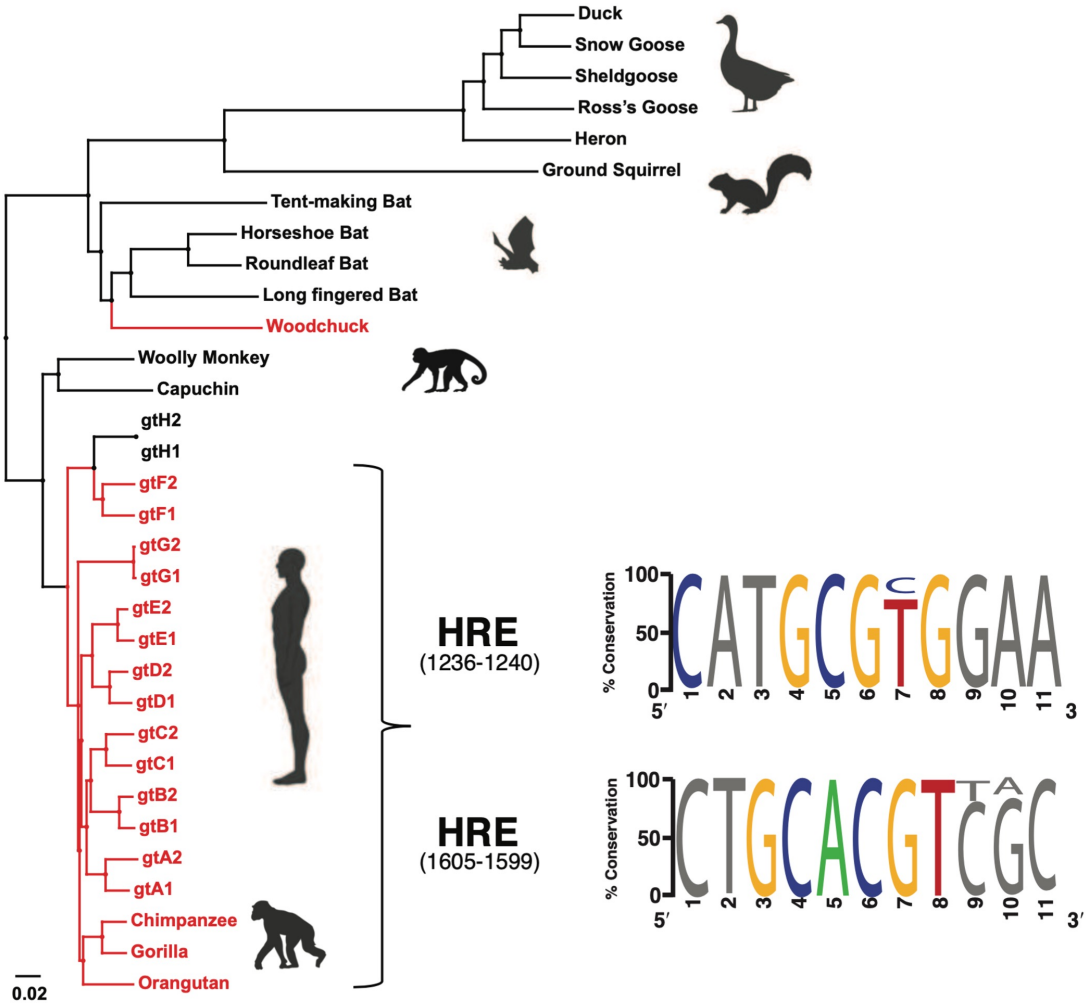


Figure 4.1. **Conservation of HREs amongst diverse HBV lineages.** Conservation of two hypoxia responsive elements (HREs), HRE-1 at position 1236-1240 on the sense strand and HRE-2 at 1605-1599 on the antisense strand, were assessed amongst 7,313 HBV sequences in the HBV database and consensus plots generated. NCBI referent full-length *hepadnaviridae* genome sequences were aligned and a neighbor-joining tree constructed. Branches in red represent HRE containing genomes. Analysis by Peter Balfe.

To understand whether the PHD-HIF pathway has a role in regulating HBV transcription, HepG2 liver hepatoma cells were treated with the PHD inhibitor FG-4592 and showed induction of both HIF-1α and HIF-2α expression (Figure 4.2a). To investigate whether FG-4592 regulates viral transcription, HepG2 cells were transfected with a HBV promoter-luciferase construct encoding firefly luciferase under the control of EnhI/II and the BCP (HBV-Luc) (Figure 4.2b)[316]. There is a significant increase in HBV-Luc activity with FG-4592 treatment at 24 hours and further time-dependent significant increase by 48 hours and 72 hours, suggesting a role for the PHD-HIF pathway in reregulating HBV transcription. As a positive control, FG-4592 treatment of HepG2 cells transfected with pGL3-HRE constructs with a luciferase reporter induced promoter activity, consistent with HBV-Luc results (Figure 4.2b). These results demonstrate that PHD inhibition regulates HBV BCP promoter activity in a transient transfection model system.

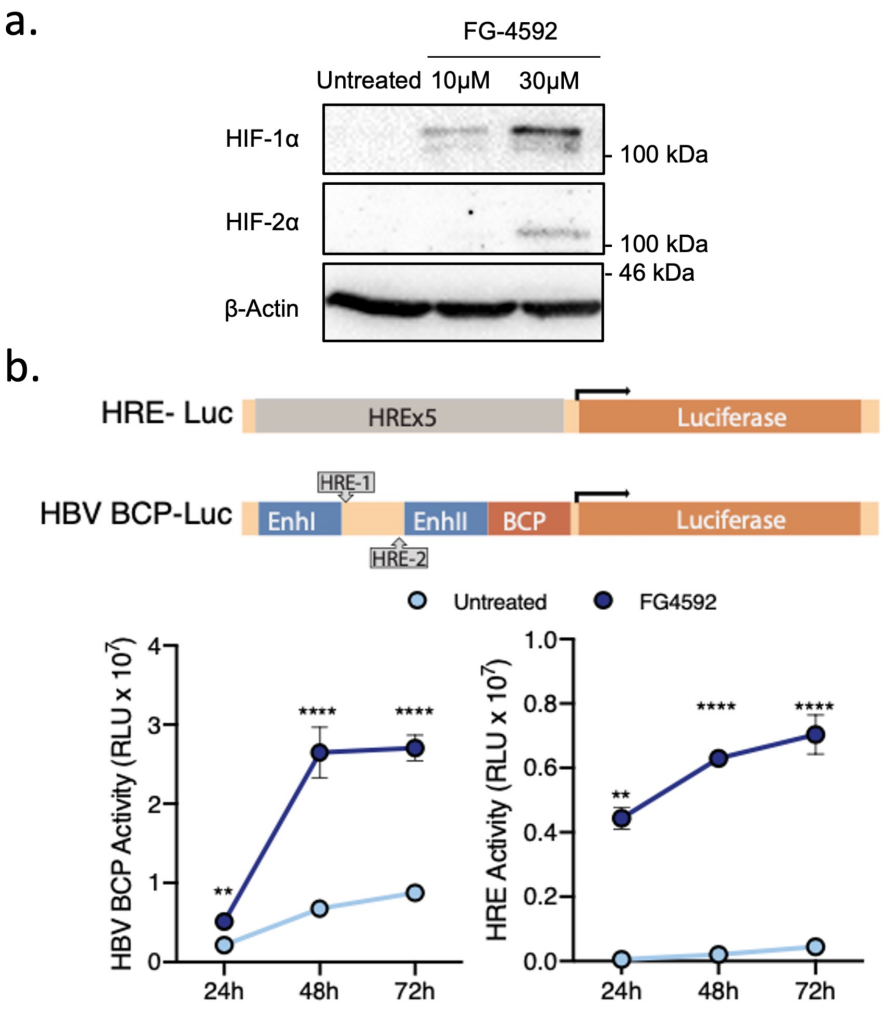


Figure 4.2. FG-4592 inhibition of PHD promotes HBV promoter activity. HepG2-NTCP cells expressing a pGL3-HRE or the HBV EnhI/II and basal core promoter construct driving luciferase (HBV-Luc) were treated with PHD inhibitor FG-4592 (30μM), induction of HIF-1α and HIF-2α expression confirmed by western blot (a) and promoter activity assessed at 24 hour intervals for 72 hours (b). Statistical analysis was performed using a 2-Way ANOVA (**p⩽0.01, ****p⩽0.0001). Data is representative of results from 3 biological repeats. Error bar represents standard deviation. Luciferase assay conducted with support from Nicholas Frampton.

To evaluate whether PHD inhibition regulates authentic HBV transcription, two *in vitro* model systems were selected: HepG2 cells with episomal replicating copies of HBV (HepG2-HBV-pEpi) and HepG2 cells expressing NTCP that support HBV replication[327]. HepG2-HBV-pEpi cells were treated with 30 μM FG-4592 for 72 hours (Figure 4.3a). FG-4592 significantly upregulated transcription of the HBV BCP regulated pC/pgRNA. As positive controls, FG-4592 induced transcription of the HIF target gene carbonic anhydrase IX (*CAIX*). In an independent system, HepG2 cells expressing NTCP (HepG2-NTCP) were infected with HBV (Figure 4.3b). Treatment with FG-4592 significantly increased pC/pgRNA levels, as well as transcripts of the positive control gene *CAIX*, consistent with results in the HepG2-HBV-pEpi model system. These data show that PHD inhibition regulates HBV transcription in episomal and *de novo* viral infection systems.

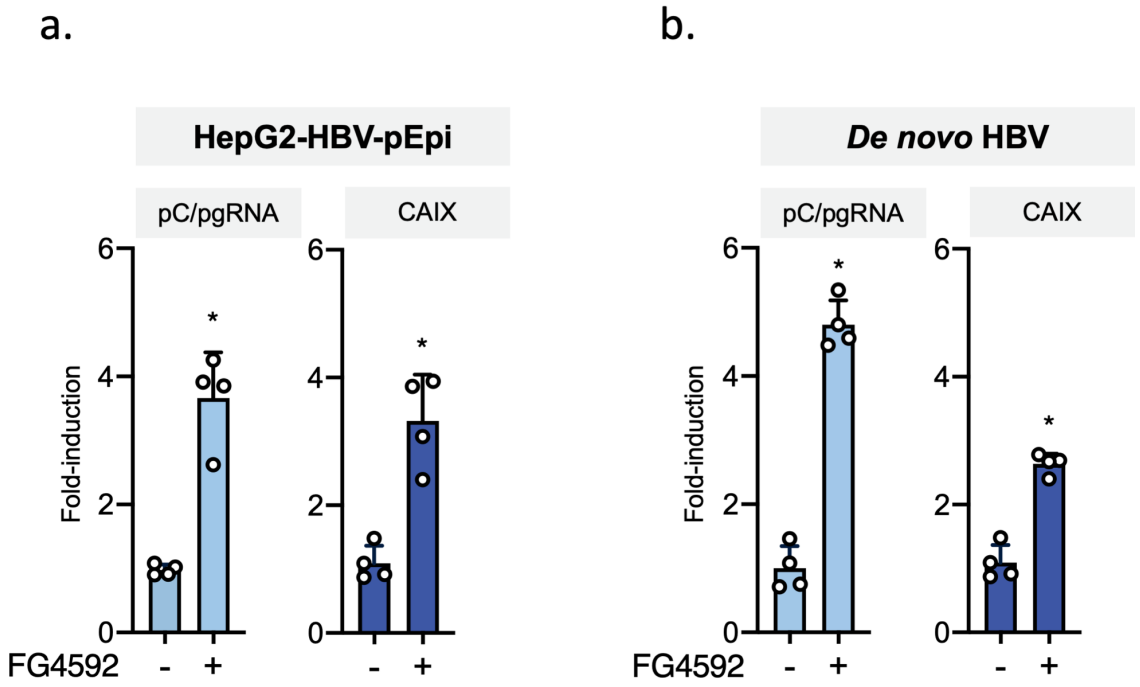


Figure 4.3. FG-4592 inhibition of PHD regulates HBV transcription activity. (a) HepG2 cells supporting episomal copies of HBV DNA (HepG2-HBV-pEpi) were treated with FG-4592 (30μM) for 72 hours. HBV pC/pgRNA and host gene transcript *CAIX*, were quantified by qPCR. (b) HBV infected (MOI 200) HepG2-NTCP cells were treated with FG-4592 (30μM) and pC/pgRNA and *CAIX* expression quantified 72 hours post-infection. Transcript data is shown relative to untreated control samples. Statistical analysis was performed using Mann-Whitney analysis (*$p \leqslant 0.05$). Data is representative of results from 4 biological repeats. Error bar represents standard deviation.

To model HBV infection and quantify HBV protein expression *in vitro*, DMSO is commonly used to arrest HepG2 cells at the G1 phase and induce liver transcription factors to facilitate HBV replication[399]. However, HBV infected DMSO arrested HepG2-NTCP cells were not responsive to FG-4592 treatment and showed no increase in pC/pgRNA (Figure 4.4a). In contrast, transcripts of the host HIF target genes *CAIX* and *VEGFA* were significantly upregulated regardless of DMSO treatment (Figure 4.4b, Figure 4.4c). These observations show that DMSO blunts FG-4592 induction of HBV transcription but had a modest effect on the host HIF target genes tested.

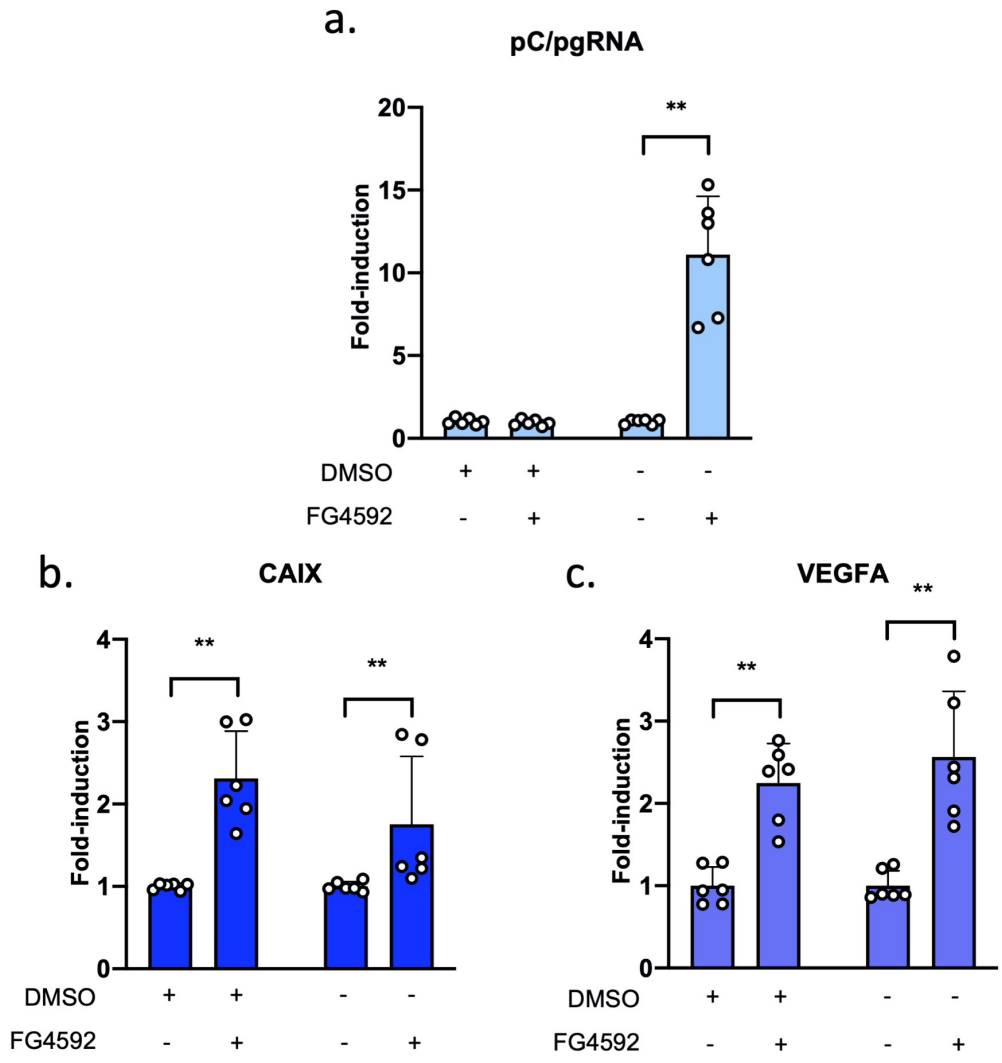


Figure 4.4. DMSO blunts FG-4592 induction of HBV transcription. DMSO (2.5%) treated HepG2-NTCP cells were infected with HBV (MOI 200) for 6 hours and treated with FG-4592 (30μM); pC/pgRNA (a), *CAIX* (b) and *VEGFA* (c) transcripts were quantified 72 hours later. Transcript data is shown relative to untreated control samples. Statistical analysis was performed using Mann-Whitney analysis (**p ⩽0.01). Data is representative of results from 6 biological repeats. Error bar represents standard deviation.

4.2.2 HIF-1α and HIF-2α positively regulate HBV transcription *in vitro*

To further dissect the role of HIF-1α, HIF-2α and HIF-1β in regulating HBV transcription, HBV infected HepG2-NTCP cells were transfected with control siRNA or siRNA against HIF-1α, HIF-2α, or HIF-1β and treated with FG-4592 (Figure 4.5a). Western blot analysis confirmed successful silencing of HIF-1α, HIF-2α, or HIF-1β in a target specific manner (Figure 4.5b, Figure 4.5c). Silencing HIF-1α significantly reduced FG-4592-dependent increase in pC/pgRNA, whereas silencing HIF-2a had a modest non-significant effect (Figure 4.5d). Interestingly, combined silencing of both HIF-1α and HIF-2α led to further downregulation of FG-4592 induced pC/pgRNA. Consistent with this result, silencing HIF-1β, which can heterodimerise with both HIF-1α or HIF-2α to form a functional HIF domain, ablated the FG-4592 dependent increase in pC/pgRNA levels. The HIF-1 target gene *CAIX* responded accordingly, showing a significant reduction by HIF-1α or HIF-1β silencing but no effect of HIF-2α silencing (Figure 4.5e). As *VEGFA* is co-regulated by HIF-1α and HIF-2α, its transcript level in response to HIF silencing was tested. Silencing HIF-1α, HIF-2α or HIF-1β reduced VEGFA transcripts that were induced by FG-4592 (Figure 4.5f).

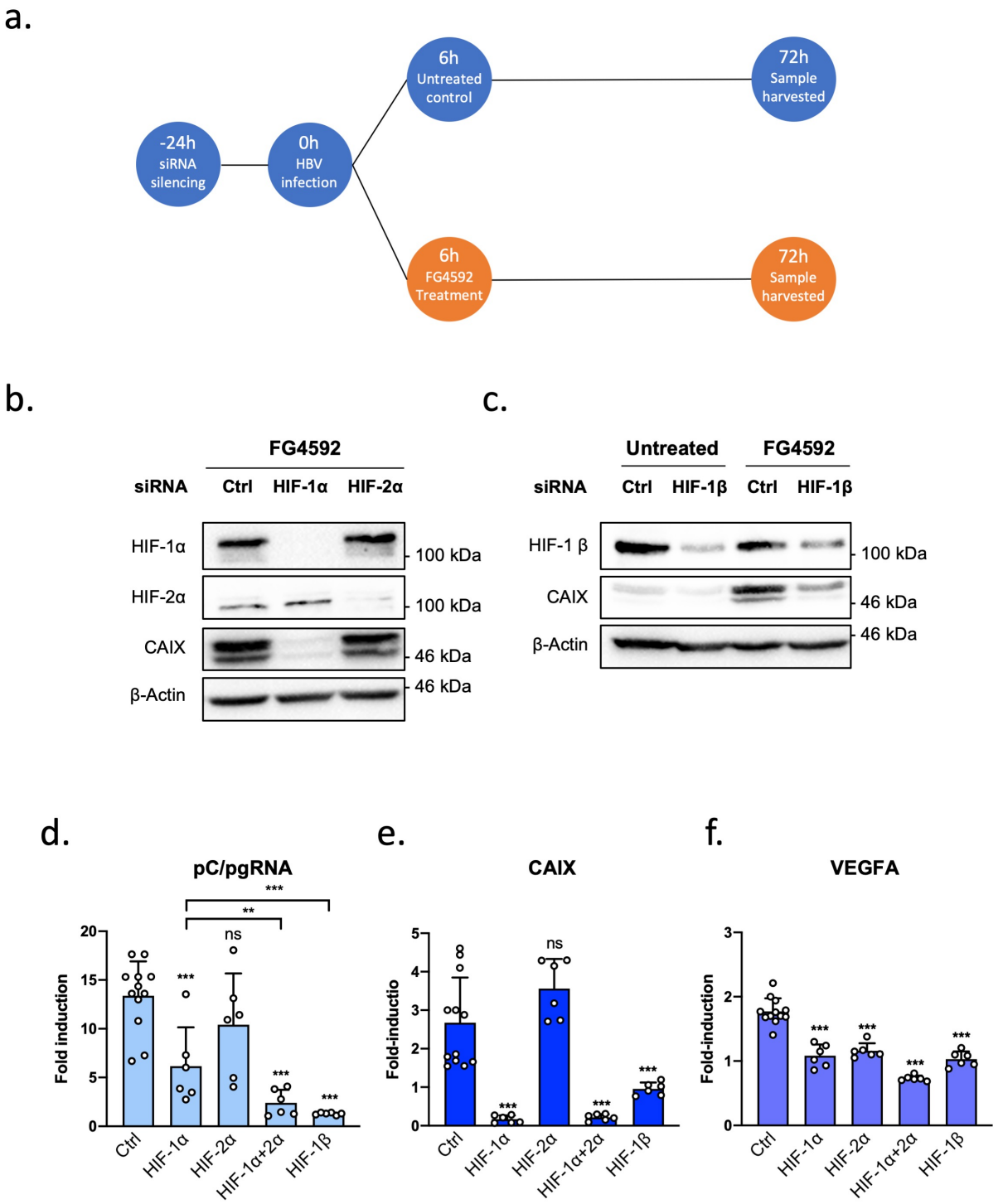


Figure 4.5. HIFs regulate HBV transcription. Figure legend on the following page.

Figure 4.5. HIFs regulate HBV transcription. (a) schematic illustration of experimental procedure. Briefly, siRNAs targeting HIF-1α, HIF-2α, or HIF-1β were delivered into HBV infected HepG2-NTCP cells and treated with FG-4592 (30μM) for 72 hours. SiRNA silencing was confirmed by western blot for HIF-1α, HIF-2α, HIF-1β, and CAIX protein expression (b,c). Transcription of HBV pC/pgRNA (d), HIF-1α dependent target gene *CAIX* (e) and HIF-1α and HIF-2α dependent target gene *VEGFA* (f) were quantified by qPCR. Data is presented as fold induction relative to control untreated cells. Statistical test was performed using One-Way ANOVA followed by Tukey's multiple comparison analysis comparing Ctrl with other treatment groups unless otherwise indicated (**$p \leqslant 0.01$, ***$p \leqslant 0.001$). Data is representative of results from at least 6 biological repeats. Error bar represents standard deviation.

Silencing HIF-2α had a modest effect on pC/pgRNA transcripts in HBV infected HepG2-NTCP cells. A HIF-2α specific inhibitor, PT2385 that was recently assessed for ccRCC treatment through a clinical trial, was used to extend these studies and to understand a role for HIF-2α in regulating pC/pgRNA levels[400]. Treating HepG2-HBV-pEpi cells or HBV infected HepG2-NTCP cells with PT2385 in the presence or absence of FG-4592 showed a modest effect of the HIF-2α inhibitor on viral transcripts (Figure 4.6a, Figure 4.6b). As controls, FG-4592 dependent induction of *VEGFA* mRNA was reduced by PT2385 treatment while mRNA levels of the HIF-1 specific target gene *CAIX* did not change (Figure 4.6a, Figure 4.6b). These observations are consistent with the siRNA silencing results and suggest a modest role for HIF-2α in regulating HBV transcription. Collectively these results suggest that HIF-1α has a dominant role in regulating HBV transcription while HIF-2α plays a supplementary role.

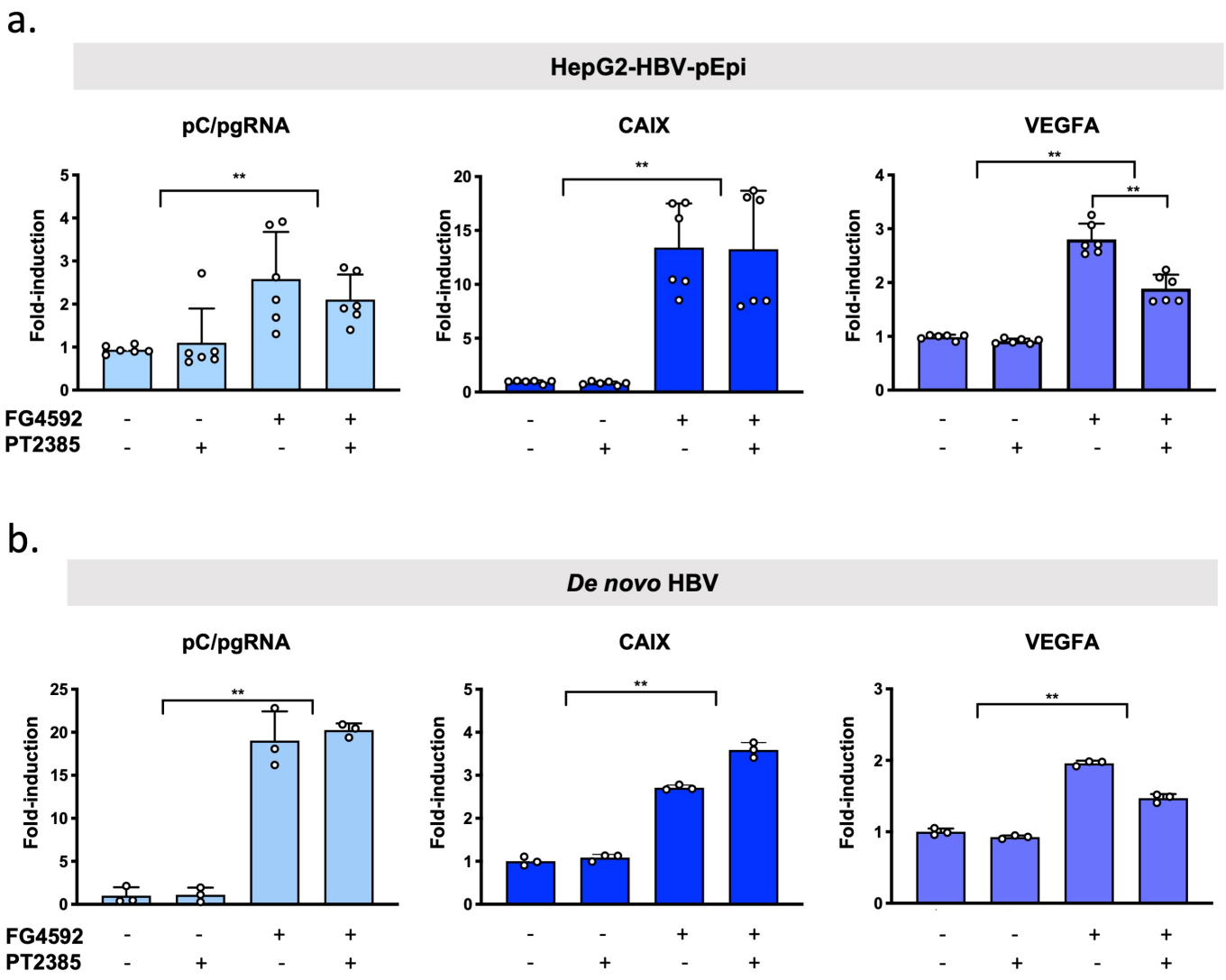


Figure 4.6. PT2385 has a minimal effect on HBV transcription. (a) HepG2 cells supporting episomal copies of HBV DNA (HepG2-HBV-pEi) or (b) HBV infected HepG2-NTCP cells were treated with FG-4592 (30μM) ± PT2385 (10 μM) for 72 hours and pC/pgRNA along with host genes *CAIX* and *VEGFA* transcripts quantified by qPCR. Data is presented as fold induction relative to the untreated control cells. Statistical analysis was performed using Mann-Whitney test (**$p \leqslant 0.01$). Panel (a) data is representative of results from 6 biological repeats and panel (b) data is representative of results from 3 biological repeats. Error bar represents standard deviation.

4.2.3 Minimal role for AhR-HIF-1β in regulating HBV transcription

HIF-1β is known to dimerise with AhR and bind XRE to regulate target genes in addition to binding to the oxygen sensitive HIF-1α and HIF-2α subunits[401]. A conserved XRE motif was identified at position 1235-1240 of the HBV genome (Figure 4.7a). To understand if the greater reduction in pC/pgRNA from HIF-1β siRNA silencing compared to HIF-1α/2α silencing resulted from an AhR-dependent mechanism, HBV infected HepG2-NTCP cells were treated with the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and antagonist BAY213. TCDD induced transcription of the AhR target *CYP1A1* in a dose-dependent manner and this effect was abolished by BAY213 co-treatment, demonstrating a functional AhR signaling pathway is present in HepG2 cells (Figure 4.7b)[402]. TCDD and BAY213 treatment had no effect on HBV pC/pgRNA or total RNA levels, suggesting a minimal role for the AhR-HIF-1β pathway in regulating HBV transcription (Figure 4.7c, Figure 4.7d).

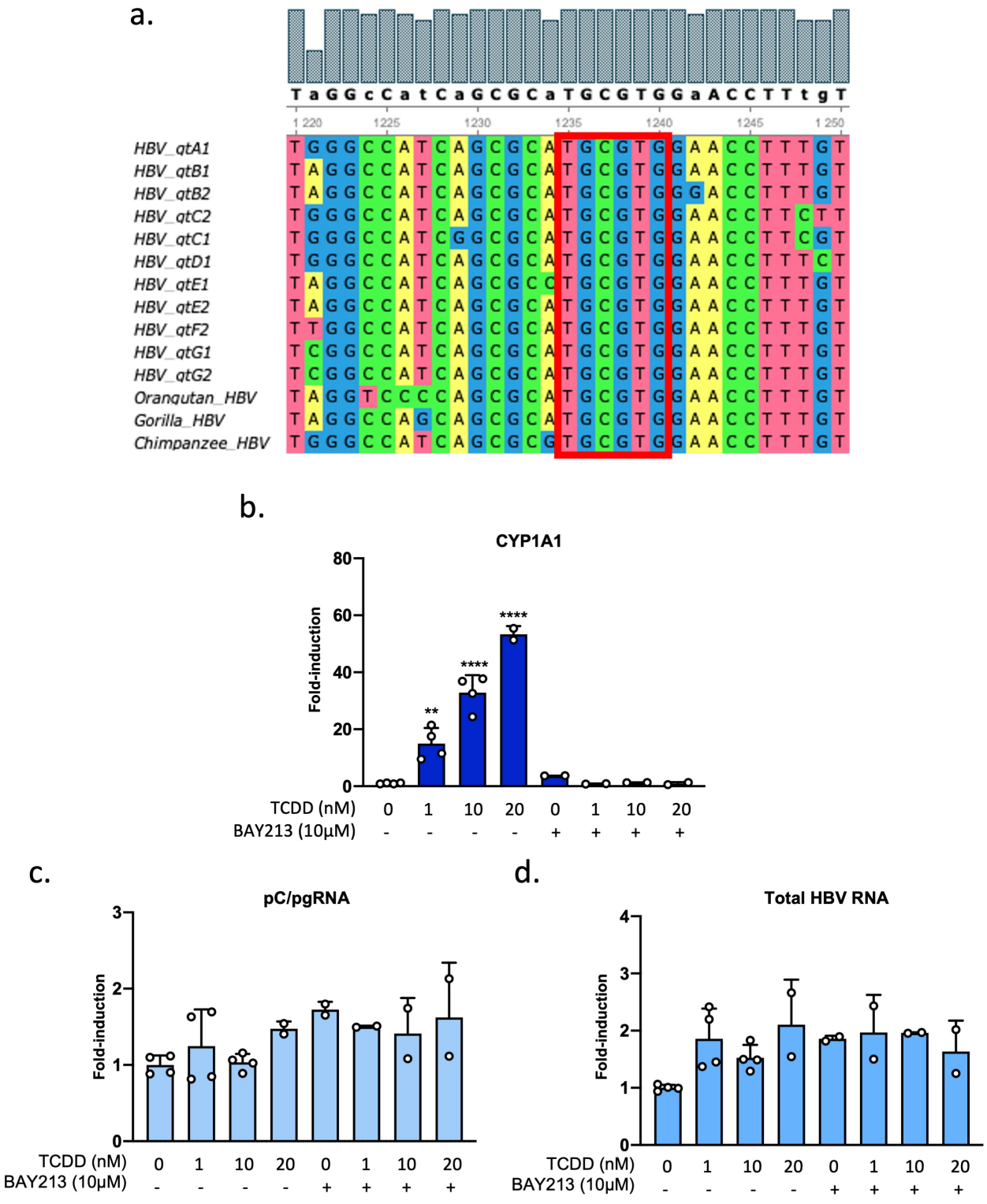


Figure 4.7. Effect of AHR-HIF-1β agonist and antagonist on HBV transcription. Figure legend on the following page.

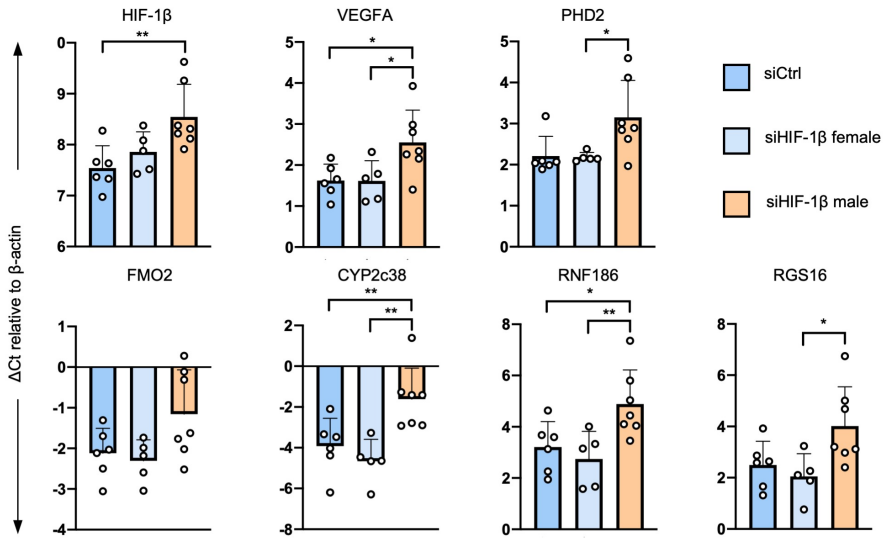
Figure 4.7. Effect of AHR-HIF-1β agonist and antagonist on HBV transcription. (a) Illustration of the conserved xenobiotic response element (XRE) motif identified at position 1235-1240 of the HBV genome through alignment of NCBI referent full-length *hepadnaviridae* genome sequences. HBV infected HepG2-NTCP cells were treated with the AHR-HIF-1β agonist TCDD at the indicated doses ± the AHR-HIF-1β antagonist BAY213 (10 μM) for 72 hours. Transcript levels of the host AHR-HIF-1β target CYP1A1 (b), pC/pgRNA (c) and total HBV RNA (d) were quantified by qPCR. Data is presented as fold induction relative to the untreated control for each condition. Statistical test was performed using a One-Way ANOVA followed by Tukey's multiple comparison analysis (*p⩽0.05, **p⩽0.01, ***p⩽0.001, ****p⩽0.0001). Data is representative of results from 2 to 4 biological repeats. Error bar represents standard deviation.

4.2.4 HIF-1β siRNA silencing in HBV transgenic mouse: effect on host gene expression

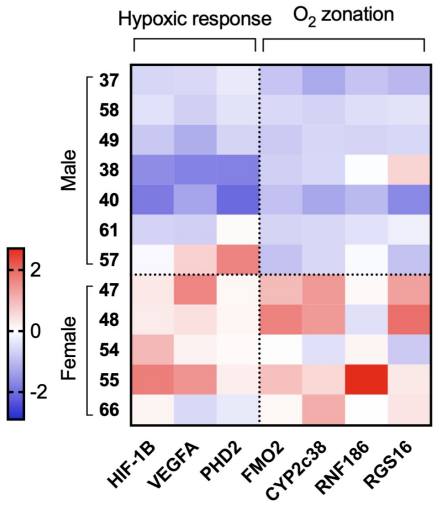
As *in vitro* results suggest that HIFs have a role in regulating HBV transcription, a transgenic C57BL/6J mouse model with 1.3-fold overlength HBV genome integrated in host genome was utilised to validate this observation *in vivo*. Male and female mice between 12-15 weeks old were treated with siRNA targeting HIF-1β (siHIF-1β) or control siRNA (siCtrl) complexed with Invivofectamine 3.0 reagent through tail vein injection at 1μg/g body weight. Halpern *et al.* identified a panel of oxygen responsive genes that were differentially regulated in the mouse liver, as oxygen tension changes across a gradient from the perivenous to the pericentral regions[116]. To validate the efficacy of HIF-1β silencing and its functional implications, relevant transcript levels were assessed using qPCR including *HIF-1β*, the HIF targets *VEGFA* and *PHD2*, and a panel of oxygen responsive genes, including dimethylaniline monooxygenase [N-oxide-forming] 2 (*FMO2*), cytochrome P450 2C38 (*CYP2c38*), E3 ubiquitin-protein ligase RNF186 (*RNF186*), and regulator of G-protein signaling 16 (*RGS16*) (Figure 4.8a). There is no significant difference in ΔCt values for *HIF-1β*, *VEGFA*, *PHD2*, *FMO2*, *CYP2c38*, or *RNF186* between siCtrl and siHIF-1β female mice, suggesting that female mice did not respond to HIF-1β silencing (Figure 4.8a). In contrast, ΔCt values for *HIF-1β*, *VEGFA*, *PHD2*, *FMO2*, *CYP2c38*, or *RNF186* are significantly different between siCtrl and siHIF-1β male mice (Figure 4.8a). To better illustrate and compare differences in HIF-1β silencing response between male and female mice, transcript levels were normalised to Z-scores and illustrated as a heatmap (Figure 4.8b). *HIF-1β* reduction in male mice (mouse number 37, 58, 49, 38, 40, 61, and 57) corresponds with downregulation in transcripts of the HIF target genes *VEGFA* and *PHD2*, as well as the mouse hepatocyte low oxygen response genes *FMO2*, *CYP2c38*, *RNF186*, and *RGS16*. In contrast, female mice (mouse number 47, 48, 54, 55, and 66) showed minimal response to HIF-1β silencing and failed to show any uniform

reduction in HIF target and low oxygen response genes. Further statistical analysis shows a correlation between *HIF-1β* and *VEGFA*, *PHD2*, *FMO2*, *CYP2c38*, *RNF186*, or *RGS16*, suggesting that HIF target and hypoxia responsive gene transcripts align with HIF-1β transcript levels in the liver (Figure 4.8c). These data suggest that HIF-1β siRNA silencing was effective in male mice but ineffective in female mice. Therefore, results from female mice could be used as a comparison to help facilitate investigations on the effect of HIF-1β silencing on HBV replication in HBV transgenic mice.

a.



b.



c.

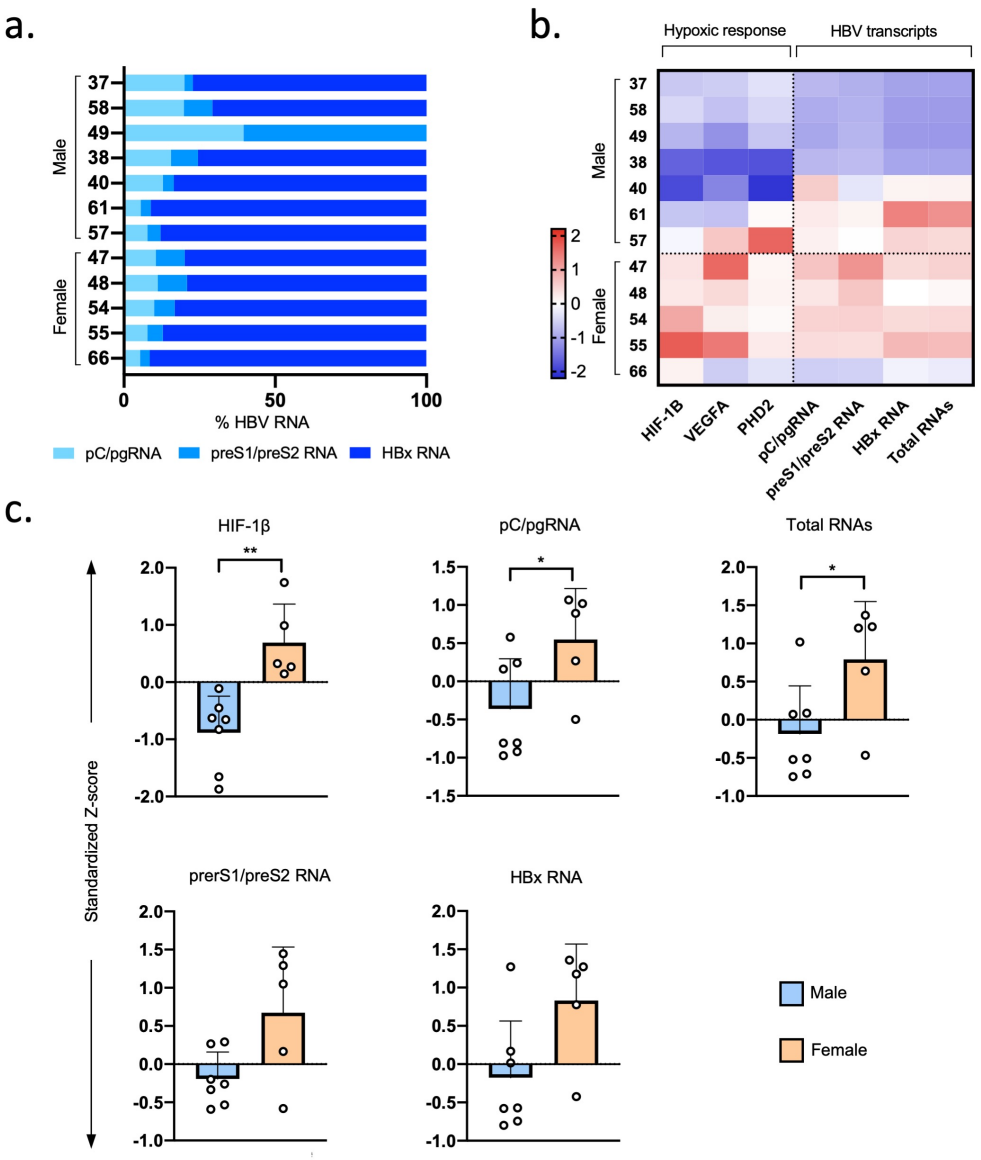
Transcript name	Spearman's rank correlation coefficient	P value
VEGFA	0.8163	<0.0001
PHD2	0.7564	0.0001
FMO2	0.6202	0.0030
CYP2c38	0.6381	0.0022
RNF186	0.5555	0.0083
RGS16	0.4283	0.0381

Figure 4.8. Effect of siRNA HIF-1β silencing in HBV transgenic mice on host genes. Figure legend on the following page.

Figure 4.8. Effect of siRNA HIF-1β silencing in HBV transgenic mice on host genes. SiRNAs targeting HIF-1β (siHIF-1β) or control siRNA (siCtrl) were delivered into male or female HBV1.3 transgenic mice and animals culled after 1 week. RNA was isolated from liver biopsies and transcript levels of HIF-1β along with oxygen sensitive host genes quantified with qPCR. (a) ΔCt values relative to β-actin for transcripts of interest in siCtrl, siHIF-1β female, or siHIF-1β male groups are presented. High ΔCt values correspond to decreased relative gene expression levels while low ΔCt values represent increased relative gene expression levels. Statistical analysis was performed using Mann-Whitney test (*p⩽0.05, **p⩽0.01). Error bar represents standard deviation. (b) The amount of each transcript was normalised (Z-score) and presented as a heat map. Blue represents decreased relative gene expression levels while red represents increased relative gene expression levels. (c) Spearman's rank order correlation statistical analysis was conducted for each oxygen sensitive host gene transcript compared to HIF-1β transcript including *VEGFA, PHD2, FMO2, CYP2c38, RNF186*, and *RGS16*, with correlation coefficient and P value presented. Thomas Michler supported and conducted siRNA delivery into mice.

4.2.5 HIF-1β siRNA silencing in HBV transgenic mouse: effect on HBV replication

Upon validating the impact of siRNA silencing of HIF-1β in a transgenic mouse model, quantification of HBV transcripts pC/pgRNA, preS1/preS2 RNA and HBx RNA was conducted by qPCR. Most mice showed similar proportions of HBV transcripts. HBx RNA was the dominant transcript accounting for 70-91% of total HBV RNA, with the exception of mouse 49 where HBx RNA was undetectable (Figure 4.9a). Other HBV transcripts including pC/pgRNA accounted for 5-20% of total HBV RNA and preS1/preS2 RNA for 3-10% with the exception of mouse 49. Four out of six male mice with effective HIF-1β silencing showed reduced HBV transcripts (Figure 4.9b). Male mouse 57 showed less effective HIF-1β silencing, which is further validated by its higher *VEGFA* and *PHD2* mRNA levels. Consistent with this result, HBV transcripts were not downregulated in mouse 57. In contrast, female mice, which showed ineffective HIF-1β silencing showed no change in HBV transcripts with the exception of mouse 66. Female mouse 66 showed a modest reduction in HBV transcripts and its *HIF-1β, VEGFA* and *PHD2* transcript levels were the lowest among all female mice. Further statistical analyses comparing *HIF-1β*, pC/pgRNA or total HBV RNAs between male and female mice show a significant decrease in these RNA quantities in males where HIF-1β siRNA silencing was effective (Figure 4.9c). Although preS1/preS2 RNA or HBx mean RNA values were reduced in male mice compared to females, the limited number of animals and heterogeneity within the model limits the statistical power of any analysis. Given the variable levels of HIF-1β silencing efficacy among mice, further statistical analysis was performed and showed a significant correlation between *HIF-1β* mRNA levels and pC/pgRNA, preS1/preS2 RNA, HBx RNA or total HBV RNAs in liver tissues of HBV transgenic mice (Figure 4.9d). Collectively, these results suggest that there is a positive association between HIF and HBV transcription in a HBV transgenic mouse model.



Transcript name	Spearman's rank correlation coefficient	P value
pC/pgRNA	0.4750	0.0232
preS1/preS2 RNA	0.4654	0.0258
HBx RNA	0.4407	0.0336
Total RNAs	0.4840	0.0209

Figure 4.9. Effect of siRNA HIF-1β silencing in HBV transgenic mice on viral transcription.

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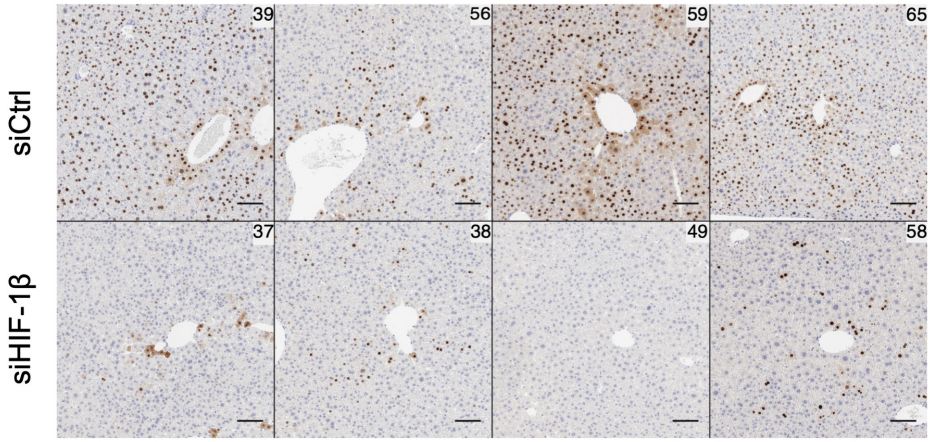
Figure 4.9. Effect of siRNA HIF-1β silencing in HBV transgenic mice on viral transcription. SiRNAs targeting HIF-1β were delivered into HBV1.3 transgenic mice and animals culled after 1 week. RNA was isolated from liver biopsies and transcript levels of HIF-1β along with HBV transcripts quantified by qPCR. (a) The proportion of HBV transcripts pC/pgRNA, preS1/preS2, and HBx RNA relative to total viral RNAs are presented. (b) The amount of viral, *HIF-1β*, *VEGFA*, and *PHD2* transcripts were normalised (Z-score) and presented as a heat map, to aid comparison of results, selected hypoxia responsive genes from figure 4.8b are presented. (c) HIF-1β and viral transcript RNA levels are compared between males and females. Statistical analysis was performed using Mann-Whitney analysis (*$p \leqslant 0.05$, **$p \leqslant 0.01$). Error bar represents standard deviation. (d) Spearman's rank order correlation statistical analysis was conducted for HBV pC/pgRNA, preS1/preS2 RNA, HBx RNA and total RNAs compared to HIF-1β with correlation coefficient and P value presented. Thomas Michler supported and conducted siRNA delivery into mice.

To further understand the impact of successful HIF-1β silencing on viral antigen expression in HBV transgenic mice, HBcAg expression in the liver and serum HBeAg, HBsAg and alanine aminotransferase (ALT) levels were evaluated (Figure 4.10a). Four mice with effective HIF-1β silencing and HBV transcript downregulation, and four mice treated with control siRNA were assessed. There was a significant downregulation of HBcAg liver staining in the transgenic mice with silenced HIF-1β compared to those treated with control siRNA (Figure 4.10b, Figure 4.10c). It is important to note that HBcAg liver staining showed a pericentral pattern, consistent with this area of the liver presenting a low oxygen environment (Figure 4.10b). There is also a decrease in serum HBeAg or HBsAg mean values in HIF-1β silenced mice compared to control (Figure 4.10d, Figure 4.10e). ALT is a measure of liver injury and comparable ALT levels among all mice were observed, suggesting negligible cytotoxic effects of HIF-1β siRNA silencing (Figure 4.10f)[403]. Taken together, these results suggest that there is a positive association between HIF and HBV antigen expression in HBV transgenic mice.

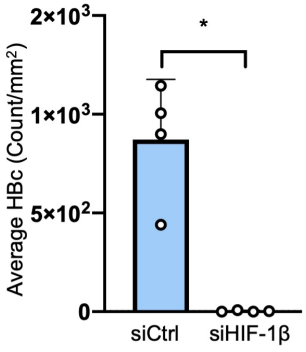
a.

Mouse (Sex)	siRNA	Average HBc Count/mm^2 (x10^2)	HBeAg (x10^2 IU/ml)	HBsAg (x10^2 IU/ml)	ALT (IU/ml)
39 (m)	siCtrl	11.44	2.40	39.3	42.0
56 (f)	siCtrl	4.41	0.14	0.57	32.1
59 (m)	siCtrl	10.05	2.25	43.87	44.0
65 (f)	siCtrl	8.99	1.03	7.08	33.9
37 (m)	siHIF-1β	0.03	0.32	1.19	61.2
38 (m)	siHIF-1β	0.08	0.47	2.18	69.2
49 (m)	siHIF-1β	0.00	0.00	0.00	37.8
58 (m)	siHIF-1β	0.00	0.22	0.02	46.4

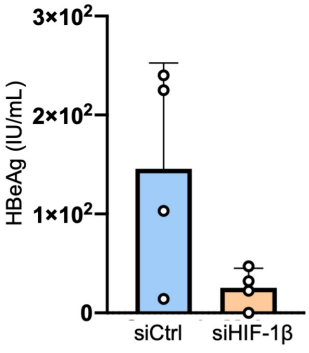
b.



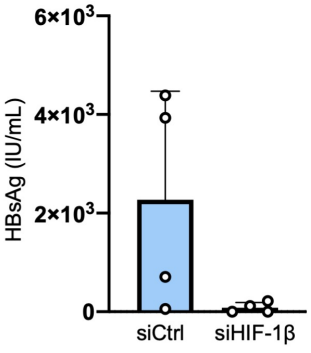
c.



d.



e.



f.

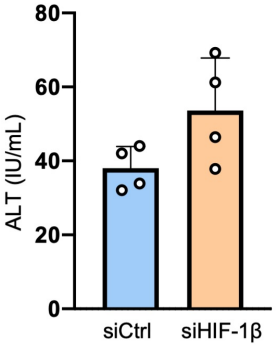


Figure 4.10. Effect of siRNA HIF-1β in HBV transgenic mice on viral protein expression.

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Figure 4.10. Effect of siRNA HIF-1β in HBV transgenic mice on viral protein expression. SiRNAs targeting HIF-1β (siHIF-1β) or control siRNAs (siCtrl) were delivered into HBV1.3 transgenic mice and animals culled after 1 week. Liver sections from male mice with response to siHIF-1β and siCtrl mice were stained for HBcAg and serum HBV e antigen (HBeAg), HBsAg or ALT values presented (a). Representative HBcAg liver stained images are shown (b). HBcAg (c), HBeAg (d), HBsAg (e), or ALT (f) quantification are compared between siCtrl and HIF-1β mice. Statistical analysis was performed using Mann-Whitney analysis (*p⩽0.05). Error bar represents standard deviation. Thomas Michler supported and conducted siRNA delivery into mice, liver sample extraction, circulating viral antigen quantification, and liver tissue staining.

4.3 Discussion

4.3.1 HIF transcription factor functional activity

Understanding how HIF stabilisation and low oxygen can impact HBV replication could lead to the identification of novel therapeutic targets for the treatment of HBV and associated disease[175]. These rationales prompted the exploration of HIF signaling's role in regulating HBV in this chapter.

FG-4592 is a pharmacological PHD inhibitor stabilising both HIF-1α and HIF-2α and has been approved for the treatment of chronic kidney disease and anaemia in China and Japan[404,405]. FG-4592 is a more target-specific PHD inhibitor for studying the PHD-HIF pathway whereas other traditionally used mimetic agents such as cobalt chloride and dimethyloxalglycine are known to activate other oxygen-independent effects[175,241,242]. FG-4592 treatment activated HBV BCP which contains two HREs (Figure 4.1, Figure 4.2). In two independent HBV model systems, FG-4592 increased pC/pgRNA levels which is regulated by BCP (Figure 4.3). HIF-1α, HIF-2α, and HIF-1β siRNA silencing suggests that HIF-1α has a dominant role in regulating viral transcription while HIF-2α has a supplementary role (Figure 4.5, Figure 4.6).

ChIP experiments showed that under 18% oxygen tension, HIF-1β does not bind HBV genomes extracted from HepG2-HBV-pEpi cells that support episomal copies of HBV DNA[72]. FG-4592 treatment or 1% oxygen incubation induced HIF-1β binding to the HBV genome in HepG2-HBV-pEpi cells. Further ChIP experiments confirmed that HIF-1α, HIF-2α, and HIF-1β bound HBV genomes replicating in human hepatocytes, that were isolated from infected liver chimeric mice.

However, despite the ability of both HIF-1α and HIF-2α to bind the HBV genome, the mechanism underlying a dominant HIF-1 driven HBV transcription pattern remains unclear.

The differential effects of HIF-1α and HIF-2α on HBV transcription could be attributed to HRE binding preference, functional activity, level of expression, and hypoxic response timeline. Studies show that HIF-1α and HIF-2α can bind HRE motifs (RCGTC) in the promoter region of a number of overlapping genes[406]. A large number of HIF target genes bind both HIF-1 and HIF-2, as both HIF-α subunits have similar HIF-1β dimerisation conformations[407,159,408]. However, HIF-1 binding near the promoter usually has higher transcriptional activity than HIF-2 under acute hypoxia[160,406]. Past studies show that HIF-1 transcriptional programming is predominantly associated with acute hypoxia response of under 24 hours *in vitro* and *in vivo* while HIF-2 is associated with chronic hypoxia of over 24 hours[409]. As 72 hours of sustained FG-4592 treatment may not recapitulate the same kinetic of PHD-HIF regulation compared to hypoxia, results on the dominant role of HIF-1α HBV regulation could represent a bias of the model system used.

Both HIF-1 and HIF-2 can bind HREs in mutually exclusive target genes and loci[160,406]. Although three-dimensional DNA conformation and epigenetic regulation have been proposed as potential factors for predicting the location of HRE binding, the precise mechanism underlying HIF-1 and HIF-2 HRE selectivity remains unclear[337,406]. As two HREs were discovered in distinct regions of the HBV genome, in EnhI (1236-1240) and between EnhI and EnhII (1604-1599) near promoter region of the BCP, there could be a preference for HIF-1 binding at one or both loci.

The varying binding activity and cofactor interaction of HIF-1 and HIF-2 in transcription regulation could also be relevant to their role in HBV regulation. HIF binding to HREs might not always lead to an upregulation in transcription[406,410]. HIF-α mutagenesis studies show that N-TAD is critical in determining HIF-1α and HIF-2α target gene specificity[410]. Changing HIF-2α N-TAD to that of HIF-1α results in a HIF-1 functional phenotype. HIF regulation of target genes is dependent on cofactor interaction, and the N-TAD domain is an important platform for cofactor interaction. Particular cofactors including ETS transcription factor (ELK) interacts with HIF-2 but not HIF-1 to modulate HIF-2 specific target genes. In contrast, HIF-1 complexes with SMAD family member 3 (SMAD3) to mediate transcription regulation[411]. Transcription cofactor expression and functional activities vary between cells and tissues[412]. The transcription efficiency of transcription complexes also varies, resulting in different degrees of target gene induction. Therefore, the dominant role of HIF-1 and supplementary role of HIF-2 in HBV regulation could arise from differential efficiency of HIF transcription induction and transcription cofactor related mechanisms. As a result, HIF and associated cofactors are worth exploring as potential novel therapeutics for HBV infection and pathogenesis.

4.3.2 Limitations of using DMSO for studying the interaction between HBV and HIF

DMSO treatment is commonly used in HBV *de novo* infection model systems to arrest cell proliferation and induce liver transcription factors[22,413]. Results from this chapter suggest that DMSO blunts positive regulatory effects of FG-4592 on HBV transcription (Figure 4.4). In contrast, HIF target genes tested, *CAIX* and *VEGFA*, were induced by FG-4592 regardless of DMSO treatment. However DMSO, especially at high concentrations used for HBV *de novo* infection experiments, is known to broadly alter host gene expression[414,415]. In human and rat hepatocytes, DMSO affects gene expression related to a myriad of mechanisms, from proteasomal degradation and ubiquitin mediated proteolysis, to oxidative phosphorylation and epigenetic regulation[415]. Earlier studies in the McKeating group show that DMSO negatively regulates a panel of HIF target genes, which did not include *CAIX* or *VEGFA*[72]. Therefore, DMSO treatment could limit studies on the PHD-HIF pathway and HBV replication. In fact, Hallez *et al*., using a DMSO dependent model system, reported that hypoxia modestly reduced HBV RNA and DNA[416]. This is consistent with observations from this chapter that DMSO may preclude an accurate understanding of HIF's role in regulating HBV replication, because of its broad-spectrum impact on host gene expression.

Although the liver harbours a physiological low oxygen environment, HBV related studies have traditionally been conducted under atmospheric oxygen tension, where hypoxia response mechanisms are not functionally active[115]. Studying the HBV life cycle under low oxygen could recapitulate a more physiologically relevant model system for studying viral replication in the liver. To this end, further investigations presented in the following chapter will explore the role of low oxygen tension in HBV replication.

4.3.3 HIF regulation of HBV transcription *in vivo*

In a HBV transgenic mouse model with siRNA targeted against HIF-1β or control siRNA, there is significant correlation between *HIF-1β* mRNA and HIF target gene transcripts, *VEGFA* and *PHD2*, as well as hypoxia response genes that are differentially regulated by an oxygen gradient in the mouse liver, including *FMO2*, *CYP2c38*, *RNF186* and *RGS16* (Figure 4.8). Further assessments show that *HIF-1β* mRNA associates with HBV transcript levels in mouse livers (Figure 4.9), and successful HIF-1β silencing in male mice led to a corresponding downregulation of HBV transcripts in four of six mice. Further statistical analysis shows that *HIF-1β* mRNA associates with HIF target genes or HBV transcripts in mice treated with siRNA against HIF-1β or control siRNA (Figure 4.8, Figure 4.9). Successful HIF-1β silencing with corresponding downregulation in HBV transcripts also led to significant downregulation of HBcAg expression in the liver (Figure 4.10). These *in vivo* results align with ChIP data discussed previously. Taken together, this chapter identified a positive role for HIFs to regulate HBV transcription through interaction with the BCP promoter region.

4.3.4 Sex-related differential response to hypoxia and HBV infection.

Results in this chapter suggest that siRNA silencing of HIF-1β is effective in male HBV transgenic C57BL/6J mice but not in females (Figure 4.8, Figure 4.9). Differential response to HIF-1β silencing suggests that the particular siRNA used is ineffective in female mice or biological sexes have differential responses to PHD-HIF signaling.

The role of biological sex in determining differential response to low oxygen conditions has been documented in humans and *in vivo* model systems. As HIF is an evolutionary conserved oxygen sensor in mammals, evidence suggests that differential response to hypoxia and HIF signaling between sexes is present in a number of species[417]. For example, past studies show that male rats and swines are more sensitive to chronic hypoxia than females[418-420]. A recent proteomics study on the molecular basis of sex difference in zebrafish in response to hypoxia observed complex networks of underlying pathways[421]. These results highlight the complexity underlying sex related differential response to hypoxia in animal model systems.

Mechanism underlying sex-related differences in hypoxia and HIF response in humans is likely complex and multifaceted. In ischemic stroke patients, there were 571 to 3,437 genes that were differentially regulated in a female-specific manner[422]. Human males exposed to high-altitude hypoxic environments have significantly higher chemoreflex sensitivity to hypoxia and more frequent nocturnal periodic breathing than females[423,424]. When Acetazolamide is administered, which inhibits carbonic anhydrase including the HIF target CAIX, apnea-hypopnea index symptoms at high altitude were improved especially for males[423]. Estrogen signaling has been reported to inhibit both HIF-1α transcriptional activity and HBV transcription, which is a mechanism potentially underlying sex-associated differential response to hypoxia[66,425].

Interestingly, it has been well documented that males with HBV have higher viral load and are disproportionally more likely to develop chronic infection, HCC, and more accelerated and severe pathological outcomes than females[426-430]. Males are five to seven times more likely to develop HCC than females[430]. In comparison to the disproportionate number of males with HBV-related HCC, both male and female have similar risks for developing HCV-related HCC upon infection. Female patients who develop HBV-related HCC have significantly lower estradiol to testosterone ratio[427]. These sex-related differential responses to HBV infection are even more pronounced in transgenic mouse models[426,431,432]. Male transgenic mice with HBV HBsAg and HBx genes integrated into their genomes developed HCC three months before females[431]. Estrogen treatment in male mice inhibited HBV transcription but was less effective in females[433]. Estrogen receptor α (ER-α) has been found to interact with EnhI region of the HBV genome, inhibit HNF-4α transcription factor binding, and reduce viral transcription[66]. Given well documented evidences on the cross-talk between HIF and estrogen signaling, further investigation is required to better understand the role of sex hormones in hypoxic response and HBV replication[434-436].

4.3.5 Use of drugs targeting the PHD-HIF pathway in HBV infection.

Portions of the texts below have been published as a part of a review manuscript in Viruses[175].

Results from this chapter suggest a role for HIFs to positively regulate HBV replication with important implications for indications and contraindications for drugs targeting the PHD-HIF pathway. The PHD inhibitors Roxadustat (FG-4592, FibroGen), Daprodustat (GSK1278863, GlaxoSmithKline), Molidustat (Bay8503934, Bayer), Vadadustat (AKB-6548, Akebia) have been assessed in phase II and III clinical trials with Roxadustat approved for chronic kidney disease and anaemia treatment in China and Japan[175,404,405,437]. Regulatory effects of Roxadustat on HBV transcription reported in this chapter suggest that patients with a positive status for HBV should be recommended Roxadustat with caution. This is especially important given that CHB is associated with chronic kidney disease, which leads to anaemia[438]. Indeed, positive HBsAg status was listed as an exclusion criterion for patients in a phase III multicentre Roxadustat trial[405].

A number of HIF inhibitors are undergoing clinical trial in patients with advanced or refractory cancers[439]. The HIF-1α synthesis inhibitor Digoxin is undergoing phase II clinical trial for Kaposi's Sarcoma[440]. CRLX101, a HIF-1α expression inhibitor, has completed a phase II trial in combination with bevacizumab, an antibody that targets VEGF, for the treatment of recurrent platinum-resistant ovarian, tubal, and primary peritoneal cancers and reduce tumour frequency and size [441,442]. However, a randomised phase II trial in patients with advanced renal carcinoma did not show any improvement in progression-free survival[443]. A phase II trial for CRLX101 in combination with capecitabine and radiotherapy for locally advanced rectal cancer is ongoing[444]. The HIF-2α specific dimerisation inhibitors PT2385[445,446] and PT2977[447] are being tested for the treatment of recurrent glioblastoma, non-metastatic VHL-associated, and advanced ccRCC[448-450].

Results from this chapter suggest that PT2385 treatment has a minimal effect on HBV replication (Figure 4.6). Given the dominant role of HIF-1α in regulating HBV transcription, future studies could investigate the effect of HIF-1α inhibiting drugs such as Digoxin and CRLX101 on HBV replication and HIF inhibitors may serve as potential novel treatments for HBV infection. Further assessment on the relationship between anaemia, CHB, and patient outcome through large-scale epidemiological studies could reveal further insight on the interaction between HIF and HBV.

4.4 Summary

This chapter demonstrated a role for HIFs in regulating HBV replication. The discovery of two functionally active HREs in the HBV genome prompted further experiments to investigate a role for PHD-HIF signaling in regulating HBV transcription. Pharmacological PHD inhibition and siRNA silencing of HIFs *in vitro* show that HIF-1α has a dominant role with HIF-2α playing a supplementary role in regulating HBV pC/pgRNA transcription. Silencing of the HIF-1β subunit, which can dimerise with both HIF-1α or HIF-2α, abolished HBV transcript upregulation induced by PHD inhibition. Further experiments show that DMSO blunts the effects of PHD inhibition on stimulating HBV transcription. The HIF-2α inhibitor PT2385 has minimal effects in regulating HBV transcription. Investigations using agonists and antagonists for AhR-HIF-1β signaling suggest AhR signaling has a minimal role in HBV transcription. Using HBV transgenic mice, successful siRNA silencing of HIF-1β led to downregulation of HBV transcription and HBcAg expression in the liver.

Results from this chapter can help propel a number of research directions in PHD-HIF regulation of HBV replication and pathogenesis. Firstly, the mechanism underlying HIF-1 dominant regulation and HIF-2 supplementary regulation of HBV transcription remains unclear. Further studies can investigate the binding preference, affinity, and functional effects of HIF interaction with HREs present in the HBV genome. In addition, the impact of PHD-HIF signaling on HBV transcription demonstrated in this chapter warrants investigations on the contraindication of PHD inhibitors and repurposing of HIF inhibitors for treating patients with HBV infection and related pathologies. In conclusion, this chapter presented HIF signaling as an important regulation mechanism for HBV transcription and identified novel directions of future research in this domain.

5. HYPOXIA AND HIF INDEPENDENT REGULATION OF HBV TRANSCRIPTION

5.1 Introduction

The previous chapter has identified HIF as a positive regulator for HBV transcription. Although the PHD-HIF pathway is the most well studied oxygen sensing mechanism, a number of other mechanisms have been identified which respond to dynamic changes in oxygen tension. For example, the newly discovered ADO oxygen sensing pathway stabilises targets such as IL-32 under hypoxia[120]. As IL-32 was recently reported to repress HBV transcription, low oxygen could induce a myriad of processes including HIF and IL-32 stabilisation, which may have different effects on HBV replication[120,451]. However, HIF independent mechanisms that could potentially contribute to HBV regulation under low oxygen remain to be assessed.

This chapter investigates the role of low oxygen tension and HIF independent mechanisms in regulating HBV transcription. Results demonstrate that a low oxygen environment induces HBV transcription in three independent *in vitro* model systems. Low oxygen incubation time dependently upregulated HBV transcription, and 1% oxygen tension induced HBV transcription to a higher degree than 3%. Silencing HIF subunits confirms that both HIF dependent and HIF independent mechanisms regulate HBV transcription under low oxygen in infected HepG2-NTCP cells. Further experiments exploring HIF independent mechanisms reveal that hypoxia downregulates the HBV restriction factor Smc5/6 complex and may inhibit anti-viral responses related to IL-32. These results collectively demonstrate the complex interplay between diverse oxygen sensing mechanisms and their impact on HBV replication.

5.2 Results

5.2.1 Hypoxia regulation of HBV transcription

To investigate whether hypoxia has a role in regulating HBV transcription, three distinct *in vitro* model systems were deployed: HBV infected HepG2-NTCP cells, HepG2-HBV-pEpi cells with episomal replicating HBV, and HepG2.2.15 cells with integrated copies of transcriptionally active HBV. Culturing cells under 1% oxygen tension for 24 hours induced HBV pC/pgRNA transcription by 5.3±1.1 fold, 2.9±0.5 fold, and 1.5±0.1 fold in the various model systems (Figure 5.1a). To assess the cellular response to low oxygen, *VEGFA* mRNA levels were quantified and showed an increase of 6.3±1.3 fold in the infected HepG2-NTCP cells, 5.6±1.0 fold in HepG2-HBV-pEpi and 3.2±0.6 fold in HepG2.2.15 (Figure 5.1a).To understand the kinetics of hypoxia regulation of HBV transcription, HepG2-HBV-pEpi cells were cultured at 1% oxygen tension for 24 hours, 48 hours or 72 hours. There is a 3.5±1.5 fold or 12.5±12.4 fold change in pC/pgRNA after 48 hours or 72 hours of incubation under 18% atmospheric oxygen tension, respectively, compared to 24 hours as a baseline (Figure 5.1a). In contrast, 1% oxygen tension induced pC/pgRNA by 5.7±2.9 fold, 31.8±7.7 fold, or 62.1±17.7 fold at 24 hours, 48 hours, or 72 hours of incubation, respectively, showing a time-dependent increase in viral transcription. In contrast, induction of the HIF target genes *CAIX* and *VEGFA* remained consistent over the duration of the experiment (Figure 5.1a). Western blot confirms that HIF-1α protein was expressed at all time points in the experiment, indicating that hypoxic regulation was active in HepG2 cells (Figure 5.1c). Since the maximal increase in hypoxia mediated HBV pC/pgRNA transcription was observed after 72 hours, this time point was selected for further experiments.

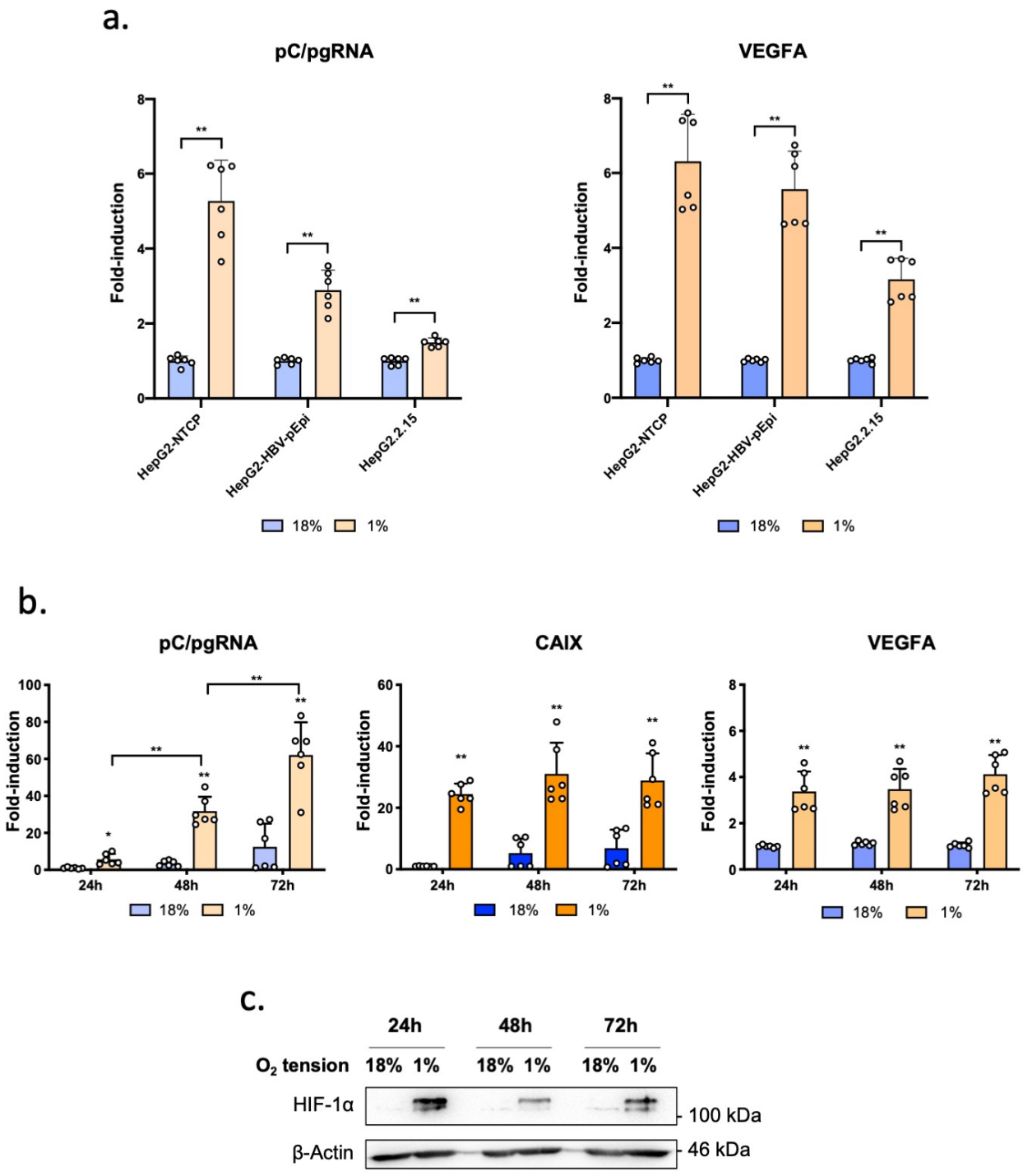


Figure 5.1. Hypoxia regulation of HBV transcription is time-dependent. Figure legend is on the following page.

Figure 5.1. Hypoxia regulation of HBV transcription is time-dependent. (a) HepG2-NTCP cells infected with HBV (MOI 200), HepG2 with episomal HBV DNA (HepG2-HBV-pEpi) and HepG2 with integrated HBV DNA (HepG2.2.15) were cultured at 18% or 1% oxygen tension for 24 hours. HBV pC/pgRNA and the HIF target gene *VEGFA* transcripts were quantified by qPCR and data plotted relative to respective normoxic samples. (b) HepG2-HBV-pEpi cells were cultured at 18% or 1% oxygen tension for 24 hours, 48 hours or 72 hours. HBV pC/pgRNA, *CAIX* and *VEGFA* transcripts were quantified by qPCR. Mann-Whitney test was conducted for statistical analysis (*$p \leqslant 0.05$, **$p \leqslant 0.01$). Data is representative of results from 6 biological repeats. Error bar represents standard deviation. HIF-1α protein expression was assessed using western blot to confirm hypoxic response induction (c).

To understand whether oxygen tension can differentially regulate HBV pC/pgRNA transcription, infected HepG2-NTCP cells were incubated at 3% or 1% oxygen tension for 72 hours. Oxygen tension at 3% models moderate physiological hypoxia near the pericentral region of the liver while 1% represents severe hypoxia which can occur under pathological conditions such as inflammation[115,452]. At 3% oxygen tension, there is a significant 4.4±0.8 fold induction in pC/pgRNA transcription (Figure 5.2a). At 1% oxygen tension, there is a further 36.6±14.2 fold induction in pC/pgRNA, which is significantly higher than pC/pgRNA levels at 3% and 18% oxygen tension. In contrast, *CAIX* was induced by 9.0±1.7 fold or 6.9±1.5 fold at both oxygen tensions (Figure 5.2b). Similarly *VEGFA* was induced by 4.2±1.3 fold or 7.2±2.3 fold at 3% or 1% oxygen tension (Figure 5.2c). *CAIX* and *VEGFA* transcript levels are not statistically different at 3% or 1% oxygen tension. Further western blot analysis confirms that HIF-1α is stabilised at both 3% and 1% oxygen tensions in HepG2 cells (Figure 5.2d, Figure 5.2e).

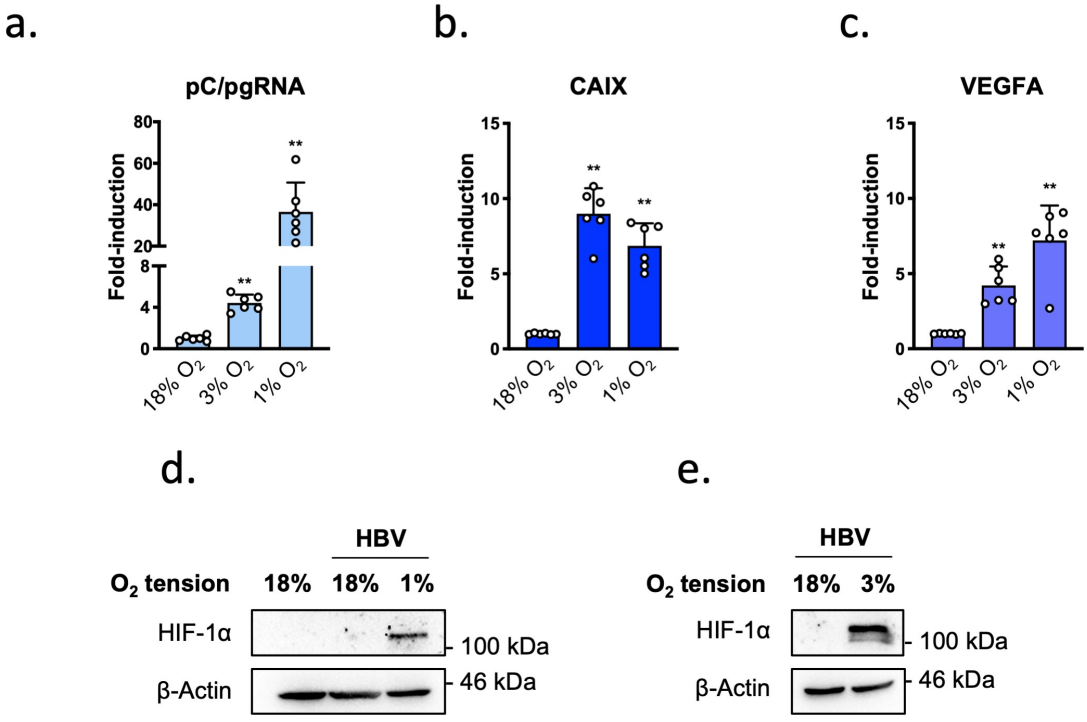


Figure 5.2. Regulation of HBV transcription by different oxygen tensions. HepG2-NTCP cells were infected with HBV (MOI 200) and cultured at 18%, 3%, or 1% oxygen tension for 72 hours. HBV pC/pgRNA (a), the transcripts of HIF-1α dependent target gene *CAIX* (b), and the HIF-1α and HIF-2α dependent gene *VEGFA* (c) were quantified by qPCR. Data is presented as fold induction relative to the 18% oxygen cultured control. Mann-Whitney test was conducted for statistical analysis comparing samples cultured under low oxygen with samples cultured at 18% oxygen tension (**$p \leqslant 0.01$). Data is representative of results from 6 biological repeats. Error bar represents standard deviation. HIF-1α expression was assessed using western blot to confirm hypoxic response induction at 1% (d) or 3% (e) oxygen tension.

The previous chapter identified a role for HIFs to positively regulate HBV transcription. To validate whether HIF can regulate HBV transcription under hypoxia, siRNAs targeting HIF-1α, HIF-2α, or HIF-1β were delivered into infected HepG2-NTCP cells cultured under 18% or 1% oxygen tension (Figurer 5.3a). Western blot assessment confirms that HIF-1α, HIF-2α, or HIF-1β proteins were downregulated in a target specific manner by respective siRNAs under 1% oxygen tension (Figure 5.3b, Figure 5.3c). Low oxygen increased HBV pC/pgRNA by 36.6±14.2 fold and silencing HIF-1α or HIF-2α individually had a modest non-significant effect on the viral RNA levels (Figure 5.3d). In contrast, silencing both HIF-1α and HIF-2α or HIF-1β significantly reduced pC/pgRNA induction under 1% oxygen tension, suggesting a role for both HIF isoforms in regulating viral transcript levels. As expected, mRNA and protein of the HIF-1 specific target *CAIX* were downregulated by siRNA targeting HIF-1α or HIF-1β under 1% oxygen tension but not by siRNA targeting HIF-2α (Figure 5.3b, c and e). Silencing HIF-1α, HIF-2α, or HIF-1β downregulated transcripts of the HIF-1 and HIF-2 target gene *VEGFA* under low oxygen (Figure 5.3f). Individually silencing HIF-1α or HIF-1β abolished low oxygen induction of *CAIX* mRNA. HIF-1α and HIF-2α double silencing or HIF-1β silencing abolished low oxygen induction of *VEGFA* (Figure 5.3e, Figure 5.3f). In contrast, neither HIF-1α and HIF-2α double silencing or HIF-1β silencing completely abolished pC/pgRNA induced by 1% oxygen tension, suggesting that HIF independent mechanisms could play a role in regulating HBV transcription (Figure 5.3d).

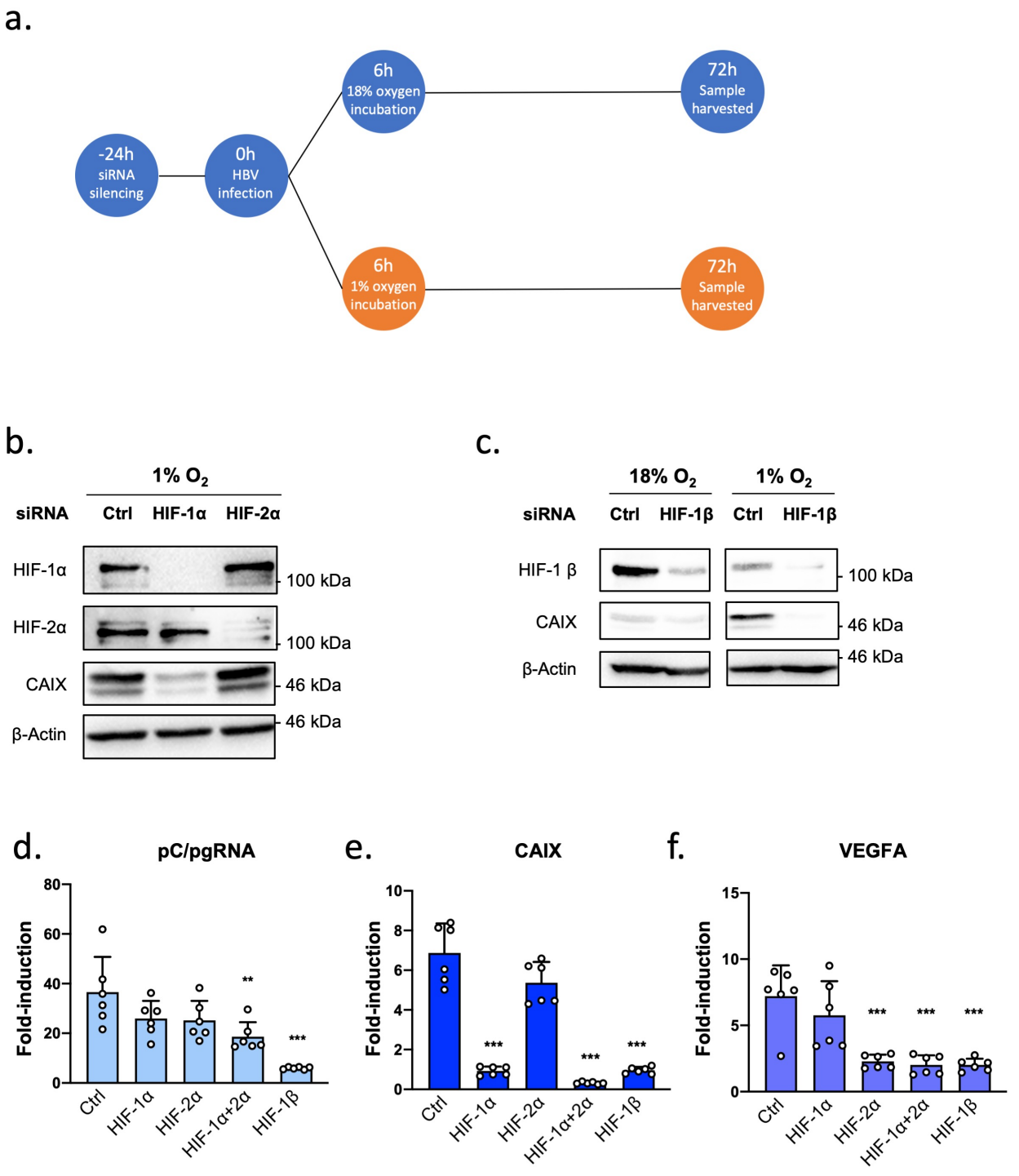


Figure 5.3. HIF regulation of HBV transcription under hypoxia. Figure legend on the following page.

Figure 5.3. HIF regulation of HBV transcription under hypoxia. (a) Schematic illustration of experimental procedure. Briefly, siRNAs targeting HIF-1α, HIF-2α, or HIF-1β or control siRNA (Ctrl) were delivered into HepG2-NTCP cells that were infected with HBV (MOI 200) and cultured at 18% or 1% oxygen tension for 72 hours. SiRNA silencing was confirmed by western blot probing for HIF-1α, HIF-2α, HIF-1β, and CAIX protein expression (b, c). HBV pC/pgRNA (d), transcripts of HIF-1α target gene *CAIX* (e), and HIF-1α and HIF-2α co-regulated gene *VEGFA* (f) were confirmed by qPCR. Data is presented as fold induction relative to the 18% oxygen control for each condition. Statistical analysis was performed using a One-Way ANOVA followed by multiple comparison analysis (*$p \leqslant 0.05$, **$p \leqslant 0.01$, ***$p \leqslant 0.001$). Data is representative of results from 6 biological repeats. Error bar represents standard deviation.

5.2.2 Hypoxia downregulates Smc6 at the post-transcriptional level

Smc5/6 is an essential host restriction factor that limits HBV transcription and HBx binds the host Cul4-DDB1 E3 ubiquitin ligase complex to target Smc5/6 for proteasomal degradation[48]. To explore other potential mechanisms for hypoxia to regulate HBV transcription, studies were conducted to examine Smc5/6 expression under hypoxic conditions. HepG2 cells were cultured at 18%, 1%, or 0.1% oxygen tension for 24 hours or 48 hours (Figure 5.4a) and expression of Smc6 and Nse4 proteins, components of Smc5/6, were reduced under hypoxic conditions. Oxygen tension at 0.1% models severe hypoxia that may occur in pathological conditions such as within a solid-state tumour[453]. *Smc6* transcript levels are comparable between 18%, 1%, and 0.1% oxygen tension, with a modest increase at 0.1%. These results suggest a post-transcriptional mechanism for the hypoxic regulation of Smc6 (Figure 5.4b).

To understand whether atmospheric oxygen reperfusion can rescue hypoxia downregulation of Smc6 and Nse4 proteins, HepG2 cells were cultured under 1% oxygen tension and exposed to 18% oxygen tension for 10 minutes, 4 hours or 6 hours (Figure 5.4c). Low oxygen-dependent Smc6 and Nse4 protein downregulation was rescued by 4 hours of oxygen reperfusion and were comparable to the normoxic samples. Since Smc6 or Nse4 protein depletion leads to destabilisation and depletion of the entire Smc5/6 complex, these results suggests that low oxygen tension downregulates the Smc5/6 complex at the protein level and this phenotype can be rescued by oxygen reperfusion[48,50].

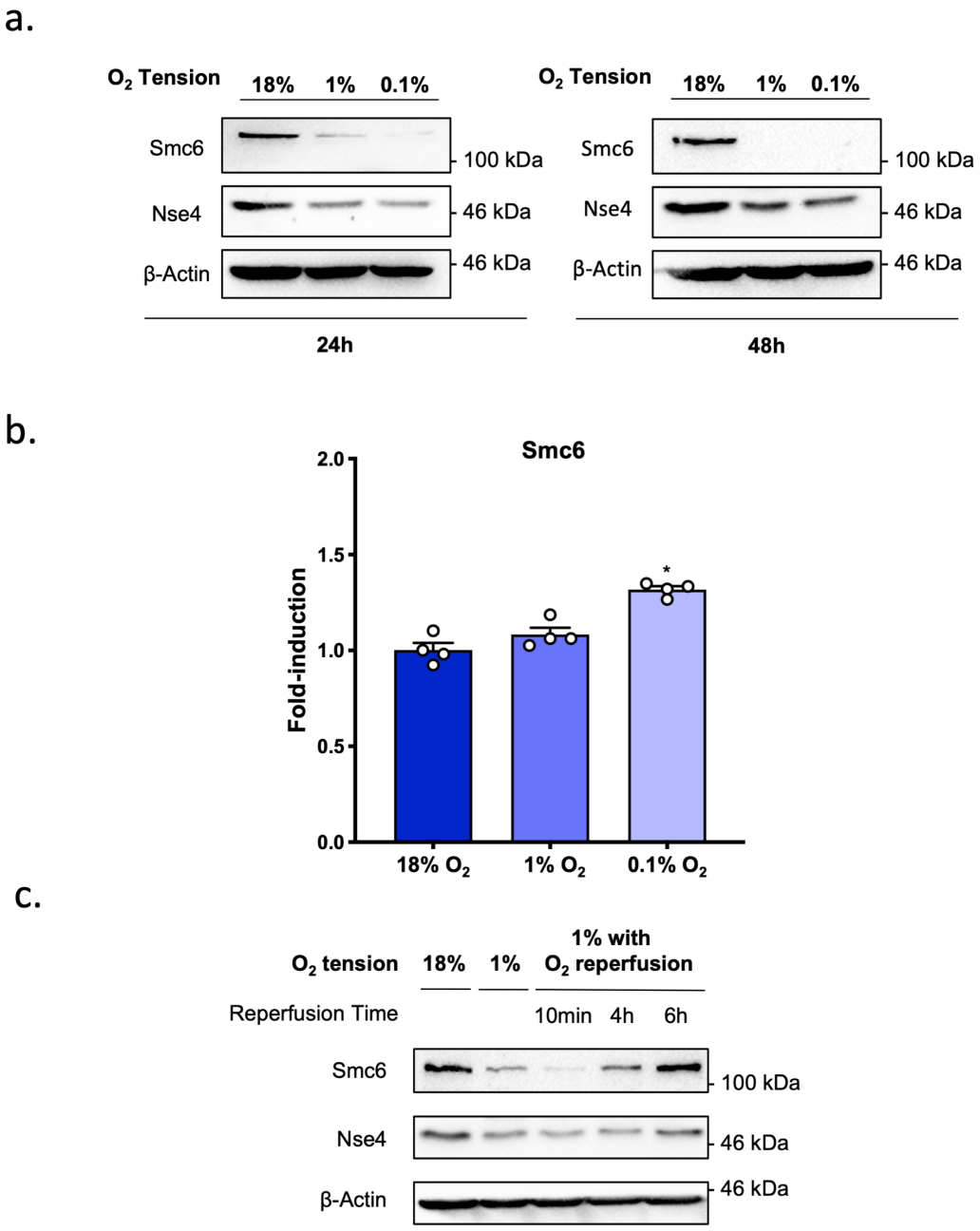


Figure 5.4. Hypoxia downregulates Smc6 and Nse4 in HepG2 cells. (a) HepG2 cells were cultured at 18%, 1%, or 0.1% oxygen tension for 24 hours or 48 hours and Smc6 and Nse4 expression assessed with western blot. (b) Transcript levels of Smc6 under 18%, 1%, or 0.1% oxygen tension for 24 hours in HepG2 cells was quantified with qPCR. (c) HepG2 cells were cultured at 18% or 1% oxygen tension for 48 hours and samples reperfused with 18% oxygen for 10 minutes, 4 hours, or 6 hours. Protein expression of Smc6 or Nse4 was assessed with western blot. Statistical analysis was performed using Mann-Whitney test with $p \leqslant 0.05$ as significant. Data is representative of results from 2-4 biological repeats. Error bar represents standard deviation.

To understand whether hypoxia regulates Smc6 and Nse4 expression in cells of other origins, U2OS cells derived from bone tissue and HEK293T cells derived from embryonic kidney were studied. Western blot confirms that 1% and 0.1% oxygen tensions downregulate Smc6 and Nse4 proteins in both cell types (Figure 5.5).

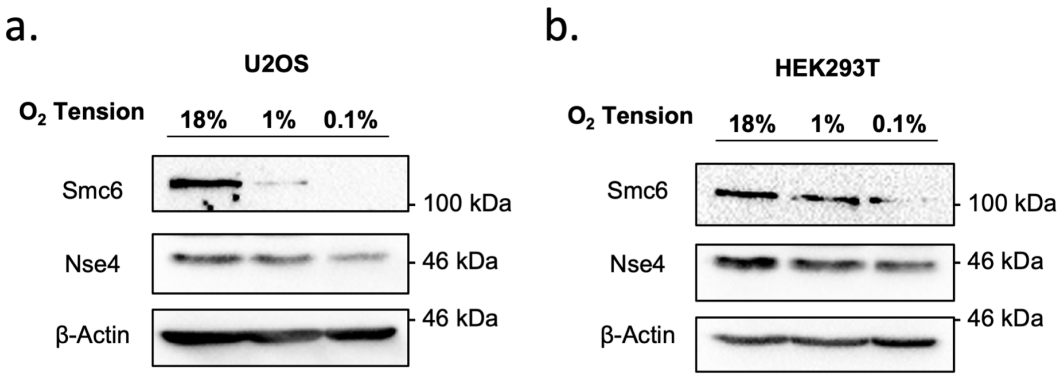


Figure 5.5. Hypoxia downregulates Smc6 and Nse4 in diverse cell types. U2OS (a) or HEK293T (b) cells were cultured at 18%, 1%, or 0.1% oxygen tension for 24 hours and Smc6 and Nse4 expression assessed by western blot. Data is representative of results from 3 biological repeats.

5.2.3 VHL and PHD regulate Smc6

The VHL-PHD-HIF pathway is the most well studied oxygen sensing mechanism and their roles in the hypoxic regulation of Smc6 were investigated. The role of VHL in regulating Smc6 and Nse4 proteins was evaluated using the 786O cell line which has a loss of function VHL mutation and constitutively expresses HIF-2α but not HIF-1α[454]. 786O cells expressing a functional VHL gene (786O-VHL) or empty vector (786O-VA) were cultured at 18%, 1%, or 0.1% oxygen tension. Results confirmed that 786O-VA cells express HIF-2α regardless of oxygen tension (Figure 5.6a). Culturing 786O-VHL cells under 1% or 0.1% oxygen tension stabilised HIF-2α protein, demonstrating that VHL is functionally active. Under 1% or 0.1% oxygen tension, Smc6 and Nse4 proteins were downregulated in 786O-VHL cells but not in 786O-VA cells (Figure 5.6a).

The role of PHD in regulating Smc6 and Nse4 was studied using PHD inhibitors. The PHD inhibitors FG-4592, GSK1278863, and Molidustat stabilised HIF-1α in HepG2 cells and all drugs induced a loss of Smc6 and Nse4 expression (Fig. 5.6b). Collectively, these results suggest a potential role for VHL and PHD to regulate Smc6 and Nse4 expression.

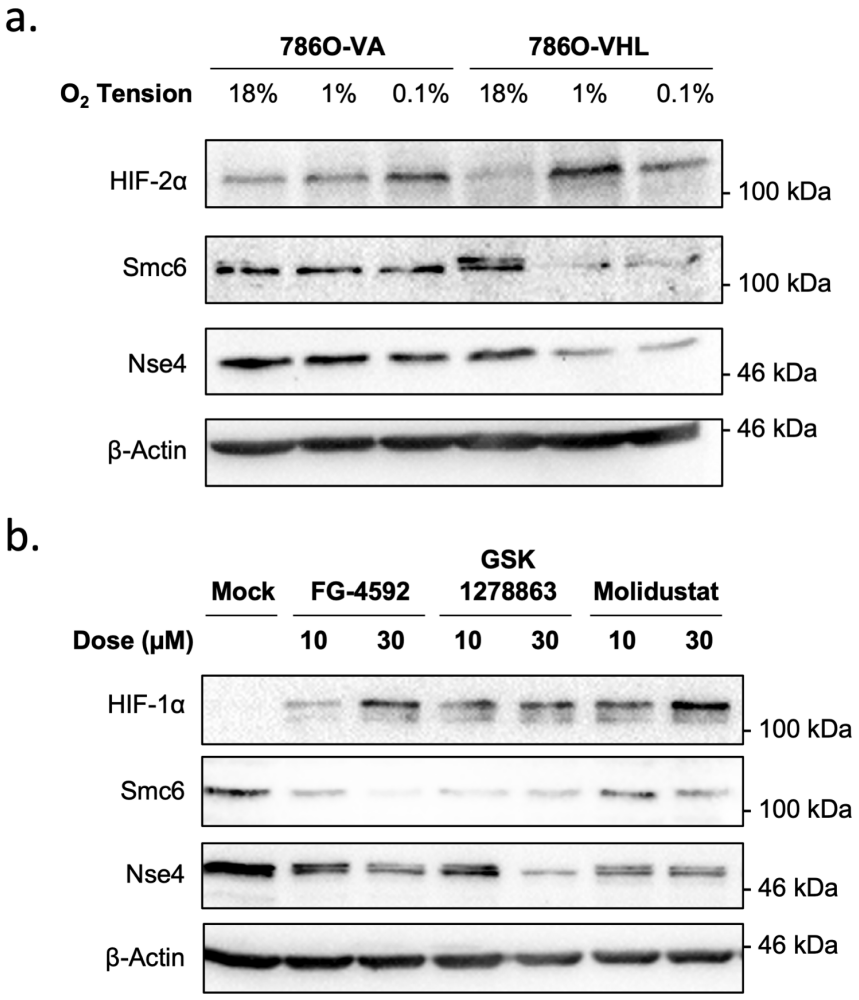


Figure 5.6. Smc6 and Nse4 are regulated by VHL and PHD. (a) 786O cells engineered to express VHL (786O-VHL) or empty plasmid (786O-VA) were cultured at 18%, 1%, or 0.1% oxygen tension for 48 hours. HIF-2α, Smc6 or Nse4 protein expression was assessed using western blot. (b) HepG2 cells were treated with HIF PHD inhibitors FG-4592, GSK1278863, or Molidustat at 10 μM or 30 μM for 48 hours and HIF-1α, Smc6, or Nse4 expression assessed with western blot. Data is representative of results from 3 biological repeats.

5.2.4 Hypoxia regulates Smc6 through HIF independent proteasomal degradation

VHL and PHD regulate their substrates through the proteasomal degradation pathway. To investigate if proteasomal degradation has a role in hypoxia regulation of Smc6, HepG2 cells were treated with the proteasomal inhibitor MG-132. MG-132 stabilised ubiquitinated HIF-1α in a dose-dependent manner (Figure 5.7a) and rescued Smc6 and Nse4 protein expression under hypoxic conditions. HIF is a substrate of PHD and subject to proteasomal degradation under hypoxia. To understand if HIF has a role in regulating Smc6, control siRNA or siRNA targeting HIF-1α or HIF-2α were delivered into HepG2 cells. Western blot confirms that siRNA effectively silenced HIF-1α and HIF-2α and reduced protein expression of the HIF target gene CAIX (Figure 5.7b, Figure 5.7c). However, HIF-1α and HIF-2α silencing did not abolish hypoxia downregulation of Smc6 protein. These results suggest that hypoxia targeting of Smc6 is mediated via a HIF independent proteasomal degradation pathway.

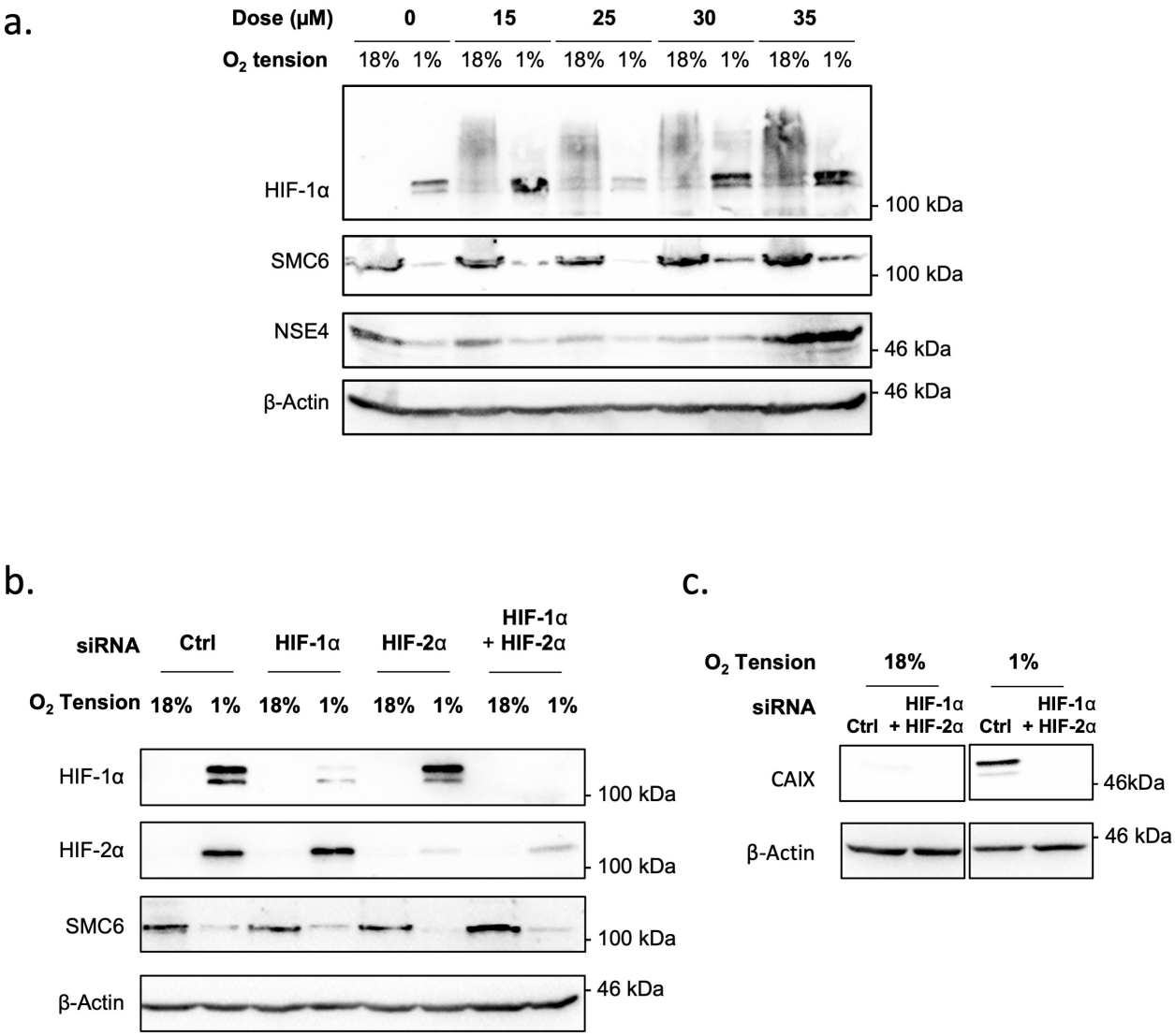


Figure 5.7. HIF independent proteasomal degradation of Smc6. (a) HepG2 cells were cultured at 18% or 1% oxygen tension for 48 hours and treated with increasing dose of the proteasome inhibitor MG-132 for 4 hours. HIF-1α, Smc6 or Nse4 expression was assessed by western blot. (b) SiRNA targeting HIF-1α or HIF-2α were delivered into HepG2 cells and cultured at 18% or 1% oxygen for 48 hours and HIF-1α, HIF-2α or Smc6 expression assessed by western blot. (c) HIF target gene CAIX expression in HepG2 cells with siRNA double silencing of HIF-1α and HIF-2α was assessed by western blot. Data is representative of results from 3 biological repeats.

5.2.5 Hypoxia regulation of HBV lacking HBx

HBV that lacks HBx (HBV_{X-}) cannot degrade the Smc5/6 complex and has a limited capacity to transcribe and replicate. Experiments were conducted to assess whether hypoxia-reduced Smc6 expression can rescue HBV_{X-} transcription. As noted in chapter 3 (Figure 3.8) transducing HepG2-NTCP cells with Ad-HBx rescued HBV_{X-} transcription (Figure 5.8a). In contrast, hypoxic conditions had no significant effect on viral transcripts in HBV_{X-} infected HepG2-NTCP cells (Figure 5.8b), demonstrating a role for HBx in the hypoxic regulation of HBV. The multifaceted and complex role that HBx has in facilitating HBV infection beyond Smc5/6 degradation likely contributed to this result[455,456]. Further investigations are essential to establish the implications of hypoxia downregulation of Smc5/6 on HBV replication.

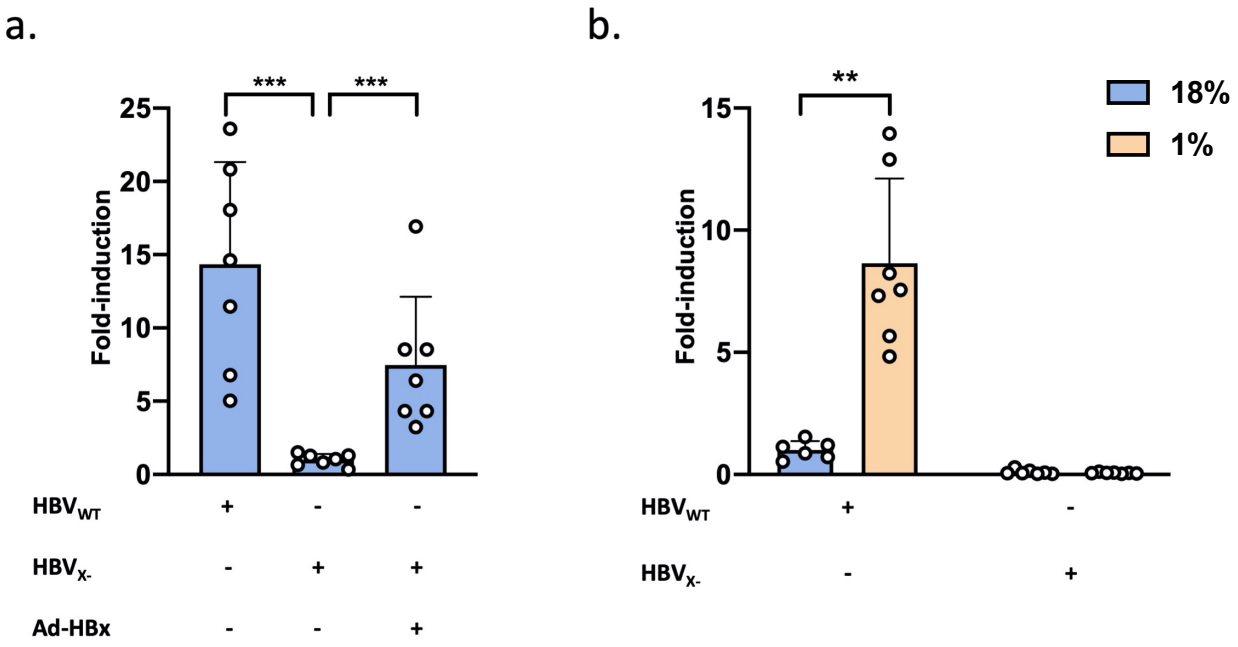
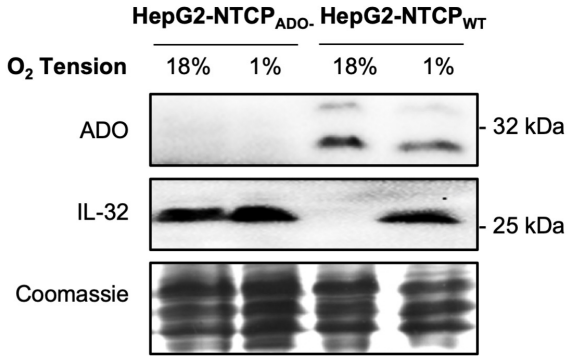


Figure 5.8. Effect of hypoxia on transcription of HBV lacking HBx. HepG2-NTCP cells were infected with wildtype HBV (HBV_{WT}) or HBV lacking HBx (HBV_{X-}) and adenovirus vector containing HBx (Ad-HBx) for 72 hours. HBV pC/pgRNA was assessed by qPCR (a). HepG2-NTCP cells were infected with HBV_{WT} or HBV_{X-} for 72 hours under 18% or 1% oxygen tension. Data is presented as fold induction relative to the 18% oxygen control for each condition. Statistical analysis was performed using Mann-Whitney test (*p⩽0.05, **p⩽0.01). Data is representative of results from 6-7 biological repeats. Error bar represents standard deviation. Experiment conducted with support from Peter Wing.

5.2.6 Hypoxia stabilises the ADO target IL-32 but inhibits its anti-viral activity against HBV

To explore the role of other HIF independent oxygen sensing mechanisms in HBV replication, the newly discovered ADO oxygen sensing pathway was assessed, because its target IL-32 was reported to regulate HBV transcription[120,451]. To explore the role of ADO and IL-32 signaling in regulating HBV, HepG2-NTCP cells with ADO gene deletion were generated. Western blot confirms ADO gene deletion (HepG2-NTCP$_{ADO-}$) and the ADO knock out (KO) cells constitutively express IL-32 at 18% or 1% oxygen tensions (Figure 5.9a). As NTCP is a HBV entry receptor critical for the establishment of *de novo* viral infection, fluorescent microscopy with a synthetic GFP tagged PreS1 peptide that binds NTCP (Myrcludex B) demonstrates comparable NTCP expression (Figure 5.9b). Huh-7 cells that lack NTCP were used as a control to show Myrcludex B specificity for NTCP.

a.



b.

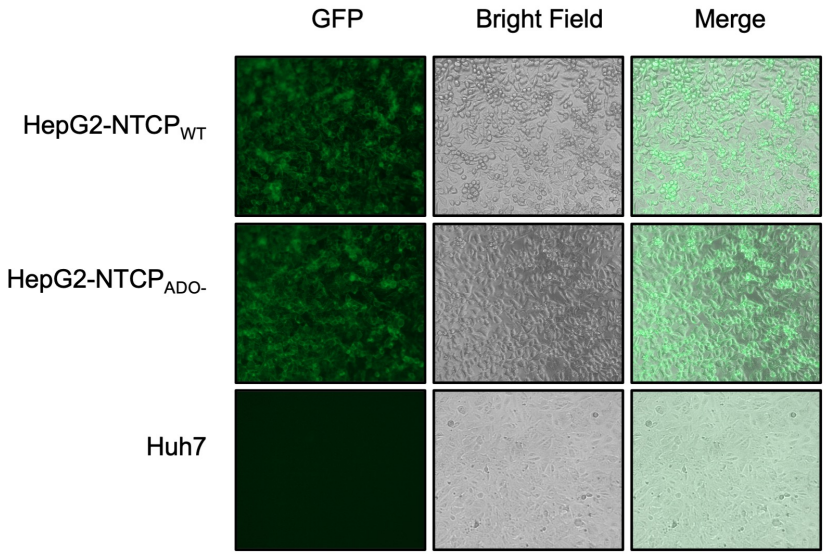


Figure 5.9. Validation of HepG2-NTCP cell phenotype with ADO knockout. (a) Wildtype (HepG2-NTCP$_{WT}$) or ADO KO HepG2-NTCP cells were assessed for ADO protein and its target protein IL-32 by western blot. (b) The NTCP inhibitor Myrcludex B tagged with GFP was used to assess NTCP expression level in HepG2-NTCP$_{WT}$ and HepG2-NTCP$_{ADO-}$ cells, as well as the negative control Huh-7 cells which do not express NTCP. Representative images at 40x magnification are shown. ADO and IL-32 western blot assessment conducted with support from Thomas Keeley.

Culturing infected HepG2-NTCP$_{WT}$ and HepG2-NTCP$_{ADO-}$ cells under 1% oxygen tension increased viral RNA levels in both the parental and ADO knock-out cells (Figure 5.10a). The HIF target genes *CAIX* and *VEGFA* were induced to comparable levels in both lines, demonstrating similar scales of HIF target gene response in both lines (Figure 5.10b, Figure 5.10c). However, there is no significant difference between HBV RNA copy numbers in HepG2-NTCP$_{WT}$ and HepG2-NTCP$_{ADO-}$ at 18% or 1% oxygen tension (Figure 5.10a).

As IL-32 has been reported to restrict HBV replication, its anti-viral activity in HepG2-HBV-pEpi cells was evaluated by transient transfection of pcDNA vectors expressing IL-32 (pcDNA-IL32) or GFP (pcDNA-GFP). IL-32 expression was confirmed (Figure 5.10b) and this associated with a modest but significant reduction in HBV RNA compared to control pcDNA-GFP expressing cells (Figure 5.10b). To study the response of this model system under hypoxia, HepG2-HBV-pEpi cells transfected with pcDNA-IL32 or pcDNA-GFP were cultured under 18% or 1% oxygen tension for 72 hours. Although IL-32 expression reduced HBV RNA transcription at 18% oxygen tension, there is no significant difference in viral RNA levels between pcDNA-IL32 and pcDNA-GFP transfected HepG2-HBV-pEpi cells at 1% oxygen tension, suggesting that hypoxia may supress IL-32 antiviral activity (Figure 5.10c).

Collectively, these results demonstrate that hypoxia could regulate HBV replication through both HIF dependent and independent mechanisms. Evidences presented show that HIF is partially responsible for positive regulation of HBV transcription under hypoxia. Hypoxia could downregulate the HBV restriction factor Smc5/6 complex through a HIF independent pathway and may inhibit antiviral effects of the viral restriction factor IL-32, which is a target regulated by the ADO oxygen sensing pathway.

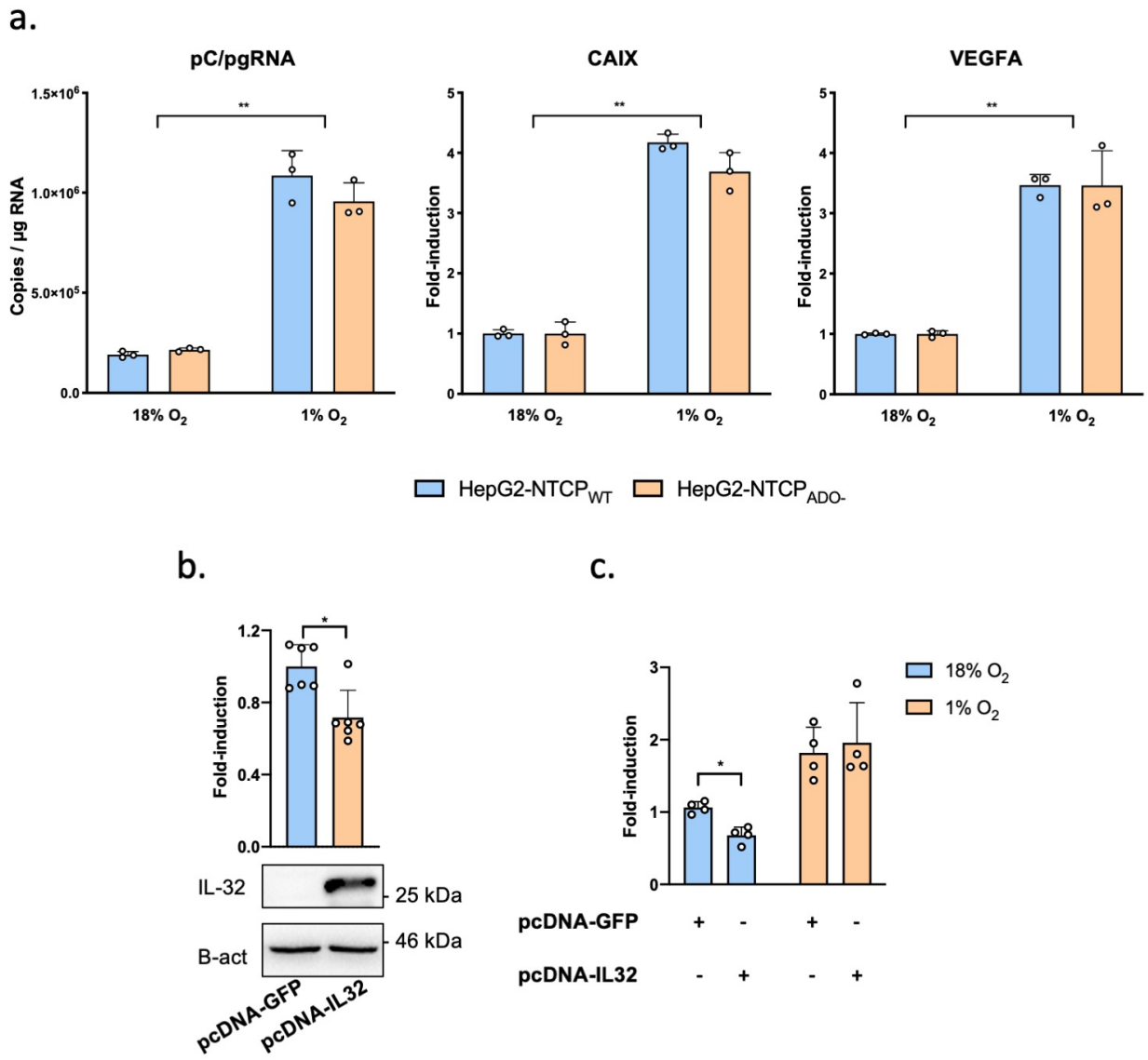


Figure 5.10. Effect of ADO, IL-32, and hypoxia on HBV transcription. Figure legend on the following page.

Figure 5.10. Effect of ADO, IL-32, and hypoxia on HBV transcription. (a) Wildtype (HepG2-$NTCP_{WT}$) or ADO KO HepG2-NTCP cells were infected with HBV (200 MOI), cultured under 18% or 1% oxygen tension for 72 hours. HBV pC/pgRNA and transcripts of the HIF target genes *CAIX* and *VEGFA* were assessed with qPCR. (b) pcDNA vectors expressing IL-32 (pcDNA-IL32) or GFP (pcDNA-GFP) as control were delivered into HepG2-HBV-pEpi cells. 72 hours later, HBV pC/pgRNA was assessed by qPCR and IL-32 expression confirmed by western blot. (c) HepG2-HBV-pEpi cells were transfected with pcDNA-IL32 or pcDNA-GFP and cultured under 18% or 1% oxygen tension for 72 hours. HBV pC/pgRNA was assessed with qPCR. Statistical analysis was performed using Mann-Whitney test (*p⩽0.05, **p⩽0.01). Panel (a) data is representative of results from 3 biological repeat. Panel (b) data is representative of results from 6 biological repeats. Panel (c) data is representative of results from 4 biological repeats. Error bar represents standard deviation.

5.3 Discussion

5.3.1 The dynamic and complex role of hypoxia in regulating HBV transcription

The previous chapter confirmed a role for HIFs in regulating HBV transcription. Although the PHD-HIF pathway is the most well studied oxygen sensing mechanism, a number of other cellular oxygen sensing modalities have evolved in response to changes in oxygen tension in humans and animals[457]. This chapter explored a role for low oxygen in regulating HBV transcription. Low oxygen tension significantly induced HBV transcription in three distinct *in vitro* model systems (Figure 5.1). Further studies show that hypoxia regulates the host restriction factor Smc5/6 through a HIF independent mechanism and limits the anti-viral activity of IL-32 (Figure5.4, Figure 5.10). These case studies illustrate an important but complex role for low oxygen in the regulation of HBV transcription.

Hypoxia induction of HBV transcription is a time-dependent phenomenon, where pC/pgRNA increases across 72 hours under hypoxia incubation in HepG2-HBV-pEpi cells (Figure 5.1). In contrast, the hypoxic induction of host HIF target genes *CAIX* and *VEGFA* remained relatively consistent across 72 hours. This observation could reflect a negative feedback loop for host HIF target genes and the self-replicating capacity of HBV genome. For example, both PHD2 and PHD3 have been identified as HIF target genes with putative HIF binding sites present near their promoter regions[384,458]. Induction of PHDs by HIFs provides a negative feedback loop and may contribute to the observation that HIF target gene transcripts were expressed at comparable levels over time. Additional feedback mechanisms including metabolic regulation, inflammatory response, and HIF

target gene feedback have been reported to tightly regulate the PHD-HIF oxygen sensing pathway[459-461]. Differential stability between host and HBV transcripts may also have a role underlying observation from this chapter. Time dependent upregulation of HBV transcripts by hypoxia suggests that HBV is able to overcome the impact of negative feedback loops on HIF. Unlike host target genes, the HBV genome is a self-replicating unit once inside the host, which could explain the time-dependent increase in HBV transcription under hypoxia[175].

HBV transcription was induced under both physiological oxygen tension at 3% and more severe hypoxia at 1% *in vitro* (Figure 5.2). However, HBV pC/pgRNA was induced by a significantly higher degree under 1% oxygen tension compared to 3%. In contrast, the HIF target genes *CAIX* and *VEGFA* were induced by comparable levels at 3% or 1% oxygen tension with no significant difference. These results suggest that additional oxygen sensing mechanisms that are active at 1% oxygen tension but not 3% could contribute to HBV regulation. Indeed, siRNA HIF silencing experiments under 1% oxygen tension confirm that the PHD-HIF pathway is partially responsible for regulating HBV transcription under hypoxia (Figure 5.3). Different degrees of hypoxia are known to induce distinct transcriptional responses. For example, incubation under 1%, 4% and 9% oxygen tension induces distinct transcriptional programs in astrocytes, with cardiovascular and haemostatic signaling being more active at physiological oxygen tension, whilst cell cycle, immune and cancer associated pathways become significantly more upregulated under severe hypoxia[462]. Another study suggests that moderate and severe hypoxia differentially regulate circadian programming and blood pressure regulation[463]. Moderate hypoxia only modestly lowered the amplitude of circadian rhythm while severe hypoxia completely abolished endogenous circadian blood pressure patterns. Furthermore, *Drosophila* with null mutation in their functional homologue of HIF-α remain viable and fertile compared to wildtype under atmospheric oxygen

tension, suggesting possible redundancy in oxygen sensing mechanisms[464]. These results align with the observation that both HIF dependent and independent mechanisms are responsible for regulating HBV transcription under different oxygen tensions. As the liver harbours an oxygen gradient with different hypoxic gene transcription profiles, the different degree of HBV transcription induction between 1% and 3% oxygen tension suggests a potential role for hypoxia in determining viral tropism[116,175]. This is further supported by observations from the previous chapter, where HBcAg is expressed in a pericentral pattern in mouse livers, consistent with a preference for a lower oxygen environment.

5.3.2 Implications of hypoxia downregulation of Smc6

Results from this chapter suggest that hypoxia downregulates the HBV restriction factor Smc6 through a HIF independent mechanism. Smc5/6 is indispensable for genome stability by resolving stalled replication forks and repairing double-stranded DNA damage[51-53]. The Smc5/6 complex has an essential role in the alternative lengthening of telomere pathway, which is the major telomere maintenance process utilised by astrocytoma with isocitrate dehydrogenase 1 (IDH1) mutations[465]. Hypoxia has been identified as an important regulator of IDH1 and can enhance telomere extension[466,467]. These observations provide a physiological relevance for findings in this present study.

Surprisingly, oxygen reperfusion, proteasomal inhibition, PHD inhibition and VHL deletion experiments suggest that hypoxia degrades Smc6 through a VHL and PHD dependent proteasomal pathway. This observation suggests that novel PHD substrates could regulate Smc6. A number of non-HIF substrates of PHD have been reported in the literature. For example, studies have reported that PHD1 and PHD3 hydroxylate the key tumour suppressor P53 protein at position p142 and p359, respectively[468,469]. PHD2 has been reported to hydroxylate the HIF target gene NDRG3 at position p294[470]. However, when Cockman *et al.* deployed a rigorous *in vitro* biochemical and mass spectrometry approach to validate reported PHD substrates in the literature, they found that HIF-α subunits were the only proteins that could be hydroxylated on their proline residues by PHDs[471,472]. As a result, the existence of primary PHD substrates other than HIFs awaits further validation. Since PHD hydroxylation of non-HIF substrates may require different co-factors and enzymes in cells and animals, *in vivo* validation will provide further confirmation on whether HIF is the only primary PHD substrate[471-473].

To identify relevant proteins for the regulation of Smc6 under hypoxia, a list of potential PHD substrates provided by Cockman *et al.* was studied. However, no potential pathways were identified[471]. Nonetheless, both PHD and VHL have been reported to regulate HIF independent processes, which is consistent with results on Smc6 from this chapter and suggest the possibility of additional targets[472-474]. To the best of our knowledge this is the first report identifying a role for hypoxia to regulate Smc6 and establishes the foundation for further studies linking these two essential mechanisms.

The Smc family of proteins serve important roles in genome stability maintenance, replication, and repair[475]. Proteomic investigations have observed that Smc family proteins including Smc2, Smc3, Smc4, and Smchd1 are downregulated in a subtype of early-stage HCC where cells closely resemble wildtype hepatocytes[476]. However, in HCC subtypes that are characterised by upregulated proliferative proteins that resemble more of a cancer and less of a wildtype hepatocyte phenotype, Smc family proteins including Smc2, Smc3, Smc4, and Smchd1 are upregulated. Given the dynamic physiological low oxygen environment in the liver, the observation that hypoxia downregulates Smc6 has potentially important implications for HBV pathogenesis and HCC.

5.3.3 ADO and IL-32 regulation of HBV transcription

In another series of experiments to elucidate a HIF independent mechanism of HBV regulation under hypoxia, the recently discovered ADO oxygen sensing pathway was examined as a case study. The ADO target protein IL-32 was reported to restrict HBV replication (Figure 5.10)[451]. However, the stabilisation of IL-32 under hypoxia appeared to contradict observations that low oxygen positively regulates HBV transcription. Further experiments suggest that low oxygen may inhibit anti-viral effects of IL-32 on HBV transcription.

Collectively, observations on HIF dependent and independent regulatory mechanisms of HBV transcription under hypoxia suggest a complex network of potentially opposing pathways. The net outcome of these mechanisms results in the positive regulation of HBV transcription under hypoxia. While HIF stabilisation and Smc6 downregulation under hypoxia could positively regulate HBV transcription, IL-32 stabilisation may downregulate viral transcription, but its effects could be abolished by other processes that occur under hypoxia. For example, HNF4 and HNF1 bind EnhI and EnhII in the BCP region of the HBV genome to induce pgRNA[451]. Independent groups have reported that hypoxia and IL-32 downregulate HNF4α and HNF1α to inhibit HBV transcription[451,453]. In the previous chapter, two HREs were identified within EnhI and between EnhI and EnhII in the BCP, which were in similar regions to where HNF4 and HNF1 bind the HBV genomes. This observation raises the possibility that HIF transcription factor binding to the HBV genome under hypoxia could potentially compete with or displace HNF4 and HNF1, leading to an increase in HBV transcription. This scenario illustrates potential dynamic interactions between transcription factors with different binding affinity and transcription rate in regulating HBV replication under hypoxia.

5.4 Summary

This chapter presents evidence demonstrating that low oxygen tension regulates HBV transcription through both HIF dependent and HIF independent mechanisms. Low oxygen upregulates HBV transcription in a time-dependent manner, and 1% oxygen tension induced viral transcript to a greater degree than 3% oxygen tension, suggesting multiple oxygen sensing mechanisms can regulate HBV replication. This conclusion was further confirmed through HIF siRNA silencing experiments under hypoxia. Hypoxia downregulation of the viral restriction factor Smc6 and suppression of anti-HBV activity mediated by the ADO oxygen sensing pathway target IL-32 were explored as two case studies for HIF independent HBV regulation under low oxygen.

Results from this chapter raise a number of questions that can be addressed to better understand the role of low oxygen in regulating HBV transcription. Firstly, data suggesting that hypoxia could regulate the Smc5/6 complex through a PHD/VHL dependent but HIF independent proteasomal degradation mechanism warrants further investigation. Whether PHDs have non-HIF primary substrates is controversial, and a better understanding of the underlying mechanism for low oxygen regulation of Smc5/6 could contribute to identifying potential novel PHD substrates. Secondly, stabilisation of IL-32 as an ADO target and inhibition of its anti-viral activity under low oxygen demonstrate that a number of conflicting regulatory mechanisms could be active under low oxygen. Therefore, further experiments to identify the underlying cause of IL-32 anti-HBV activity suppression under low oxygen is required and could help identify potential therapeutic targets. Thirdly, the observed induction in HBV transcription represents a net positive effect arising from the impact of various active oxygen sensing mechanisms under low oxygen. Further exploration will help elucidate the complex interplay between various oxygen sensing mechanisms in HBV

regulation. Finally, the impact of hypoxia and anaemia on HBV replication should be further assessed in animal model systems to extend upon the *in vitro* observations reported in this chapter. In conclusion, this chapter establishes an important role for low oxygen in positively regulating HBV transcription and generated new directions to explore novel therapeutic targets related to oxygen sensing mechanisms.

6. DISCUSSION

6.1 CHB, Inflammation, and Hypoxia Signaling

This thesis examined the interplay between HBV replication, PHD-HIF signaling, and oxygen sensing mechanisms. Bioinformatic analysis of hypoxia gene signatures derived from HepG2, PHH and the molecular signature data base show evidence for an increased HIF transcriptional response in CHB[304]. Further inflammatory gene signature analysis in a CHB cohort suggests that HBV infection is associated with HIF target gene upregulation and inflammation. Validation in *in vitro* and *in vivo* HBV model systems, including HBV *de novo* infection and liver derived cells expressing viral proteins, HBV siRNA silencing in HBV transgenic mice, and HBV infection in chimeric liver mice did not recapitulate HIF target gene upregulation observed in CHB patients. These results suggest that existing HBV model systems do not capture the complexity and inflammatory processes underlying HBV infection and pathogenesis. Further research is required to develop more sophisticated model systems to facilitate the understanding of HBV infection and pathogenesis and enable the design and evaluation of new treatments.

Chronic HBV infection, the leading cause of liver cancer worldwide, remains incurable. Finding a cure for HBV infection is a goal at the forefront of the agenda for hepatitis research[477]. Many studies have been focusing on developing treatments that can achieve a functional cure as defined by the loss of detectable serum HBsAg[478]. The use of nucleoside or nucleotide drugs (NUCs) to inhibit HBV reverse transcription and interferon alpha (IFN-α) to suppress viral transcription as the current standard of care can achieve HBsAg loss only in a limited number of patients (5-7%)[69].

However, definitive sterilising cure characterised by the elimination of cccDNA as the HBV reservoir remains elusive and the majority of patients continue to support viral replication whilst on long term NUC treatment. The persistence of cccDNA, insufficient immune response, and lack of model systems that recapitulate the complex interaction between HBV and the host are major factors hindering the development of more effective therapeutics. Current therapies cannot fully eradicate cccDNA, likely due to its stability and replenishment through new rounds of infection to maintain a viral reservoir. HBV cccDNA half-life is estimated to be 40 days in infected HepG2-NTCP cells, 61 days in HBeAg positive infections, and 26 days in HBeAg negative infections due to greater cccDNA clearance rates[327,479]. Mathematical modelling suggests that having fewer hepatocytes infected with HBV could double cccDNA half-life as a result of reduced cytosolic immune response and lower hepatocyte mitosis rate, potentially resulting in more sustained maintenance of cccDNA reservoir. In addition to challenges associated with cccDNA reservoir elimination, the lack of effective $CD4^+$ and $CD8^+$ T-cell response against HBV limits sustained viral control[480]. As illustrated by results from chapter 3, more effective model systems to study the underlying mechanisms for the interaction between HBV and host immune pathways are essential for developing more effective treatment modalities against HBV infection. In contrast, treatments that led to a cure for HCV were developed as more effective *in vitro* and *in vivo* model systems became available to dissect the viral life cycle and identify promising therapeutic targets[481]. For example, enzymatic screening assays for specific HCV protease and polymerase were developed and enabled efficient testing of direct-acting antiviral (DAA) compounds[482]. However, the lack of approaches to produce functional recombinant HBV polymerase for enzymatic screening assays has limited drug development[483].

Results from this thesis show a complex interplay between inflammation, HBV and hypoxia that are not recapitulated with current *in vitro* and *in vivo* model systems. Humans and chimpanzees are the only two primates that are susceptible to HBV infection[392,484]. HBV infection in chimpanzees presents the highest level of similarity to humans among animal models but infection is nonetheless cleared without significant liver disease induction in the majority of cases[485,486]. Furthermore, the use of apes including chimpanzees in research is restricted in many countries globally including the UK, and other *in vivo* model systems need to be deployed[487]. As discussed in chapter 3, the woodchuck hepatitis virus, immunocompetent mouse, or immune cell co-culture model systems may be deployed to study the interplay between HBV, HIF and immune response, but each has their respective caveats[84,396,397,488]. Anti-HBV T-cell immune response has an important role in facilitating cccDNA elimination in humans and chimpanzees. Therefore, understanding the role of inflammatory pathways in the interplay between HBV and hypoxia could help contribute to developing treatments for the eradication of cccDNA[477,489].

Given that HIF signaling is activated in CHB and that the liver has a low oxygen environment, this thesis explored the role of HIFs in HBV transcription[115]. Two evolutionarily conserved HREs were discovered at EnhI (1236-1240) and between EnhI and EnhII (1604-1599), near the BCP which drives major viral transcripts including pC/pgRNA[72]. Conservation of these motifs in humans, higher primates, and woodchucks suggests a conserved function of HIFs in the viral replicative life cycle. Further experiments using *in vitro* HBV *de novo* infection models and siRNA silencing in HBV transgenic mice demonstrate a role for HIFs in HBV transcription. The presence of conserved HREs in the HBV genome and HIFs as host transcription factors that can promote HBV transcription have important implications for viral tropism and suggest that specific animal model systems could be suitable for the study of HBV and hypoxic response.

To confirm a role of hypoxia in regulating HBV, experiments using three distinct models of HBV infection show that a low oxygen environment positively regulates HBV transcription. Further studies show that hypoxia increases HBV transcription in a time-dependent manner and that 1% oxygen tension induces HBV transcripts to a significantly higher level than 3% oxygen tension. However, there is no significant difference in HIF target gene induction between 1% and 3% oxygen tension, suggesting that additional HIF independent oxygen sensing mechanisms could have a role in regulating HBV transcription. A number of enzymatic processes in humans are oxygen dependent and can be regulated at different oxygen tensions in addition to the PHD-HIF pathway. For example, the 2-oxoglutarate (2-OG)-dependent oxygenase superfamily of enzymes rely on the presence of oxygen to regulate diverse oxidative reactions with substrates including DNA, RNA, proteins and intermediate metabolites[490]. Notable examples of members of this superfamily include PHD and FIH, two important oxygen sensors regulating HIF proteins with different levels of sensitivity to oxygen availability[491]. As discussed in earlier chapters, other newly discovered oxygen sensing processes with varying dependency on oxygen availability include KDM 5/6 which regulate epigenetic modification and ADO which regulates protein substrates including IL-32 and RGS4[120,121,233]. Therefore, siRNA silencing experiments were conducted to assess a role for HIFs to promote HBV transcription under hypoxia and results suggest that HIFs are only partially responsible.

The possibility of additional HIF independent mechanisms regulating HBV transcription prompted further experiments to explore other processes. A series of experiments on Smc6 show hypoxia downregulation of this viral restriction factor is mediated by VHL-PHD dependent but HIF independent proteasomal degradation. Downregulation of Smc6 through hypoxic and viral mediated pathways could lead to important pathological consequences such as HCC, because of

the important role of Smc5/6 complex in telomere regulation and double-stranded DNA damage repair[51-53].

Another case study examining the HBV restriction factor IL-32 which is stabilised under hypoxia through the ADO oxygen sensing pathway shows that IL-32 anti-viral effects may be suppressed under 1% oxygen tension. An earlier study reported that IL-32 inhibited HBV transcription through downregulation of host transcriptional activators, including HNF4 and HNF1[451,453]. Over two decades of research on host factors that can regulate HBV transcription have identified a number of proteins that could positively or negatively regulate viral transcription[19]. Results from this thesis on the role of IL-32 in HBV transcription under hypoxia warrant further investigations to better understand how HBV transcription factors could be regulated under low oxygen environments. Collectively, these results highlight the complex interplay between HBV and oxygen sensitive pathways in the liver. Conclusions from chapter 5 suggest that the interaction between hypoxia and HBV is an important direction of research to pursue and could help illuminate novel insights on fundamental host oxygen sensing processes and HBV therapeutic targets.

This thesis has illustrated a role for HIFs to positively regulate HBV transcription. This discovery has important implications for developing new model systems for studying the viral life cycle. The majority of *in vitro* studies on HBV replication studies have been conducted under atmospheric oxygen tension, where a myriad of low oxygen and hypoxia induced mechanisms are inactive. The use of chemical compounds such as DMSO to arrest cells and maintain hepatocyte differentiation is widely used despite its broad impact on host gene expression[414,415]. Indeed, results from this thesis and past studies suggest that the high concentration of 2.5% DMSO used for HBV *de novo*

infection model systems interferes with HIF signaling[72]. Drastic alterations in epigenetic patterns and miRNA expression have been proposed as potential mechanisms for DMSO associated changes in gene expression profile[492]. Studying HBV replication under a low oxygen environment could provide a more physiologically relevant model system to studying viral-host cell interplay.

6.2 Targeting Oxygen Sensing Mechanisms as Novel Therapeutic Modalities for HBV Infection

The discovery that HIFs induce pC/pgRNA transcription has implications for viral replication. Results from this thesis suggest targeting HBV transcription by inhibiting HIF could help to reduce cccDNA reservoir in infected patients. HBV pC/pgRNA is reverse-transcribed to generate rcDNA which can be reimported to the nucleus or secreted as infectious particles[19]. Nuclear rcDNA could be repaired to cccDNA and replenish existing viral reservoir whilst rcDNA in secreted particles can initiate new rounds of HBV infection. Therefore, limiting pC/pgRNA levels by targeting HIF warrants further exploration. Because HIF target genes including PAI-1 and HO1 have important roles in inflammation in the liver, understanding the impact of HIF inhibition on HBV replication in the context of immune signaling could help guide future therapy development[130-132]. As discussed in previous chapters, a number of HIF inhibitors are undergoing clinical trials for malignancies such as Kaposi's Sarcoma, rectal cancer, and renal carcinoma[440,446,493]. Thus, existing HIF inhibitors could be available for preclinical and clinical validation as a potential treatment strategy for HBV infection. However, as HIF signaling is an essential pathway, studies should also explore the effects of long-term HIF inhibition for the treatment of chronic HBV infection.

6.3 Implications of the Interplay Between HIF and HBV in Anaemia Patients

This thesis has presented *in vitro*, *in vivo*, and patient data on the interplay between HBV replication, hypoxia and oxygen sensing mechanisms. However, implications for these interactions in various clinical settings remain unclear. One particular question of interest is whether anaemia has a role in pathogenesis and disease progression. Organs and tissues in anaemia patients are exposed to lower oxygen tensions and represent a physiologically relevant system to translate the findings of this thesis. Although aplastic anaemia has been documented in HBV infected patients, no investigations have examined whether it is linked to higher susceptibility to infection or altered disease progression[494]. Understanding the role of anaemia in chronic HBV infection through large scale epidemiological studies could support laboratory based studies on the regulation of HBV. Findings from these studies could provide important information on biomarkers that can help guide prognosis determination and treatment decision-making to impact existing guidelines and patient outcome.

7. FUTURE DIRECTIONS

7.1 Epidemiological Studies on the Interplay Between HIFs and HBV Infection

This thesis demonstrated that HIF and low oxygen tension regulate HBV transcription with *in vitro* and *in vivo* results. Published chromatin immunoprecipitation experiment showing that HIFs bind HBV DNA in human hepatocytes from infected liver chimeric mice provide further evidence on the interaction between the HBV genome and HIFs[72]. However, to understand whether these interactions have tangible impacts on HBV infection and CHB progression in humans, large-scale epidemiolocal studies are required. A number of HBV infected cohorts have been assessed longitudinally in China. For example, 1,638 HBV infected patients in the city of Qidong, China were monitored prospectively and annually for 25 years to assess causal factors for primary liver cancer[495]. Patient blood samples and medical history were recorded. Assessing patient blood biobank and medical records in this cohort could help identify whether anaemia is associated with differential HBV disease outcomes and risks for primary liver cancer. Another potential cohort to assess contains 322 biopsy-confirmed CHB patients in Shanghai, China[496]. As laboratory tests and medical records are available for this retrospective cohort, the relationship between CHB and anaemia could be assessed. A number of HIF-1α single nucleotide polymorphisms including those causing missense mutations and at least 14 types of malignancies have been identified[497]. Screening for HIF-1α polymorphisms and identifying their association with HBV infection and CHB disease progressions in established patient cohorts could help further validate and build on results from this thesis.

Another clinically relevant question to assess is the impact of increased HIF signaling in CHB progression and pathogenesis. HIF signaling is known to be associated with carcinogenesis and HCC as reviewed throughout this thesis. Although HIF-1α usually serves a tumour suppressor role in a number of tissues, reports suggest it could induce cancer cell growth and proliferation in the liver[156]. Similarly, HIF-2α is known to serve an oncogenic role and has been associated with glioblastoma, colorectal cancer, and lung cancer[143,164-166]. Therefore, the phenotypic impact of HIF induction in CHB and its role in HCC development should be further investigated. If patient liver samples are available from the Qidong cohort which focused on primary liver cancer, they could be assessed for HIF and target gene expression and association with HCC[495].

7.2 Molecular Characterisation of HBV, HRE, and Hypoxia Interactions

ChIP experiments confirmed that both HIF-1α and HIF-2α can bind the BCP region of the HBV genome[72]. However, the precise binding affinity and transcriptional activity of HIFs to each HRE in the HBV genome remain to be elucidated. Site-directed PCR mutagenesis techniques targeted at HRE-1 and HRE-2 in the HBV BCP region could be deployed to assess HIF binding and transcriptional activity specific to each HRE. This approach could help further define specific sites for targeted novel therapeutic development[498]. In addition, the role of HIF-3 in regulating HBV replication remains unknown and could be studied in future investigations.

As this thesis focused on the impact of hypoxia on HBV genotype D, future investigations could further explore the effect of low oxygen tension on different HBV genotypes. Interestingly, HBV genotype H is the only human HBV genotype without conserved HREs in its genome and data suggests it is insensitive to hypoxia regulation[72].

Further experiments could also be conducted to assess the impact of hypoxia on HBV RNA stability. Although hypoxia increases the total number of pC/pgRNA, whether RNA stability is affected remains unknown. Recent studies suggest that N6-methyladenosine (M^6A) RNA methylation sites are present on both the 5' and 3' ends of HBV pgRNA[499]. The presence of M^6A at these sites directly regulates pgRNA stability and reverse transcription. Assessing the interaction between hypoxia and M^6A methylation in HBV could reveal novel insights on viral RNA regulation.

7.3 Role of HIFs in Regulating HBV Particle Genesis, Infectivity, and Viral Spread

Data from this thesis suggests that HIF and hypoxia upregulate HBV pC/pgRNA transcripts in *de novo* HBV infection. Recently published results suggest that low oxygen tension increases the levels of extracellular HBV DNA[72]. However, whether HBV transcripts are upregulated at the single cell level or if more hepatocytes are infected under hypoxia remain unclear. Results from HBV transgenic mice in Chapter 4 show that HIF-1β silencing leads to reduced number of liver cells staining positive for HBcAg, suggesting that HIF could promote HBV infectivity and viral spread. However, further evidence is required to consolidate this observation. Fluorescent imaging and enumeration of cells expressing HBcAg protein under hypoxia in a *de novo* HBV infection model system could further clarify if hypoxia and HIFs increase HBV spread[327]. Furthermore, whether hypoxia increases the infectivity of viral particles remains to be assessed. Cell-free supernatants containing HBV particles could be extracted and applied in *de novo* infection experiments to assess the impact of low oxygen tension on viral infectivity. Other potential investigations include experiments to understand the impact of hypoxia on HBV entry, and host NTCP expression. A synchronised HBV *de novo* infection approach to assess viral entry was reported in past studies and could be applied to assess the impact of hypoxia on HBV entry[500].

7.4 HIF Independent Regulation of HBV Replication Under Hypoxia

Results from this thesis suggest both HIF dependent and independent mechanisms could regulate HBV transcription under hypoxia. The KDMs are members of the 2-OG-dependent oxygenase family of enzymes that have important roles in epigenetic regulation. Recent studies suggest that KDM5A and KDM6A are oxygen sensors and mediate chromatin methylation under hypoxia[121,122]. As HBV cccDNA is known to behave as a mini chromosome and subject to epigenetic regulation in the host, future research could examine the role of KDMs and epigenetic regulation in HBV replication under hypoxia[16].

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