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CHARACTERIZATION OF THE ROLE OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 7 (IGFBP7) USING A GENETIC KNOCKOUT MOUSE MODEL

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in Human Genetics at Virginia Commonwealth University, Richmond, Virginia, United States of America.

by:

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DEDICATION

I dedicate this dissertation to my father Abdullah Akiel and my mother, Faaten Akiel. The dedication also extends to my siblings, Nouf Akiel, Nada Akiel, and Michael Akiel. Last but not least, the dedication also reaches the new member of the family my nephew, Mansour Al-homaid.

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- **AASLD:** American Association for the Study of Liver Diseases.
- **AFP:** α-feto protein.
- **BCLC:** Barcelona Clinic Liver Cancer.
- **BrdU:** 5-bromo-2'-deoxyuridine.
- **DEN:** Diethyl- nitrosamine.
- **EGFR:** Epidermal Growth Factor Receptor.
- **EGF:** Epidermal Growth Factor.
- FDA: Food and Drug Administration.
- **GF:** Growth Factor.
- **GSK-3β:** Glycogen synthase kinase 3 β subunit.
- GPCR: G protein-coupled receptors.
- **HBV:** Hepatitis B Virus.
- **HCC:** Hepatocellular Carcinoma.
- **HCV:** Hepatitis C Virus.
- **HRAS:** Harvey Ras.
- **IGF:** Insulin Growth Factor.
- **IGFBP:** Insulin Growth Factor Binding Protein.
- **IGF-R:** IGF- receptors.
- **IGFBP7:** Insulin-like growth factor binding protein-7.
- **IL6:** Interleukin 6.

- **IL10:** Interleukin 10
- **IL1β:** Interleukin 1β.
- LRR: Leucine-Rich repeats.
- **MAPK1**: Mitogen Activated Protein Kinase 1.
- MCA: 3'-methylcholanthrene 1.
- **MDSC:** Myeloid-derived suppressor cells.
- **MHC:** Major histocompatibility complex.
- **MTT:** 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide.
- **mTOR:** mammalian Target Of Rapamycin.
- mTORC2: rapamycin-insensitive mammalian target of rapamycin 2.
- **NAFLD:** Non-alcoholic fatty liver disease.
- NK-cells: Natural Killer cells.
- **PCNA:** Proliferating cell nuclear antigen.
- PD-1: Programmed cell death protein 1.
- PD-L1: Programmed cell death protein ligand 1.
- PH: pleckstrin homology domain.
- **PI3K:** Phosphatidylinositol 3-kinases.
- PTEN: phosphatase and tensin homolog deleted on chromosome 10.
- **pRB:** pophorylated Retinoblastoma.
- RAG2: Recombination Activating 2.
- RAE-1: Ribonucleic Acid Export 1.
- SOCS-1: Suppressors of Cytokine Signaling-1.
- **STAT3:** Signal Transducer and Activator of Transcription 3.

- TLR: Toll-like receptor.
- TGF-β: Transforning Growth Factor β.
- **TNFα:** Tumor Necrosis Factor α.
- **VEGF:** Vascular Endothelial Growth Factor.
- 4E-BP1: Eukaryotic translation initiation factor 4E–binding protein 1.

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ABSTRACT

CHARACTERIZATION OF THE ROLE OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 7 (IGFBP7) USING A GENETIC KNOCKOUT MOUSE MODEL

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in Human Genetics at Virginia Commonwealth University,

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In the US, the incidence and mortality rates of hepatocellular carcinoma (HCC) are alarmingly increasing since no effective therapy is available for the advanced disease. Activation of IGF signaling is a major oncogenic event in diverse cancers, including HCC. Insulin-like growth factor binding protein-7 (IGFBP7) inhibits IGF signaling by binding to IGF-1 receptor (IGF-1R) and functions as a potential tumor suppressor for hepatocellular carcinoma (HCC). IGFBP7 abrogates tumors by inducing cancer-specific senescence and apoptosis and inhibiting angiogenesis. We now document that Igfbp7 knockout (Igfbp7-/-) mouse shows constitutive activation of IGF signaling, presents with pro-inflammatory and immunosuppressive microenvironment, and develops spontaneous tumors in lungs and liver and markedly accelerated carcinogen-induced HCC. Loss of Igfbp7 resulted in increased proliferation and decreased senescence in hepatocytes and mouse embryonic fibroblasts that could be blocked by an IGF-1 receptor inhibitor. A significant inhibition of genes regulating immune surveillance was observed in Igfbp7-/- livers which was

associated with marked inhibition in antigen cross presentation by Igfbp7-/- dendritic cells. IGFBP7 overexpression inhibited growth of HCC cells in syngeneic immune competent mice, which could be abolished by depletion of CD4+ or CD8+ T lymphocytes. Our studies unravel modulation of immune response as a novel component of pleiotropic mechanisms by which IGFBP7 suppresses HCC. Even though HCC has an immunosuppressive milieu, immune targeted therapies are beginning to demonstrate significant objective responses in clinical trials. IGFBP7 might be an effective anti-HCC therapeutic by directly inhibiting cancer cells and stimulating an anti-tumor immune response.

CHAPTER 1: INTRODUCTION

1.1 Hepatocellular Carcinoma (HCC):

Hepatocellular carcinoma (HCC) is a tumor arising from hepatocytes, the epithelial cells of the liver. Macroscopic pathology of HCC is characterized by scattered, large, and multinodular tumors. HCC represents more than 80% of liver cancers. Worldwide, it is a serious life-threatening illness with a poor prognosis. Globally, HCC ranks fifth in terms of cancer prevalence and third in cancer deaths [1, 2]. HCC is considered the fifth most prevalent cancer in males and the seventh in females, and the likelihood of developing HCC is four to five times higher in males than in females [3]. This difference in the occurrence of HCC according to sex is thought to be due to the influence of sex hormones [4, 5] – while androgen enhances the pregression of hepatitis B virus (HBV)-triggered HCC in HBV transgenic male mice, estrogen reduces the development of HCC in female mice on exposure to the hepatocarcinogen, diethylnitrosamine [4-7]. However, the regulatory action by which androgen and estrogen orchestrate the disparity between the sexes is still not fully understood. The number of reported new cases of HCC is escalating each year and HCC has a 5-year survival rate of less than 12%.

The American Cancer Society estimates that in 2016 there will be more than 39,230 new cases of HCC and more than 27,170 reported HCC related deaths in the United States of America, approximately three-fourths of which would be HCC with 2.7% increase since 1980 (updated data not yet available to confirm this estimate) [3, 8]. Chronic HBV infection constitutes 50% of HCC cases around the world (Figure 1.1). In contrast, in the USA, hepatitis C virus (HCV)-related HCC is more common than HBV-related HCC, with the former constituting 48% of all HCC cases in the USA and the latter 16% [8, 9]. Immigrants from countries where

HBV is endemic account for most of the HBV-related HCC cases in the USA [9]. The remaining percentage of cases is unequally distributed across various risk factors that modify disease, such as alcohol-related liver disease and metabolic syndromes [8, 10]. HCV, alcohol-related liver disease, and nonalcoholic fatty liver disease tend to be common risk factors in the USA [3, 8]. HCC is not a population-specific illness; however, statistical data on the incidence of HCC show that Asians, Pacific Islanders, and Native Americans have the highest rate of incidence, followed by African Americans and Caucasians in the USA [9]. The incidence of HCC in the USA has increased from 1.6 to 4.9 cases per 100,000 of US population from 1975 to 2005, an increase of more than 200% [11]. As HCV-related HCC has manifested most commonly around the age of 70, the increase of HCV-related HCC in the USA might be linked to the needle sharing involved in drug use at the time when drug abuse was prevalent (more than 30 years ago), as implied by a cohort study of HCV-infected patients [1, 12].

HCC is a highly heterogeneous cancer, which may be explained by the diversity and increased number of etiological factors [13]. The majority of risk factors leads to chronic liver disease that later progresses to HCC [3]. Patients with HCC have an extremely low health-related quality of life (a marker considered when assessing HCC prognosis) compared with chronic liver disease patients [14]. The American Association for the study of Liver Diseases endorses the use of the Barcelona Clinic Liver Cancer Staging System. The Barcelona Clinic Liver Cancer has implemented a treatment algorithm that tailors a treatment strategy based on the stage of HCC. For example, surgical resection and liver transplantation are offered to patients diagnosed at an early stage resulting in a more or equal to 75% survival rate. In most cases, HCC is diagnosed at an advanced stage at a time when symptoms start to manifest, by which time prognosis is dismal and the aforementioned conventional treatments are no longer efficacious [1-3].

The only US Food and Drug Administration-approved drug available for non-resectable advanced HCC is the multikinase inhibitor sorafenib, which provides a survival advantage of only 2.8 months compared with placebo [15]. Therefore, the identification of new diagnostic markers is mandatory to diagnose and treat patients at a very early stage when prognosis is highly favorable. Additionally, new regulatory molecules need to be identified that might be targeted to develop effective therapeutic approaches for advanced HCC, thereby providing longstanding benefits to patients.

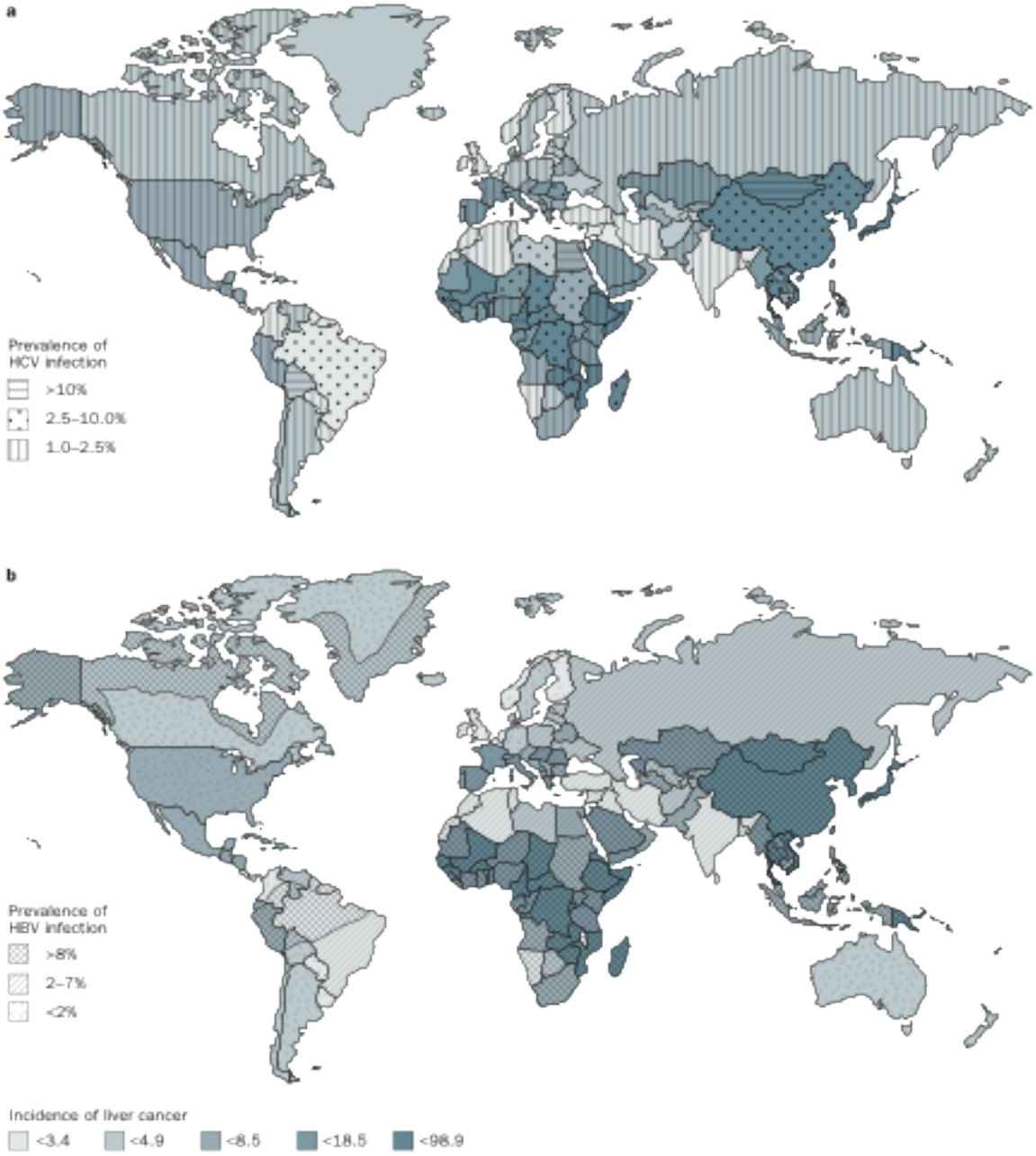


Figure 1.1:

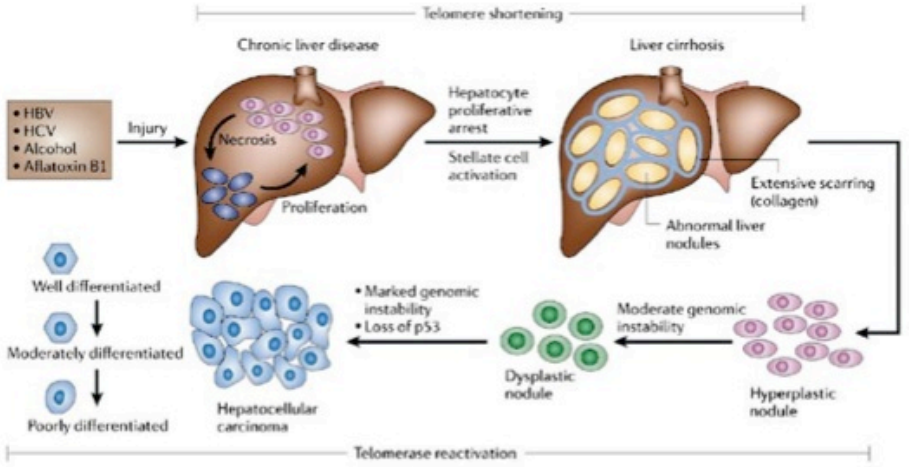
A world map representing the prevalence of HBV and HCV infection and the age adjusted incidence of liver cancer. The figure was adopted from [11]

1.2 Molecular mechanisms of HCC:

Hanahan and Weinberg described molecular deregulations in cancer as "acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate; these functions are acquired in different tumor types via distinct mechanisms and at various times during the course of multistep tumorigenesis" [16]. In HCC, genomic alterations lead to loss of cell control and transformation of a single or group of hepatocytes to proliferate, undifferentiated and ultimately migrate from a primary site to invade and hijack secondary distant organs. This process of carcinogenesis and metastasis is complicated and much of it is largely unexplored [17].

Initiation of HCC requires progressive cellular alterations starting from hepatic progenitor cells "oval cells" or differentiated hepatocyte, which ultimately leads to loss of control of key molecular pathways that regulate cellular homeostasis [18]. As a result, these catastrophic changes facilitate development of hallmarks of cancer [16-18]. In most cases HCC is believed to arise from an inflammatory cirrhotic microenvironment, which will allow the initiation of hyperplastic nodules that will progress to dysplastic nodules leading to a full blown HCC (Figure 1.2) [3, 16, 18]. For these histopathological changes to occur, a multistep deregulation of epigenetic and genetic processes that are involved in regulating cellular behavior and interaction with neighboring normal cells and microenvironment need to occur [16, 18]. Although the majority of HCC cases arise from a cirrhotic microenvironment, there are reported cases where HCC arise from a non-cirrhotic microenvironment [11, 19]. The reason is still unclear, however there are suggestions implicating the exposure of additional risks that might accelerate the process to surpass the need of cirrhosis [11]. Therefore, since Diethylnitrosamine (DEN) induced HCC does not require cirrhosis; it remains an acceptable animal model of HCC in mice.

The inherent complexity and heterogeneity of HCC might be due to the variability in the impact of risk factors [20]. Although gene expression studies of HCC demonstrated that genetic signature of every patients is different than the other, the majority of molecular changes share deregulations in pathways such as EGFR-Ras-MAPKK pathway, IGF signaling, PI3K/Akt/mTOR pathway and inflammatory pathways (such as IL-6), (reviewed in[18, 20, 21]).



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nature REVIEWS CANCER

Figure1.2:

Histopathological progression of Hepatocellular Carcinoma (HCC). This cycle predisposes a chronic liver disease with inflammation, which leads to cirrhosis, hyperplasia, dysplasia and eventually HCC

1.2.1 Ras-MAPK Pathway:

The Ras-Raf-MAPK pathway is activated by binding of growth factors to their cognate Receptor Tyrosine Kinases (RTKs). RTKs are high-affinity cell surface receptors for many polypeptide growth factors, cytokines, and hormones. RTKs are monomeric receptors that have a single hydrophobic transmembrane-spanning domain composed of 25 to 38 amino acids, an extracellular N terminal region, and an intracellular (cytosolic) C terminal regions. The N terminal region of the receptor binds growth factor ligands i.e. Epidermal Growth Factor (EGF) or Insulin-like Growth Factors (IGFs), while the C-terminal region harbors the kinase domain. Binding of the ligand to RTKs brings the monomeric sub units together resulting in autophosphorylation of the receptor to transmit downstream signaling. Closely related RTKs such as IGF1R and Insulin receptor or Erbb family receptors (Erbb 1, 2, 3 and 4), have high homology to each other and therefore they can homodimerize or heterodimerize with each other resulting in a cross talk between the signaling of growth factors, cytokines or hormones. The phosphorylation of specific tyrosine residues within the activated receptor generates a binding site for Src homology 2 (SH2) domain- and phosphotyrosine binding (PTB) domain-containing proteins. Phosphorylation and activation of these two proteins on receptor binding leads to the initiation of signal transduction pathways. Sequentially, Ras gets is activated by increased binding to GTP (mediated by Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)), Raf gets phosphorylated, and then ERK1/2 gets phosphorylated and activated. As result, ERK1/2 translocate to the nucleus to trans-activate transcription factors such as c-fos,-jun or Myc, switching gene expression to wide range of biological activities including promotion of proliferation or mitosis [21, 22].

The implication between activation Ras-MAPK pathway and HCC was observed in most of HCC cases through overexpression of growth factors such as Insulin-like Growth Factor-1 (IGF-1) or Epidermal Growth Factor (EGF) [20]. Overexpression of EGF or its RTK, EGF Receptor correlated with dismal prognosis. Recent studies demonstrated that high levels of tissue and serum EGF or IGF-1 in cirrhotic patients increased the adjusted risk for HCC compared to unaffected individuals [21, 23]. It is worth noting that, activating mutations of Ras are relatively low in HCC. Interestingly, studies on HCC showed that Ras is activated in most of HCC patients [20]. Alternatively, activation of Ras-MAPKK pathway was also seen to be through downregulation of Ras inhibitors such as Spred or Raf inhibitors such as RKIP, which act as phosphatases that deactivate the receptor as a negative feedback loop, and basically this will terminate the signaling. Loss of negative feedback control also leads to constitutive activation of this pathway [20, 24](Figure 1.3).

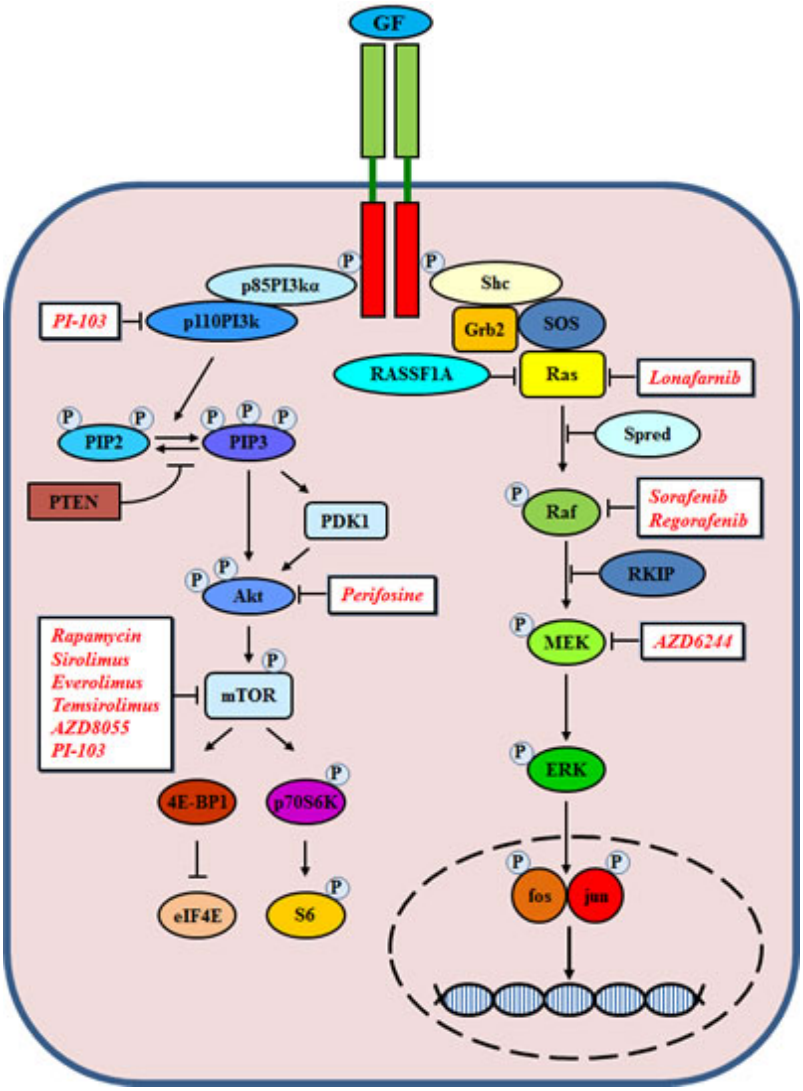


Figure 1.3:

Schematic illustration of a receptor tyrosine kinase receptor signaling upon ligand binding (GF: Growth Factor) such as binding of EGF to EGF receptor. White squares represent current drugs used to block signaling pathway in HCC. "P" denotes the event of phosphorylation. Adopted from [21]

1.2.2 PI3K/Akt/mTOR Pathway:

Phosphoinositide 3-kinase (PI3K) signaling has a pivotal role in cellular physiology, governing insulin signaling during organismal growth and mediating critical biological processes such as glucose homeostasis, protein synthesis, cell proliferation, and survival. This pathway is commonly deregulated in cancer including HCC controlling most hallmarks of cancer, including cell proliferation, survival, and metabolism [16, 20].

PI3Ks are a group of intracellular lipid kinases. PI3Ks are classified into three groups (classes I, II, and III) based on structure and substrate specificity (reviewed in [25]). Class I PI3Ks are the most commonly altered in cancer. Class I PI3Ks mainly phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). The lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) nullifies the activity of PI3Ks by dephosphorylating PIP_3 back into PIP_2, therefore, PTEN acts as a negative feedback control of PI3K. Class I_A PI3Ks are heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit which transduces upstream signals from receptor tyrosine kinases (RTKs), whereas Class I_B transmits signaling of G protein-coupled receptors (GPCRs).

PIP_3 acts as a lipid second messenger that brings cytosolic proteins containing pleckstrin homology (PH) domains to the plasma membrane to promote their activation or co-localization with other effector protein. Of the PH domain containing proteins that do bind PIP_3 is the serine/threonine protein kinase AKT. Full activation of AKT then ensues through phosphorylation by either PDK1 (3-phosphoinositide dependent kinase) at threonine 308 or at serine 473 by rapamycin-insensitive mTOR complex (mTORC2). AKT then in turns phosphorylate the transcription factor FOXO preventing the nuclear translocation of FOXO and

thereby preventing activating transcription of cell-cycle-regulatory genes such as p27Kip1 and proapoptotic genes such as FasL and Bim. By doing so, AKT can increase proliferation and survival. Additionally, AKT phosphorylates TSC1/2. TSC1 (hamartin) and TSC2 (tuberin) form a complex that block activity of the small G protein Rheb. Phosphorylation of TSC2 by AKT removes TSC2 inhibition of Rheb, resulting in activation of the rapamycin-sensitive mTOR1 complex. Activation mTORC1 enhances growth through upregulation of protein synthesis via regulation of the protein synthesis machinery, eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1) and p70S6 kinase. Furthermore, AKT regulates metabolism through phosphorylation of glycogen synthase kinase 3 (GSK3). This phosphorylation inhibits GSK3 function (synthesis of glycogen), which in turn stops storage of glycogen and alters glucose homeostasis. The activation of AKT also leads to phosphorylation of IkB, the inhibitor subunit of NFkB, leading to the release of the of NFkB that translocates into the nucleus to activate transcription of inflammatory target genes such IL-6, IL-1β and TNF-α (reviewed in [25]. In HCC the activation of PI3K/Akt/mTOR pathway takes place at different levels. Constitutive activation of PI3K might result from mutation in PIK3CA (catalytic domain of PI3K), which has been reported in HCC patients [26, 27]. An alternative mechanism of constitutive activation of the pathway was reported through epigenetic aberrations leading to downregulation of PTEN (tumor suppressor; negative regulator of PI3K) or Ras inhibitors. However, it is worth noting that PTEN mutations are low in HCC and the most possible mechanism for PTEN silencing is through loss of heterozygosity [20, 21, 28]. Downregulation of PTEN and activation of the PI3K/Akt/mTOR pathway was reported to be mediated by HBx protein [29]. The activation of the PI3K/Akt/mTOR pathway is associated with poor prognosis in HCC [21].

1.2.3 Inflammation in HCC:

Inflammation is a defensive mechanism of an adaptive immune response that is stimulated by infection or tissue injury. Clinical features of inflammation are swelling, redness, hotness and pain [30]. Acute inflammatory response induced by infection or tissue injury involves the orchestrated interplay and delivery of blood components (plasma and leukocytes) to the site of infection or injury. During initial phases of microbial infections Lipopolysachrides (LPS) on cell wall of bacteria binds to and activates Toll-like receptor (TLR) signaling in innate immune cells such as tissue resident macrophages (Kupffer cells in the liver), and mast cells. All TLRs possess an extracellular domain with a series of leucine-rich repeats (LRRs) and an intracellular domain with a conserved signaling module called a Toll/IL-1 receptor (TIR) domain. The activation transduce a conserved signaling cascade resulting in the activation of NF-kB and IFN-regulatory factor (IRF) transcription factors resulting, which leads to the production of a number of inflammatory mediators, including chemokines, cytokines such as IL-6, TNF-α and IL-1β [30, 31].

Inflammation plays a critical role in the development of cancer including HCC [3, 16]. Nearly, all risk factors (Viral hepatitis, obesity, and alcoholism) for HCC activates the inflammatory pathway, and the development of HCC in most cases is preceded by cirrhotic/chronic inflammatory microenvironment [3, 11, 32]. The dying hepatocytes increase the secretion of IL-1β, which binds to IL-1 receptor on kupffer cells leading to recruitment of MyD88, Tollip, IRAK-1, IRAK-4 and TRAF6 to form complex I. Assembly of complex I triggers IRAK-4 autophosphorylation and the phosphorylation of IRAK-1. IRAK-1then dissociates from the receptor complex with TRAF6 and binds the preformed TAK1-TAB1-TAB2/TAB3 complex (Complex II) at the membrane. After binding of TAB2, TAK1 is

activated, which subsequently phosphorylates IKKα and IKKβ. IKKα/β complex phosphorylates the inhibitory subunit of NFkB (IKβ) leading to its Lysine 48 poly- ubiquitination by SCF-bTrCP and proteasome dependent degradation of the inhibitory subunit IkB. NF-kB (p65/p50 dimers) then translocates to the nucleus to bind the promoters of NFkB target genes such as IL-6. This leads to increased secretion of IL-6 from macrophages which binds to IL-6 receptor, which subsequently activates the transcription factor STAT-3 (another regulator of inflammation), STAT-3 then translocates to the nucleus to bind to the promoters of STAT-3 target genes, which include IL-6. Through this cross talk between the hepatocytes and kupffer cells a positive feedback loop is maintained to sustain the development of chronic inflammatory state facilitates development and progression of HCC [33, 34].

The mechanism behind the modulation of chronic inflammation in HCC is not fully understood. The available data in the literature implicates the involvement of cytokines such as IL-6 and other inflammatory mediators such as tumor necrosis factor α (TNFα), prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX-2) (reviewed in [35, 36]). Moreover, STAT3, downstream of IL-6, was also implicated in HCC. Activation of STAT3 (pTyr705) induced transformation of NIH3T3 and 3Y1 immortalized fibroblasts and tumor formation in mice [37]. In HCC, NF-κB is overexpressed and aberrantly activated in many HCC cases and HCC cell lines [35, 38].

The general mechanism behind inflammation induced HCC is believed to be through the activation and enhanced secretion of pro-inflammatory factors such as cytokine that lead to the activation of STAT3 and activation and recruitment of inflammatory cells such as macrophages. Infiltration of macrophages increases the oxidative stress and genomic instability. Collectively, all these events are thought to create a favorable environment for a tumor to arise [13, 36].

1.2.4 Insulin-like Growth Factor (IGF) Signaling:

The IGF pathway is an evolutionarily conserved pathway across mammals. The binding of IGF-1 and IGF-2 to their cognate receptors leads to the activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-AKT and Ras-Raf-mitogen-activated protein kinases (MAPK) signaling through which the IGF axis regulates metabolism, tissue homeostasis, and survival [23, 39]. The role of IGF in carcinogenesis is becoming apparent, as there is increasing evidence from epidemiological studies associating IGF signaling with malignancy including HCC [23]. Additionally, activation of the IGF axis is associated with resistance to therapeutic drugs [23, 40-42]. The complexity of IGF signaling resides at the level of regulation conferred by three types of interacting proteins: ligands (IGF-1 and IGF-2), receptors (IGF-1R, IGF-2R) and insulin-like growth factor-binding proteins (IGFBPs).

1.2.4.1 Ligands:

The liver produces the majority of IGF-1 and IGF-2, which are 67% identical and share 40% identity with pro-insulin. However, IGF-1 and IGF-2 have additional domains that do not exist in insulin [43, 44]. IGF-1 is induced in the liver by growth hormone (GH), while IGF-2 seems to be GH independent [45]. In animal models, IGF-2 is termed "fetal growth factor", as IGF-2 expression increases during fetal development and decreases shortly after birth. In contrast, IGF-1 expression is detected in adults and thereby named "adult growth factor." Both IGF-1 and IGF-2 act in autocrine, paracrine, and endocrine manners [44, 46].

This developmental difference of expression in IGF-1 and IGF-2 is not the case in humans, as both IGF-1 and IGF-2 are detected in adulthood [46]. The IGF-2 gene, however, is different from IGF-1, as it is a maternally imprinted gene in humans and mice [47, 48]. Through maternal imprinting, heritable epigenetic silencing of genes based on the parent of origin, IGF-2 is monoallelic and expressed by the paternal allele. Loss of this maternal silencing leads to bi-allelic expression, as has been observed in neoplasms such as colorectal cancers and HCC [49-51]. The IGF-2 gene is developmentally regulated through highly controlled transcriptional regulation utilizing four distinct promoters (P1 to P4). During fetal development, P2, P3, and P4 are used. In adulthood, the P1 promoter is mostly used and is responsible for approximately 50% of total IGF-2 messenger RNA (mRNA) levels, and the utilization of P2 and P4 is markedly reduced [52]. In concert with this tight regulation, a study of a cohort of 104 HCC patients has shown that re-usage of the fetal promoters (P3 and P4) leads to the increased abundance of IGF-2 transcripts [41]. In HCC, IGF-2 is more commonly overexpressed than IGF-1, and given the observation that the GH receptor is downregulated in HCC, these studies might explain why the increased abundance of IGF-2, and not IGF-1, is observed in HCC [40, 53, 54].

1.2.4.2 Receptors:

Receptors that mediate IGF signaling are classified as tyrosine kinase receptors. These receptors contain α-subunits (exposed to the extracellular side) and a β-subunit (facing the cytoplasmic side) and include IGF-1R, the insulin receptor, and insulin-related receptors. These receptors are structurally similar and can homodimerize and heterodimerize with each other [23, 55]. Upon ligand binding, the receptors undergo conformational change resulting in the autophosphorylation of the cytoplasmic β-subunit of the receptors and activation of the insulin-receptor substrate (IRS) 1 and IRS2, and Shc proteins, downstream of the receptors. This process ultimately results in the activation of two major pathways – the PI3K and MAPK – which exert proliferative and survival advantages favoring transformation and cancer development [23].

1.2.4.2.1 IGF-1R:

The expression of IGF-1R in normal hepatocytes is low. The majority of IGF-1R in the liver comes from Kupffer cells, endothelial cells, and stellate cells [56, 57]. This explains why, in normal physiology, hepatocytes are not sensitive to signaling through IGF-1R and are not the major target of IGF-1 [58]. However, there is evidence that during the transformation process of hepatocytes into malignant HCC, hepatocytes overexpress IGF-1R [59]. IGF-1 and IGF-2 can bind IGF-1R with high affinity [60]. Similarly, in animal models of HCC, IGF-1R expression is increased in tumors compared with in preneoplastic lesions [61]. Through this overexpression of IGF-1R, HCC cells are able to gain a proliferative advantage and evade apoptosis because IGF-1R blockade exerts an antiproliferative effect and sensitizes cancer cells to apoptosis. These data indicate that excessive signaling through the IGF axis is a critical component in the transformation of preneoplastic cells into malignant HCC [59, 62].

1.2.4.2.2 IGF-2R:

IGF-2R, also called mannose-6-phosphate receptor, is a unique single transmembrane protein that binds only to IGF-2 with high affinity. It predominantly binds to mannose-6-phosphate containing proteins and targets them to the lysosomal compartment for degradation [63]. Hepatocytes, Kupffer cells, endothelial cells, and stellate cells all express IGF-2R [64].

In the liver, IGF-2R binds IGF-2 and facilitates its endocytosis for degradation, thereby maintaining the bioavailability of IGF-2. As a result, a role of IGF-2R as a tumor suppressor has been proposed [62]. In HCC, loss of heterozygosity and function mutation in IGF-2R are common events [65-67].

1.2.4.3 Insulin-like Growth Factor Binding Proteins (IGFBPs):

The IGFBP family encompasses six members, IGFBP1–6. Structurally, IGFBPs are clustered together with strong similarity and contain 16–18 conserved cysteines, 10–12 at the N-terminus and six at the C-terminus. At the N-terminus, IGFBPs share a common motif, termed the IGFBP motif (GCGCCXXC). IGFBPs are classified as having a high affinity for binding to IGFs, probably by forming a pocket and both the N-terminus and C-terminus contribute to this high affinity binding [68]. Secreted IGFs are generally found in the circulation in a ternary structure bound to IGFBPs and the acid labile subunit (ALS), IGFBP3 being the most abundant IGFBP in the circulation. As a result, the role of IGFBPs is generally thought to limit the bioavailability of IGFs to their cognate receptors. However, some membrane IGFBPs, such as IGFBP5, may enhance the stability of IGFs and their binding to IGF receptors [69]. IGFBPs are regulated by proteases that cleave IGFBPs to increase the bioavailability of IGFs [46, 68].

The role of IGFBP1–6 in HCC is not very well understood; however, there is evidence that IGFBP3 is downregulated by promoter hypermethylation in HCC [70]. Moreover, the addition of IGFBP3 mitigates the mitogenic effects of IGF-1 and IGF-2. IGFBPs also have IGF-independent functions; for example, IGFBP3 can still exert antiproliferative effects even after cleavage.[68, 70-72].

1.3 Insulin-like Growth Factor Binding Protein 7 (IGFBP7):

IGFBP7, also known as insulin-like growth factor-binding protein-related protein-1 (IGFBP-rp1), mac25, tumor adhesion factor (TAF), prostacyclin-stimulating factor (PSF), and angiomodulin (AGM), is a secreted protein of a family of low-affinity IGFBPs termed "insulin-like growth-factor binding protein-related proteins" (IGFBP-rp1–10) [68, 73].

The IGFBP7 locus is mapped to chromosome 4q12 [74]. Mouse IGFBP7 has a 94.4% similarity and 87.5% nucleotide identity to human IGFBP7 [75]. IGFBP7 shows 40%–45% similarity and 20%–25% identity to other IGFBPs [76]. At the N-terminus, IGFBP7 contains the IGFBP motif (GCGCCXXC) in a region of eleven conserved cysteines. IGFBP7 is different from the other IGFBPs in that it lacks the conserved cysteines at the C-terminus and has only one cysteine (Figure1.4) [76]. Moreover, it further differs from the other IGFBPs in that it has 100-times less affinity for binding to IGF-1 and is the only family member that binds insulin with strong affinity, limiting insulin binding to the insulin receptor [77]. Contradictory to this notion, most recent data show that IGFBP7 binds IGF-1R and block it's activation rather than binding to the IGF-1 [78].

Additionally, there is evidence of differences in posttranslational modifications of the other IGFBPs compared with IGFBP7. For example, conserved serines (residues 101, 119, and 183), known to enhance growth-factor response to IGF-1 in IGFBP1 via phosphorylation, are not found in IGFBP7. IGFBP7 has 25 serines of which only two are conserved in other IGFBPs. It is worth noting that the ability of IGFBP7 to inhibit insulin binding to its receptor was tested under supraphysiological conditions. The average amount of IGFBP7 in serum is around 33 ng/mL, and insulin binding was analyzed at 100 pmol [76, 79, 80]. This difference in structure and

posttranslational modifications might suggest that IGFBP7 has unique functions that are primarily IGF independent [68].

1.3.1 Physiological expression of IGFBP7:

IGFBP7 mRNA is expressed in normal tissues of the brain, liver, heart, small intestine, spleen, kidney, placenta, lung, skeletal muscle, thymus, prostate, testis, ovary pancreas, and colon, as detected by northern blotting [81]. IGFBP7 protein expression was analyzed by immunohistochemistry using an antibody directed against the decapeptide on the C-terminus. Peripheral nerves, cilia of the respiratory system, epididymis, and fallopian tubes had strong positive staining for IGFBP7 [82].

Smooth-muscle cells from gut, bladder, prostate, and endothelial cell walls also showed strong staining. While endothelial cells stained positively for IGFBP7, lymphocytes, plasma cells, and fat cells were negatively stained. In the kidney, the epithelia of distal tubules were more strongly stained than those of proximal tubules. Moreover, cells from the zona reticularis and the zona glomerulosa were more strongly stained than cells from the zona fasciculate. In the liver, using serial analysis of gene expression (SAGE), activated stellate cells were found to be the major contributor to the expression of IGFBP7, as seen by the low expression of IGFBP7 in total liver compared with in isolated activated stellate cells [83]. This discrete expression of IGFBP7 in tissue and cell specific manner might suggest that IGFBP7 might have specific functions in those organs [82].

IGFBP7

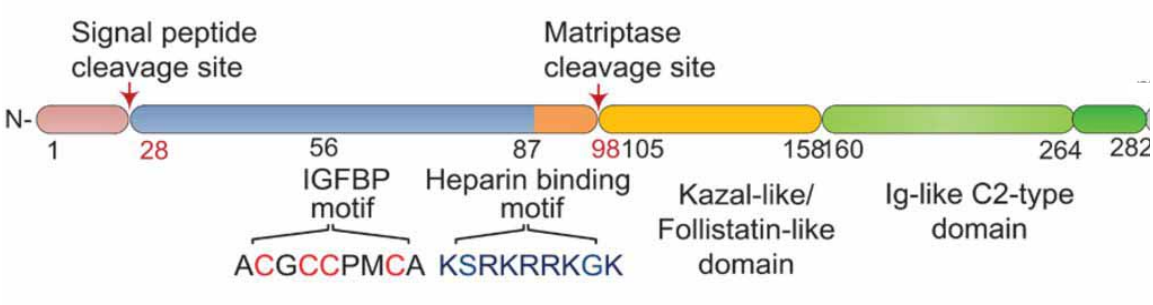


Figure 1.4:

Schematic representation of IGFBP7 protein. Sketch adopted from [78]

1.3.2 Protein–protein interactions and cell-surface interactions of IGFBP7:

In addition to the IGFBP family, IGFBP7 also shows a strong homology with follistatin, an activin-binding protein, except that IGFBP7 lacks the C-terminus of follistatin [84]. IGFBP7 binds to activin A, thus resembling the function of activated follistatin. As a result, IGFBP7 can modulate the growth-suppressing effects of the transforming growth factor (TGF)-β superfamily, as seen in the upregulation of IGFBP7 after treatment with TGF-β1 and retinoic acid. However, the mechanism by which IGFBP7 modulates TGF-β effects is not very well understood [81, 84, 85]. IGFBP7 binds to heparin sulfate on the cell surface, and, on cleavage by matriptase, the binding seems to be affected. Proteolytic cleavage at the N-terminus, containing the heparin binding motif, reduces heparin binding and the occupancy of IGF-1R [78, 86, 87]. Moreover, IGFBP7 has been shown to bind to type IV collagen on the vascular basement membrane [86-88].

Additionally, in the high endothelial venules (HEVs), IGFBP7 binds with extracellular matrix components; collagen type IV; heparan sulfate, glycosaminoglycans; secondary lymphoid-tissue chemokine (SLC; also known as chemokine [C-C motif] ligand 21 [CCL21]); interferon (INF)-g-inducible protein 10 (IP-10; also known as C-X-C motif chemokine 10 [CXCL10]); and regulated on activation, normal T cell expressed and secreted (RANTES; also known as chemokine [C-C motif] ligand 5 [CCL5]). Through these interactions at HEVs, IGFBP7 might play a role in controlling lymphocyte trafficking to lymph nodes [88]. The functional relevance of these interactions in regulating HCC development, progression, and metastasis has not been reported yet.

1.3.3 The role of IGFBP7 in cancers:

The role of IGFBP7 in carcinogenesis was first documented when it was identified as a differentially expressed gene in normal leptomeninges compared with meningiomas and in primary prostate epithelia cell lines compared with prostate cancer cell lines. Multiple studies have reported IGFBP7 downregulation in breast cancer [74, 81, 89]. IGFBP7 is suggested to be a potential tumor suppressor regulating senescence and cell proliferation. Loss of heterozygosity in the IGFBP7 4q12 locus has been reported in breast cancer in more than 50% of cells isolated from tumor tissue compared with normal tissue. This was in parallel with loss of IGFBP7 in invasive carcinoma cells and was associated with poorer prognosis in estrogen receptor-negative breast cancer [90, 91],

Similarly, a role of IGFBP7 as a tumor suppressor in colorectal and lung cancer has been proposed [92, 93]. The downregulation of IGFBP7, probably through aberrant DNA methylation, was associated with poorer prognosis [92-95]. Using tumor tissue array sections of invasive breast-cancer samples with known cell-cycle aberration status and other clinicopathological data, decreased expression of IGFBP7 in tumor tissue correlated with poor differentiation, increased cyclin E, and inactivation of retinoblastoma protein and showed an inverse association with proliferation marker Ki-67 in estrogen receptor-negative tumors [90].

Gene-expression microarray analysis of ductal carcinomas in situ documented a significant downregulation of IGFBP7 in high-grade compared with low-grade ductal carcinomas in situ [96]. Serial transplantation of primary breast tumors in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice resulted in progressive reduction of IGFBP7 expression that correlated with the increased growth and aggressiveness of the serially transplanted tumors [97].

Overexpression of IGFBP7 in triple-negative breast-cancer cell line (MDA-MB-468) abrogated their growth and migration via inhibition of phosphorylation of MAPK extracellular signal-regulated kinase (ERK)-1/2. When transplanted into NOD/SCID mice, IGFBP7 overexpressing clones had reduced tumor growth compared with controls [97]. Treatment of a triple-negative breast-cancer cell line (MDA-MB-231) with recombinant IGFBP7 protein induced G1 cell-cycle arrest and senescence, as indicated by cell-cycle analysis and β-galactosidase staining, respectively. The IGFBP7-induced cell-cycle arrest and senescence was mediated by induction of the p53–p21 axis and the activation of stress-activated p38 MAPK.

Tail-vein injection of recombinant IGFBP7 for 3–4 days in NOD/SCID mice harboring xenografted triple-negative breast-cancer tumors resulted in decreased angiogenesis, as shown by a reduction in cluster of differentiation (CD) 31 and vascular endothelial growth factor (VEGF) expression. Recombinant IGFBP7 was able to induce apoptosis as seen by caspase-3 and poly adenosine diphosphate ribose polymerase cleavages [98]. Taken together, these results suggest that IGFBP7 could act as a negative regulator of mitogenesis by suppressing ERK-1/2 and activating p38 MAPK and p53-p21 pathways to curb uncontrolled proliferation, induce senescence, and suppress angiogenesis in breast cancer.

Further studies on breast cancer have demonstrated that IGFBP7 occupies and blocks activation of the IGF-1R receptor, limiting its internalization rather than binding to IGF-1/2 or insulin [78]. This blockade was mediated by the N-terminus of IGFBP7 and resulted in reduced phosphorylation of IGF-1R and hence activation of the IGF-1R–AKT–mammalian target of rapamycin (mTOR) axis leading to dephosphorylation of 4E-binding protein-1 and activation of its translation-suppressive actions. However, both intact and truncated (lacking N-terminus) IGFBP7 was able to induce apoptosis [78].

These data suggest that IGF-1R–AKT–mTOR–4E-BP1 could be an important axis that IGFBP7 interferes with to decrease protein translation, growth, and survival. Additionally, the data also suggest an IGF-IGFR-independent action of IGFBP7 in inducing apoptosis. In melanocytes, a genome-wide small hairpin RNA screen identified IGFBP7 as an essential gene involved in oncogene-induced senescence (OIS) [99].

Overexpression of BRAF-V600E, glutamic acid to valine substitution at position 600 (a common mutation in melanomas), initially conferred a proliferative effect on normal melanocytes. However, soon after, activated BRAF induced expression of IGFBP7, which led to OIS. This was further demonstrated in melanocytic nevi, which show increased expression of IGFBP7. Downregulation of IGFBP7 in melanocytic nevi promoted their escape from OIS and facilitated their transformation into melanomas.

Treatment of melanomas harboring the BRAF-V600E mutation with recombinant IGFBP7 promoted apoptosis via induction of B-cell lymphoma-2/adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L), a pro-apoptotic B-cell lymphoma-2 family protein. Downregulation of IGFBP7 expression in melanomas was shown to be through increased methylation of the CpG island of the IGFBP7 promoter, a feature also observed in other tumors [99]. Intra-tumoral injection of IGFBP7 expressing plasmids inhibited the growth of malignant melanoma in mice via the reduction of VEGF and induction of apoptosis [100]. It has also been observed that IGFBP7 might function as a regulator of proliferation in keratinocytes.

Knockdown of IGFBP7 using RNA interference in spontaneously immortalized human keratinocytes and primary human keratinocytes increased proliferation and decreased tumor necrosis factor-α-induced apoptosis, and treatment with recombinant IGFBP7 induced apoptosis in keratinocytes [101]. Moreover, the downregulation of IGFBP7 was concomitant with the downregulation of genes involved in the calcium-dependent differentiation of keratinocytes [101]. These data suggest a potential role of IGFBP7 in regulating cell growth and differentiation.

1.3.4 The role of IGFBP7 in HCC:

In our attempt to decipher the function of astrocyte-elevated gene-1 (AEG-1), an oncogene overexpressed in more than 90% of HCC patients, we identified IGFBP7 as one of the most downregulated genes on overexpression of AEG-1. This observation suggested that IGFBP7 downregulation might be required to mediate the tumor-promoting function of AEG-1 [102]. We documented downregulation of IGFBP7 expression in human HCC patient samples and HCC cell lines compared with normal liver and hepatocytes. Additionally, an inverse correlation between IGFBP7 expression and the grades and stages of HCC was observed (Figure 1.5A) [103].

Genomic deletion of IGFBP7 leading to loss of heterozygosity was observed in 26% of patients (Figure 1.5B). In HCC, downregulation of IGFBP7 expression was associated with hypermethylation of the CpG islands in the IGFBP7 promoter in murine SV40 T/t antigen-induced hepatocarcinogenesis and in human HBV-related HCC [104, 105].

The HBV-mediated transformation of hepatocytes into HCC is thought to occur at the level of regulation and interaction with a number of genes and signaling pathways that control cell survival and apoptosis [106]. For example, hepatitis B virus X (HBx), a viral protein with multifaceted functions, can perturb the expression of a number of genes, including growth-control genes, such as Src tyrosine kinases, Ras, Raf, MAPK, ERK, c-Jun N-terminal kinase, and a number of other genes probably through the inherent capability of HBV to integrate into the genome, which might lead to the upregulation of genes such as oncogenes via strong viral-enhancer elements and the alteration of centrosome replication, leading to genomic instability or the microdeletion of genes such as tumor-suppressor genes [13, 107, 108].

HBx can bind and nullify the function of p53, a tumor-suppressor gene with multifaceted actions [109]. Similarly, HCV can trigger a cascade of events leading to the inactivation of p53 and retinoblastoma, the activation of MAPK, and the increased production of reactive oxygen species. Unlike HBV, HCV does not integrate into the host genome [110, 111]. Moreover, HBV and HCV have been shown to induce epigenetic changes via the alteration of DNA methylation and histone modifications (reviewed in Herceg and Paliwal) [112].

The functional link between viral hepatitis and the loss of IGFBP7 has not been established yet. However, it has been observed recently that the frequency of serum IGFBP7-promoter methylation in patients with HBV-induced HCC is significantly increased compared with in chronic hepatitis B patients and healthy controls [105].

IGFBP7 is a responsive gene to p53 in colorectal cancer, and it has been shown that p53 binds a p53-responsive element in the intron-1 of the IGFBP7 gene, inducing its expression. This binding is lost in colorectal cancer, probably due to the DNA methylation of CpG island at the promoter region [113]. As previously described, IGFBP7 has been shown to interfere with the MAPK-ERK pathway, and its loss was associated with epigenetic alterations or genomic deletions [91, 103-105]. Thus, it is tempting to hypothesize that HBV or HCV might require the alteration of critical nodes in signaling pathways to facilitate genomic instability and loss of tumor-suppressor genes such as IGFBP7 to facilitate the transformation of hepatocyte and the development of HCC from a chronic disease state.

The methylation of IGFBP7 in HCC patients has been associated with vascular invasion [104, 105]. Recently, it has been shown that reintroducing (SWItch/Sucrose NonFermentable)-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 (SMARCB1)/SNF, a commonly deleted core subunit of SWI/SNF chromatin remodeling complexes, in malignant rhabdoid tumors increased the expression of IGFBP7 [114].

SMARCB1/SNF5 directly regulates IGFBP7 and is essential for IGFBP7 expression. Moreover, restoring IGFBP7 expression in SMARCB1/SNF5 null cells sensitized them to apoptosis [114]. Whether the downregulation of IGFBP7 by AEG-1 overexpression is mediated by promoter methylation or alteration in chromatin remodeling remains to be determined. Further studies from our laboratory have confirmed a potential tumor-suppressor role of IGFBP7 in HCC. Stable overexpression of IGFBP7 in AEG-1-overexpressing HCC cell lines abrogated proliferation and colony formation, and induced senescence [103]. The effect was more pronounced in vivo in a nude mouse xenograft model, as shown by a marked reduction of tumor growth and Ki-67 staining in IGFBP7 overexpressing clones compared with in controls. The profound inhibitory effect of IGFBP7 in vivo was explained by the inhibition of angiogenesis, as evidenced by a reduction in CD-31 staining, decreased blood vessel formation in chick chorioallantoic membrane assays, and abrogated differentiation and tube formation of human umbilical vein endothelial cells [103].

An anti-angiogenic effect of IGFBP7 might be conferred by a reduction in phosphorylation of IGF-1R and mitogen-activated protein kinase kinase/ERK-1/2, leading to decreased VEGF expression in human umbilical vein endothelial cells and tumor cells [100, 115]. A separate study showed decreased IGFBP7 staining in 35.6% of HCC patients (104 patients studied), and this decrease was statistically associated with large tumor size, increased vascular invasion, and poor overall survival and disease-free survival rates.94 Thus, IGFBP7 downregulation might be used as a marker to assess HCC progression.

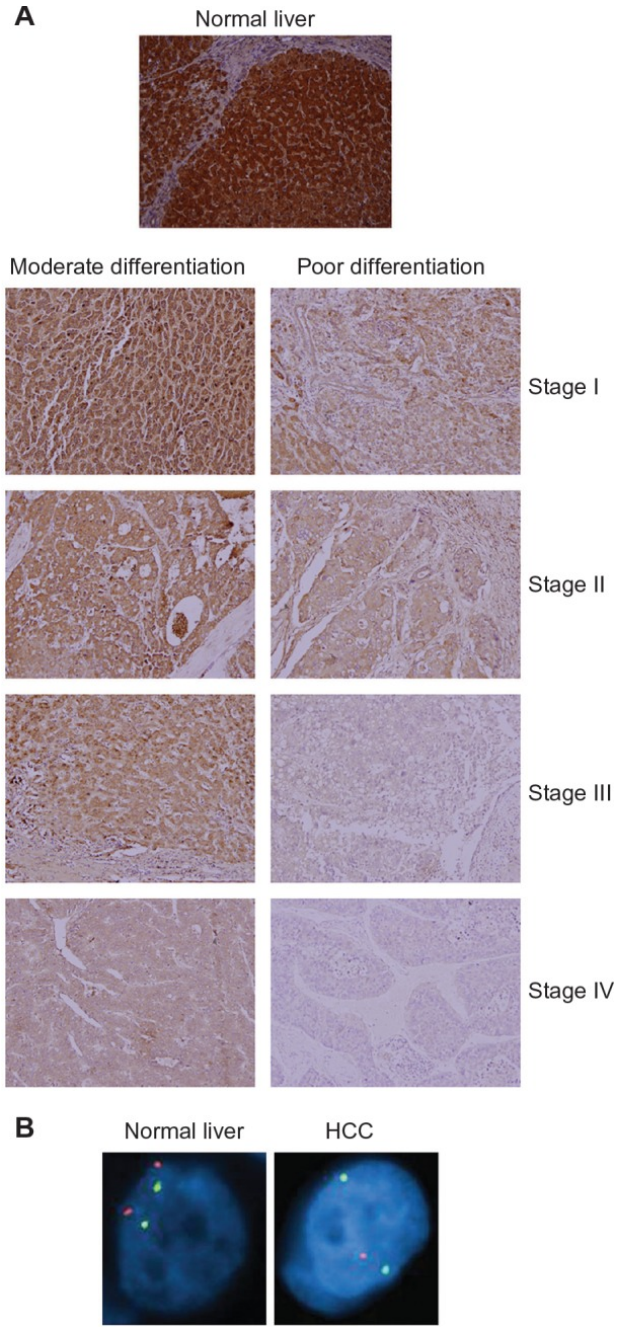


Figure 1.5: Insulin-like growth factor-binding protein 7 (IGFBP7) is downregulated in hepatocellular carcinoma (HCC). (A) Tissue microarray using immunohistochemistry examining IGFBP7 expression at/in different stages and grades of HCC. (B) Fluorescent in situ hybridization in normal liver and HCC patient samples showing loss of heterozygosity. Orange, IGFBP7; green, CEP4 (pericentromeric region). Note the loss of one IGFBP7 allele in HCC compared with in normal liver.

1.3.5 Potential role of IGFBP7 in cancer therapy:

The Sarkar lab tested the efficacy of IGFBP7 gene delivery using a replication-incompetent adenovirus (the Ad.5/3 virus) as a potential therapeutic approach for HCC [116]. Three different HCC cell lines, differing in their p53 and Rb status, were used in this study – namely, Huh7 (mutated p53 and wildtype Rb), Hep3B (p53 and Rb null), and HepG3 cells (wildtype p53 and Rb). Infection with Ad.IGFBP7 in all cell lines abrogated proliferation and induced apoptosis but not senescence, which might be explained by the accumulation of a large amount of IGFBP7 on Ad.IGFBP7 infection, shifting the balance from senescence to apoptosis. Ad.IGFBP7 infection resulted in the generation of reactive oxygen species; the induction of a DNA damage response, as evidenced by the phosphorylation of Ataxia telangiectasia mutated, Ataxia telangiectasia and Rad3 related, checkpoint kinase-1, checkpoint kinase-2, and p53; and the activation of the p38 MAPK pathway. Treatment with an antioxidant or the inhibition of p38 MAPK provided significant protection against IGFBP7 mediated apoptosis.

The growth-inhibiting effect and apoptosis induction by Ad.IGFBP7 was also demonstrated in vivo. Human HCC xenografts were established orthotopically in nude mouse livers. Intravenous injections of Ad.IGFBP7 at a dose of 1×10^9 viral particles/injection for a total of five injections over a 2-week period significantly inhibited tumor size and growth and reduced levels of alpha-fetoprotein (Figure 1.6A). Ad.IGFBP7 did not adversely affect the adjacent normal liver, only the xeno-transplanted tumor that showed hemorrhagic necrosis. Similar effects were also observed when subcutaneous xenografts were implanted in both flanks of nude mice (Figure 1.6B).

Ad.IGFBP7 was injected only on one side and the growth-inhibiting effect and apoptosis induction were evident in both sides, as evidenced by a reduction in Ki-67 and CD31 staining. Moreover, conditioned media from Ad.IGFBP7-infected cells could induce apoptosis in freshly plated human HCC cells [116]. These findings suggest that IGFBP7 protein might enter the circulation from the initial site of injection and inhibit the growth of distal tumors (illustrative of metastasis). Thus, Ad.IGFBP7 might be an effective therapy for both primary and metastatic HCC.

Targeted molecular therapy has ushered in strategies for the treatment of advanced HCC. For example, sorafenib, a multikinase inhibitor, increased the survival of advanced HCC patients by 2.8 months [3, 15, 21]. Unfortunately, systemic therapy is limited in its efficacy, reinforcing the need for the characterization of additional molecular mechanisms driving the progression of HCC that could be targeted in combination with systemic therapy. A previous study on HCC demonstrated that IGFBP7 mediated sensitivity to IFN-α treatment in HCC [117]. IFN-α-resistant HCC cell lines were established by repetitive exposure to IFN-α. Microarray analysis of the IFN-α-resistant cells showed a significant downregulation of IGFBP7. Knocking down IGFBP7 using RNA interference further augmented resistance to IFN-α. In contrast, IGFBP7 overexpression restored sensitivity to IFN-α in resistant cells. The mechanism by which IGFBP7 enhances sensitivity to IFN-α-resistant cells might be via the modulation of apoptosis, although this aspect has not been well studied [117].

IGFBP7 is downregulated in hypopharynx cancer cells by low-dose treatment with paclitaxel [118]. This finding suggests that a reduction in IGFBP7 might be essential in acquiring resistance to paclitaxel. However, it is worth noting that, in that study, the authors did not establish resistant clones, and the role of IGFBP7 in acquired resistance to paclitaxel requires

further investigation. In an experimental mouse model of metastatic melanoma, tail-vein injection of recombinant IGFBP7 in athymic nude mice suppressed the growth of metastatic melanoma cells and increased the survival rate of IGFBP7-injected animals [119]. These findings further strengthen the notion of using IGFBP7 as a biomarker to predict drug resistance and as a potential target for cancer therapy.

Although the tumor-suppressor function of IGFBP7 is established in diverse cancers, the molecular mechanism by which IGFBP7 exerts its effect remains to be clarified. Is IGF-1 inhibition the primary mechanism of IGFBP7 action? How does IGFBP7 mediate its IGF-independent functions? Is there a cell-surface receptor for IGFBP7 similar to that for IGFBP3? [120] What is the consequence of insulin inhibition by IGFBP7? These questions might be addressed by analyzing an IGFBP7 knockout mouse. The efficacy of IGFBP7 as a therapy needs to be stringently evaluated in transgenic mouse models of cancer, either alone or in combination with conventional therapy. The studies so far on IGFBP7 are very promising and future in-depth analysis will establish the utility of this molecule as a biomarker and therapeutic agent.

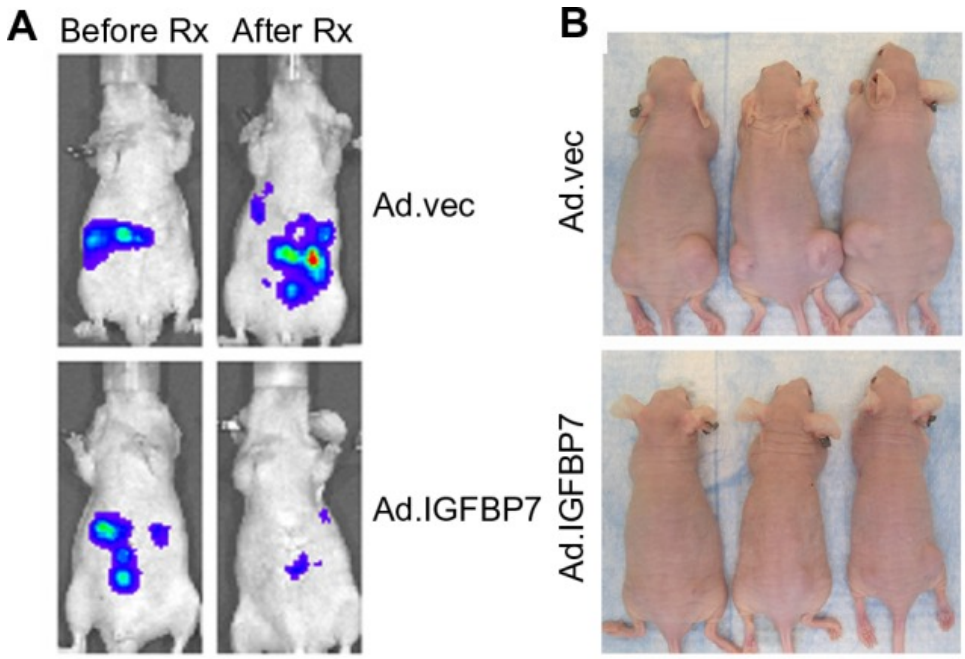


Figure 1.6: Ad.IGFBP7 is an effective therapy for hepatocellular carcinoma (HCC).
(A) Bioluminescence imaging of nude mice with established orthotopic tumors in the liver using human HCC cells before (left) and after (right) treatment with Ad.IGFBP7. (B) Photograph of nude mice bearing subcutaneous xenografts from human HCC cells on both flanks treated with control Ad.vec (top) or Ad.IGFBP7 (bottom). Only the left-sided tumors were injected with Ad. Note the almost complete disappearance of both left- and right-sided tumors in Ad.IGFBP7-treated mice. **Abbreviation:** Rx, treatment. Adopted from [116]

1.4: Cancer Immune Surveillance:

1.4.1 Immune editing in cancer:

In 1909 Paul Ehrlich said that cells with cancer causing mutation must be appearing in a continuous basis during development. However, the immune system must be the one that provided control over the propagation of cells harboring these cancer causing mutations. This was the first report to implicate the immune system with control over tumorigenesis [121]. The increased knowledge on the composition of the immune system and the presence of tumor antigens led to increased understanding on how the immune system control the development of cancer. Thomas and Burnet first introduced the hypothesis that cancer immune surveillance is the sentinel behind the development of cancer in immune competent organisms [122, 123].

Subsequently, Stutman refuted this hypothesis when the susceptibility of immunocompetent mice to spontaneous and carcinogen induced tumors were similar to that of the immune compromised mice [124, 125]. At that time, the theory of cancer immune editing was not understood. Therefore, this led to the abandonment of Thomas and Burnet hypothesis on control of cancer development by the immune system. The introduction of improved immunodeficiency mouse models on pure genetic background strains in the 1990s helped investigators to analyze and reassess the involvement of immune system in the progression of carcinogenesis.

Several discoveries reintroduced the role of immune system in control of cancer development. For example, findings that Interferon-γ (IFN-γ) is critical in inducing tumor rejection of transplanted tumors i.e. mice lacking IFN-γ responsiveness by either genetic deletion of the IFN-γ receptor or the transcription factor STAT-1 (necessary for signaling through IFN-γ receptor) or mice lacking adaptive immunity (RAG2−/− mice, lacking T cells, B cells, and natural killer T cells) were more susceptible to spontaneous primary tumor formation and carcinogen induced

malignancies [126], and the finding that tumors that arose in immunocompromised mice are eliminated when injected in immune competent mice, while aggressiveness of cancer cells increases when immune competent mice are re-challenged with same cancer cells further implicated the role that the immune system not only control cancer growth but also helps to evolve and select for cancer cells capable of immune evasion, a term called cancer immune editing [126]. Cancer immune editing encompasses three phase: Elimination, Equilibrium and Evasion.

1.4.1.1 Elimination:

Elimination is the process when both the innate and the adaptive immune system help to kill and destroy cancer cells from the tissue microenvironment before they manifest clinically (Figure1.5). Several alert signals help the immune system to recognize the developing tumor. For example, Type I Interferons, which is induced early during tumor development, activate dendritic cells and facilitated the formation of adaptive immune response against the developing tumor [127]. Also expression of ligands on cell membrane such as Ribonucleic Acid Export 1 (RAE-1) and Major Histocompatibility IA/B (MICA/B in human) (H60 in mice) activate the innate immune response cells such as NK-cells, which then secrete pro-inflammatory and immunomodulatory cytokines to facilitate the development of adaptive immune response and tumor killing. The mechanisms behind the "elimination" phase is still largely unknown and its implication in cancer immunotherapy is a hot area of research.

1.4.1.2 Equilibrium:

During the elimination phase, cancer cells that escaped the killing by the innate immune system cells (e.g. NK-cells) undergo a state of equilibrium and are kept "in check" by cells of the adaptive immune system ($CD8^+$ and $CD4^+$ cells). The discovery that immunocompetent mice treated with low dose of 3' -methylcholanthrene (MCA) did not develop palpable tumors (at the site of MCA injection) until immune cells from these mice were eradicated confirms the presence of pre-existing dormant cancer cells that were controlled by the immune system [128]. As a result, the adaptive immune systems prevents the clonal expansion of malignant cells [128, 129]. This process of tumor dormancy or "Equilibrium" is the longest period during malignancy. Unfortunately, this negative pressure by the immune system on cancer cells helps malignant cells to evolve and acquire novel mechanisms by which a cancer cell can escape the immune system.

1.4.1.3 Escape:

During the process of tumor dormancy, the immune system helps to edit these dormant cancer cells. Rare population of these dormant cancer cells acquire enough editing that allows the cancer cell to escape the control of the immune system resulting in clonal expansion and tumor relapse. There are many different mechanisms by which cancer cells are edited to escape the immune recognition. Most common mechanisms in cancer cells include: loss of tumor antigen presentation to immune cells by downregulation of MHC-class I molecules or by downregulation of tumor antigen process such as downregulation of the immunoproteasome LMP2 or LMP7 or by downregulation of antigen transport system such as TAP-1 in cancer cells[130, 131]. Another mechanism, is by overexpression of anti-apoptic effector molecules such as overexpression of B-cell lymphoma 2 (BCL2) or the overexpression of the pro-oncogenic transcription factor STAT-3, which regulates the expression of a variety of genes regulating survival and oncogenesis [132]

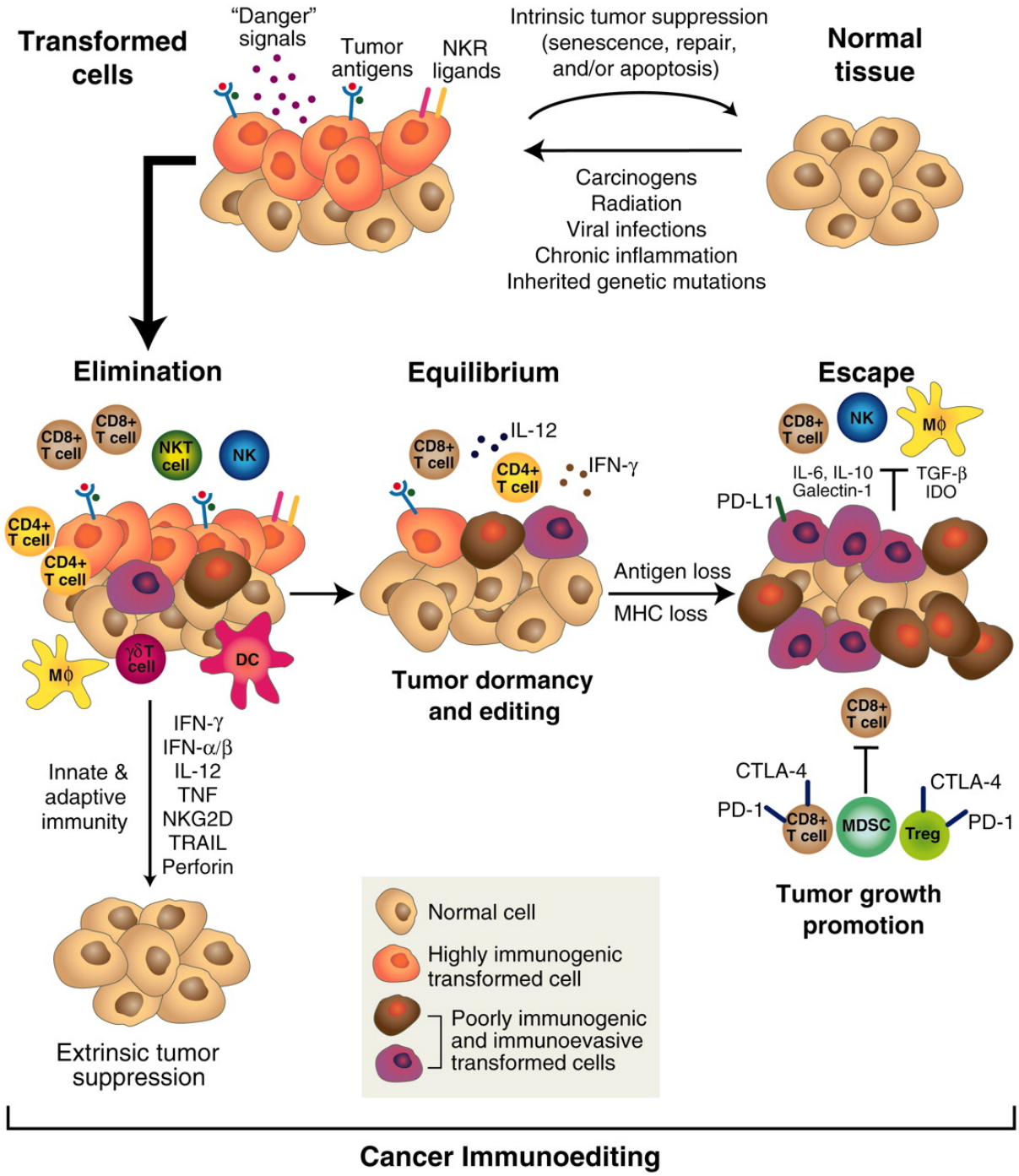


Figure 1.7:

The process of cancer immune editing during carcinogenesis. Sketch adopted from [131]

1.4.2 Immune evasion in HCC:

The liver is the organ in which the uptake of blood borne pathogens and excretion of toxic agents from the gastrointestinal tract are carried out. As results, this exposes the liver to an enormous number of antigens. In order to prevent an autoimmune disease, the liver evolved to develop immune tolerance [133]. For instance, under normal conditions the resident macrophages of the liver "Kupffer cells" secrete IL-10, which induces immune suppression and tolerance [134]. Moreover, the release of transforming growth factor β (TGF-β) from liver endothelial cells and kupffer cells also provide an immune suppression and tolerance [135].

In HCC, the mechanisms facilitating tolerance in the liver include a reduction in recognition of malignant cells and suppression of immune system function in the liver microenvironment [131]. Downregulation of MHC class I molecules and/or failure of antigen processing was reported [136, 137]. Also, increases in the level of Tolerogenic regulatory T-cells (T-regs) and Myeloid Derived Suppressor cells (MDSCs) were reported in HCC [138-140]. A variety of cytokines associated with immune regulation in the tumor microenvironment was reported in HCC [141]. Hepatocytes express receptors for a number of cytokines, which makes the hepatocytes a target for these cytokines. Cytokines are involved not only in the physiological functioning of the liver, development, and regeneration, but also aid in chronic liver inflammation and progression of HCC. For example, deregulation of cytokines that were associated with poor prognosis of HCC include an increase in immunosuppressive cytokines (e.g. IL-4, IL-5, IL-8, and IL-10), and suppression of immune activating cytokines (e.g. IFN-γ) [141]. Mechanisms regulating the immune tolerance and immune evasion in HCC are diverse and largely unknown. Therefore, targeting multiple strategies of immune regulation is probably the best approach to target this deadly and highly virulent disease.

1.4.2.1 Inflammatory and anti-inflammatory cytokines in HCC:

As discussed earlier in the chapter, inflammation is a critical component of cancer including HCC [16, 35, 142]. An increase in the secretion of the proinflammatory cytokine TNF-α was reported in HCC, serum levels of TNF-α was shown to be significantly higher in patients with HCC compared to healthy controls [143, 144]. Furthermore, TNF-α was also know to increase the expression of the T-cell inhibitory checkpoint PD-L1 on macrophages and by doing so TNF-α can inhibit and suppress anti-tumor $CD8^+$ cells [145]. Similarly, increased expression of IL-1β from the dying hepatocytes helps to increase the expression and production of another proinflammatory cytokine called IL-6 in the macrophages. This cross-talk between the hepatocytes and macrophages is mediated by NF-kB, the major culprit of inflammation [34, 146].

The most well-studied anti-inflammatory cytokine in HCC is IL-10. Overexpression of IL-10 has been reported in the tumor microenvironment of HCC patient compared to healthy controls [147]. Moreover, levels of Th2 (Immune suppressive type of T-cells) cytokines such as IL-4 and IL-5 were found to be increased in the tumor microenvironment of HCC patients, and this facilitated the shift from Th1 (anti-tumor T-cell) to Th2. This shit was necessary for progression into highly metastatic HCC [141]. Furthermore, increased secretion of TGF-β in HCC facilitates the induction of an immune suppressive microenvironment through which suppression of responsiveness to INF-γ signaling by downregulation of INF-γ gamma receptor, which in turn decrease phosphorylation of STAT-1 (active state) and therefore inhibit the expression of STAT-1 target genes which include: antigen presentation machinery and processing cluster of genes such as MHC-class I, LMP2 and LMP7, respectively.

1.4.2.2 Immune therapeutic applications in HCC:

The presence of tumor associated antigens during carcinogenesis allows for the generation and selection of vaccines to prevent cancer in high risk individuals [148]. In HCC, alpha fetoprotein (AFP) was the first identified HCC associated antigen and then was the first vaccine to be introduced for clinical trials [149]. However, results from this clinical trial was not promising as only immunologic responses detected in HCC patients due to either reduced effector functions of $CD4^+$ or limited number of antigens used [149]. To get around this failure, investigators used Dendritic Cells (DCs) pulsed with lysates from HCC cells to expose and load DCs with HCC associated antigens. Through this approach patients' survival extended from 6 to 16 months [150].

As highlighted earlier, alternative mechanisms that HCC cells use to evade immune response is to overexpress Programmed Cell Death protein 1 ligand (PD-L1), which binds the PD-1 receptor on T-cell and induce apoptosis in T-cells. The use of blocking antibodies capable of block the activation of this inhibitory pathway was used in HCC and results in HCC patients were very promising [151]. Moreover, the use of blocking antibody of another immune inhibitory check point called CTLA-4 was also used. Both Anti PD-1 and Anti-CTLA-4 antibodies were approved by Food and Drug Administration (FDA) and are currently being used in cancer patients including HCC patients [151].

Results from pre-clinical studies on the use multiple immune targeted therapeutic approaches for HCC has ushered novel and beneficial therapeutic approach to target this dire disease. As a results, multiple clinical trials on immune therapies in HCC were conducted [152]. Combinatorial treatment of targeted molecular therapies and targeted immune therapies is an active area of research and results from such studies would provide novel and effective therapeutic stratigies for HCC.

1.4.2.3 Interferon-γ (IFN-γ) signaling in HCC:

Infiltrating antitumor immune cells in HCC microenvironment ($CD4^+$, $CD8^+$) secrete IFN-γ as a counter mechanism to stop the proliferating cancer cells. IFN-γ is a type II Interferon and it signals through Tyrosine kinases of the Janus family (JAK). Jak1 and Jak2 are associated with the Interferon-γ receptor 1 and 2 (IFNGR1) and (IFNGR2) receptor subunits of IFN-γ, respectively.

Binding of IFN-γ to its receptors leads to activation of kinase domains of the related Jaks through autophosphorylate of tyrosine residues in the intracellular domains of the receptor subunits that serve as recruitments sites for STAT-1. Phosphorylation on tyrosine 701 on STAT-1 by JAK1/2 of STAT-1 is essential for activation of STAT-1 and phosphorylation of serine 727 by protein kinase C delta is optimal for STAT-1 function. STAT-1 then translucates to the nucleus where it binds to IFNγ-activated (GAS) sequences in the promoters of cluster of genes regulating antigen presentation and processing such as HLA-Class I and II and LMP7 and LMP2, respectively.

Moreover, IFN-γ also induces the expression of the transcription factor interferon regulatory factor-1 (IRF-1) through which IFN-γ induces apoptosis and autophagy [153,

154]. IRF-1 plays an important role in IFN-γ induced apoptosis in tumor cells through which it regulates expression of a number of genes such as iNOS , p21 and p53 upregulated modulator of apoptosis (PUMA) [154-157]. As a feedback mechanism of IFN-γ signaling STAT-1 is negatively regulated by : Suppressors of Cytokine Signaling-1 (SOCS-1), which acts as a phosphatase to suppress activation of STAT-1 [153].

CHAPTER 2: MATERIALS AND METHODS

2.1 Generation of Igfbp7$^{-/-}$ mouse:

Igfbp7$^{-/-}$ mouse was created in a pure C57BL/6J background using a Cre-loxP strategy (Figure 2.1). The targeting vector used to delete promoter, exon1 and part of intron 1 of Igfbp7 gene. A Loxp (L83) and a FNFL (Frt-Neo-Frt-Loxp) cassette were inserted to flank the promoter,exon 1 and part of intron 1 (about 1.9kb) of the Igfbp7 gene to generate the "floxed/neo" Igfbp7 allele.

The gene targeting vector was constructed by retrieving the 5kb Left homology arm (5' to L83), the 1.9kb floxed sequence, the FNFL cassette, and the 2kb Right homology arm (end of FNFL to 3'). The FNFL cassette conferred G418 resistance during gene targeting in PTL1 (129B6 hybrid) ES cells. Several targeted ES cells were identified and injected into C57BL/6 blastocysts to generate chimeric mice (chimeras).

Male chimeras were bred to ACTB (Flpe/Flpe) females and EIIa (Cre/Cre) females to transmit the floxed Igfbp7 allele (L83/FL146 allele with neo cassette removed by Fple recombinase) and Igfbp7-null allele (L85 allele with promoter, exon 1 and neo cassette removed by Cre recombinase) through germline. Mice carrying heterozygous Igfbp7-null allele Igfbp7$^{+/-}$ were intercrossed to generate homozygous Igfbp7-/- mice. Pups were weaned at 4 weeks and tail DNA was obtained to genotype the animals.

Genotyping was carried out using conventional PCR using the following primers:

(8894-8915): 5' CTCTGCAACGGCTTCCCACATG 3'

(11126-11104): 5' CCCAATGTCAAAGCCATTCCAGC 3'

(9175-9152): 5' GAGTATGTATGTTTGTGGGTCCTC 3'

All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and were performed in accordance with the Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals, and the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training. Mouse models was created by the help of Precision Targeting Lab, LLC, NJ and the VCU Transgenic/Knockout Mouse Core

2.2 Primary cells isolation and culture conditions:

Primary mouse hepatocytes were isolated and cultured in Williams E Medium containing NaHCO3, L-glutamine, insulin (1.5 mmol/L), and dexamethasone (0.1 mmol/L) as described [158]. Kupffer cells were isolated from liver homogenates by centrifuging at 500 RPM for 10 min. The supernatant containing immune cells was sorted for CD11b+F4/80+ cells using FACSAria II (BD Biosciences).

Mouse embryonic fibroblasts (MEFs) were isolated as described from (E13.5 embryos) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) [159]. Bone marrow-derived macrophages and peritoneal macrophages were isolated according to standard protocols [160]. Bone marrow cells were isolated from femurs of C57BL/6J mice and were differentiated into macrophages using RMPI-1640 medium supplemented with 10% heat-inactivated FBS and 20% L929 conditioned media for 7 days.

At day 7, the media was changed to complete RPMI-1640 containing 10% heat-inactivated FBS. Macrophages were cultured in complete media for at least 12 hours prior to using for experiments. Stellate cells were isolated according to standard protocol [161].

All primary cells were isolated from male mice of 6-12 weeks of age, were cultured at 37C° and in 5%CO_2 with 100% humidity and were used for experiments at 60-80% confluence. Cells from DEN-treated animals were obtained at 20-32 weeks of age.

2.3 N-nitrosodiethylamine (DEN) induced HCC:

A single dose of 10 μg/g body weight of DEN was administered intraperitoneally (i.p.) to 14 days old male pups. The animals were monitored and euthanized at 32 wks of age. Serum liver enzymes were analyzed in the Molecular Diagnostic Laboratory, Department of Pathology, VCU using standard procedures.

2.4 RNA extraction, cDNA synthesis and Quantitative Real-time PCR:

Total RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN; Cat#74104). 2 μg of RNA was used for cDNA synthesis using ABI cDNA Synthesis Kit (Applied Biosystems). Q-RT-PCR was performed using an ABI ViiA7 fast real-time PCR system, and Taqman gene expression assays using predesigned best coverage Taqman probes for standard gene expression (Applied Biosystems) according to the manufacturer's protocol. All mRNA levels were normalized by GAPDH mRNA levels.

2.5 RNA Sequencing (RNA-seq):

RNA, extracted from livers of 3 adult mice per group, was used. RNA-Seq library was prepared using Illumina TruSeq RNA Sample Preparation Kit and sequenced on Illumina HiSeq2000 platform. RNA-Seq libraries were pooled together to aim about 25 to 40 million read passed filtered reads per sample. All sequencing reads were aligned with their reference genome (UCSC mouse genome build mm9) using TopHat2.

Bam files from alignment were processed using HTSeqcount to obtain the counts per gene in all samples. The counts for all samples were read into R software using DESeq package. For each condition a pairwise test was performed using the functions in DESeq and plot distributions were analyzed using Reads Per Kilobase Million (RPKM) values. Data were filtered on the basis of low count or low RPKM value (<40 percentile). Genes showing log2 fold-change of >1.5 or <-1.5, FDR of <0.1 and p-value of <0.05 were selected. A complete list of differentially regulated genes is available in GSE85427 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ctoloyiiznwbtgt&acc=GSE85427)

2.6 In vitro antigen cross presentation of bone marrow-derived dendritic cells (BMDCs) and T-cell priming:

BMDCs were isolated as described [162]. Bone marrow-derived cells were differentiated into dendritic cells by culturing in RPMI-1640 medium containing granulocyte macrophage colony-stimulating factor (GM-CSF; 20ng/ml) for 8 days. Day 8 Igfbp7+/+ and Igfbp7-/- BMDCs were loaded with 1μg/mL gp100 (25-33a.a.) peptide for 3h, followed by 500 ng/mL lipopolysaccharide (LPS) for 2 h (priming). LPS was washed before co-culturing the BMDCs with pmel-17 T lymphocytes at a molar ratio of 1:10, 1:20 and 1:40 (DC:TC) for 60 hours. In a second experiment, gp100-loaded BMDCs were treated or not with rIGFBP7 protein (R&D) for 3 h followed by OSI-906 (4 μmol/L) for 2 h and LPS (500 ng/mL) for 2 h. The cells were washed and co-cultured with pmel-17 T cells for 60 h. Supernatants were collected at 48 h for ELISA. Cells were pulsed with 0.5μCi/well [3H]-thymidine for the last 16 h of incubation. Proliferation was measured by [^{3}H]-thymidine incorporation in triplicate wells.

2.7 Establishment of stable Igfbp7 overexpressing clone in Hepa1-6 cells:

Hepa-1-6 cell line (CRL-1830) was obtained from ATCC and was cultured as instructed. Mouse Igfbp7 (NM_001159518) expression plasmid was obtained from Origene Technologies, Inc. (MR222256). Hepa1-6 cells were transfected with 8 μg of mouse Igfbp7 expression plasmid DNA and corresponding empty vector using FuGENE® HD transfection reagent at a ratio of 3.5:1 (reagent:DNA). Transfected cells were selected in 450 μg/ml G418 for 4 weeks to establish BP7-OE pooled clone.

2.8 Xenograft studies in syngeneic mice:

C57L/J mice were purchased from The Jackson Laboratory. Control and BP7-OE pooled clones of Hepa1-6 cells ($4x10^6$) were injected subcutaneously into the right dorsal flank of C57L/J mice. Five mice per group were used. Tumor volume was measured by the following formula: $\pi/6$ x (small diameter)^2 x (large diameter). Mice were monitored twice a week and euthanized after 4 weeks at which point the tumors were harvested. Immune cells infiltration was determined by FACS as described [163].

2.9 CD8+ and CD4+ depletion assays:

S.c. xenografts were established using BP7-OE clones in C57L mice. Depletion of $CD8^+$ and $CD4^+$ cells was performed by injecting 200 μg of neutralizing antibodies i.p. on days 1, 5, 12 and 19 as described [163]. Tumors were collected 4 weeks after tumor implantation. Neutralizing antibodies were obtained from Bioxcell, $CD8^+$ Ab (Clone: 2.43 Catalog #BP0061), $CD4^+$ Ab (Clone: GK1.5 Catalog #BP0003-1).

2.10 Immunohistochemistry (IHC) and immunofluorescence (IF) assays:

IHC was performed on formalin-fixed paraffin-embedded (FFPE) sections as described [164] using the following antibodies: AFP (Santa cruz: sc-15375), PCNA (Cell signaling #13110), c-Myc (Cell signaling #13987), CD31 (Dako #JC70A), F4/80 (AbD Serotec #MCA497RT) and IGFBP7 (R&D systems #MAB21201). IF was performed on cells cultured in 4- chamber slides for hepatocytes (collagen-1 coated) and MEFs using antibodies against p65 (Cell signaling #8242) and γ-H2AX (Cell signaling #5438). The images were taken by a confocal laser scanning microscope. Antibody dilutions were used as recommended by the manufacturer.

2.11 Senescence-associated β-galactosidase (SA-β-Gal) assay:

Hepatocytes were cultured for 8 days and SA-β-Gal activity was measured as described [103].

2.12 Cell proliferation, BrdU incorporation and colony formation assays:

Cells ($1x10^3$) were plated in each well of a 96 well plate for measuring proliferation by a standard MTT assay [103]. BrdU incorporation was measured using BrdU Cell Proliferation Assay Kit (Cell signaling #6813) according to the manufacturer's protocol. Colony formation assay was performed by plating 1x104 MEFs on 6 cm dishes and culturing for 4 weeks.

2.13 Western blotting analysis:

Cell lysates and tissue extracts were prepared and Western blotting was performed as described (3). The primary antibodies used were: p-AKT (Cell signaling #4060), AKT (Cell signaling #4685), pERK (Cell signaling #9101), ERK (Cell signaling #4695). p-IGF-Rβ (Cell signaling #6113) GAPDH (Santa Cruz #sc-166545), EF1α (Millipore #05-235), p-GSK3β (Cell Signaling #5558), GSK3β (Cell Signaling #12456), p65 NF-κB (Cell signaling #5438), p-p65 (Cell signaling #8242), Cyclin D1 (Cell Signaling #2978), Cyclin E1 (Cell signaling #4129), Arginase-1 (Santa cruz #sc-18355), Myc-tag (Cell signaling #2276), p-STAT1 (Cell signaling #9167), STAT1 (Cell signaling #14994), Igfbp7 (R&D systems #MAB21201) and β-actin (Sigma Aldrich #A5316). Antibodies were diluted as recommended by the manufacturer. Densitometry analysis was performed by ImageJ software.

2.14 In vitro treatment of cells:

Recombinant mouse IGF-1 (R&D systems #Q8CAR0) was used at 20ng/ml for signaling experiments and 100ng/ml for cell proliferation experiments. LPS (sigma Aldrich #L3024) was used at 200ng/ml. OSI-906 (Selleckchem #S1091) was used at 4 μmol/L. In vitro DEN was used at 10ng/mL.

2.15 Cell cycle analysis:

MEFs ($1x10^5$) were synchronized in serum-free media for 18 h and released into complete growth media. Cells were washed with PBS and fixed in 70% ethanol for 30 min at -20°C. The fixed cells were washed three times with PBS and were incubated with PBS containing 10 μg/mL RNase A for 30 min at 37C° following which cells were incubated with 30 g/mL propidium iodide (PI) for 30 min in the dark. The samples were acquired in a FACSCanto II system. For each measurement 10,000 cells were acquired and the data were analyzed using the FACSDiva software.

2.16 Statistical Analysis:

Data were presented as the mean ± SD and analyzed for statistical significance using two-tailed student t-test. For canonical pathway analysis the p-value was calculated using the right-tailed Fisher Exact Test.

2.17 Fluorescence-activated cell sorting (FACS) analysis:

To analyze immune cell population livers from 5 months old DEN-injected littermates were perfused and resuspended into a single cell suspension as described [158]. The cell suspension was subjected to FACS analysis as described [163]. The s.c. xenograft tumor tissues were digested with collagenase D (10 mg/mL) and DNase I (100 mg/mL), and cell suspensions were filtered through a 70 μm cell strainer prior to FACS analysis (2). Antibodies were obtained from Biolegend: CD4 (GK1.5), CD8 (2.43), INF-γ (XMG1.2), Ly6G (1A8), Ly6C (HK1.4), CD80 (16-10A1), CD86 (GL-1), CD11b (M1/70), CD11c (N418), F4/80 (BM8), CD3 (17A2), NK1.1 (PK136), B220 (RA3-6B2) and Gr1 (RB6-8C5).

2.18 NF-κB luciferase reporter assay:

NF-κB luciferase reporter assay was performed in Igfbp7$^{+/+}$ and $^{-/-}$ hepatocytes exactly as described [164]. Experiments were performed in triplicates with two independent experiments.

2.19 Co-injection of HCC cells and bone marrow-derived macrophages in NOD scid Gamma (NSG) mice:

Dihxy cells (1X10^6), developed from DEN-injected C57BL/6 mice in Dr. Karin's laboratory (6), were co-injected with bone marrow derived macrophages (10x10^4 or 5x10^4) from Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ mice subcutaneously in 7 weeks old male NSG mice and tumor development were monitored for 7 weeks. Tumor volume was measured with calipers using the formula: (Width)^2 x length/2. For each group 5 mice were used.

CHAPTER 3: GENETIC DELETION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 7 (IGFBP7) PROMOTES HEPATOCELLULAR CARCINOMA (HCC)

3.1 Introduction:

Hepatocellular carcinoma (HCC) is a highly virulent and dire neoplasm [3]. The incidence of HCC is increasing in the West, and it is the third highest cause of cancer related deaths globally [3, 165]. Most HCC cases are diagnosed at an advanced stage at which treatment modalities are ineffective and prognosis is dismal [3]. Thus there is an immense need for developing effective therapies for HCC. Evasion from immune surveillance is an important mechanism facilitating tumor development and progression [166].

Three equally important events are necessary for immune evasion, the masking of neo-antigens, downregulation of antigen presentation machinery, and irresponsiveness to Interferon-γ (IFN-γ)-mediated killing by immune cells [167]. Tumor antigenic peptides are generally produced in the cytosol via processing by proteasomes that contain interferon-γ-inducible subunits LMP-2, LMP-7 and LMP-10 [168].

The 8-9 amino acids antigen peptides thus produced are translocated by transporters associated with antigen processing (TAP-1 and TAP-2) to the endoplasmic reticulum (ER) where the peptides are assembled with MHC class I heavy chain and β2- microglobulin light chain and are transported to the cell surface to be presented to $CD8^+$ cytotoxic T lymphocytes (CTL).

The components of the antigen presentation pathway, such as TAP1/2 and LMP2/7, are downregulated in cancers, including HCC, resulting in a loss of immune-surveillance and

initiation and progression of the disease [169]. Insulin-like growth factor (IGF) signaling plays an important oncogenic role in hepatocarcinogenesis [40].

Insulin-like growth factor binding protein7 (IGFBP7) is a secreted protein that binds to IGF-1 receptor (IGF-1R) and blocks activation by IGFs [78]. IGFBP7 functions as a tumor suppressor in a variety of cancers, including HCC, where its expression is markedly downregulated [99, 103, 170]. Genomic deletion and promoter hypermethylation cause downregulation of IGFBP7 in HCC [103, 105].

Negative IGFBP7 staining in HCC patients statistically correlated with large tumor size, increased vascular invasion, and poor overall survival and disease-free survival rates [171]. Recombinant IGFBP7 (rIGFBP7) protein induces senescence and/or apoptosis and inhibits angiogenesis in diverse cancers either in vitro or in nude mice xenograft models [99, 119, 170].

Stable overexpression of IGFBP7 in aggressive human HCC cells led to inhibition in IGF signaling, induced senescence, inhibited proliferation and resulted in profound inhibition in xenograft growth in nude mice which was accompanied by marked inhibition in angiogenesis [103]. Intratumoral injection of an adenovirus expressing IGFBP7 (Ad.IGFBP7) eradicated both injected tumors as well as non-injected tumors established in the other flank of nude mice indicating that IGFBP7 not only has direct effect on primary cancer but also exerts a 'by-stander' anti-tumor effect [116]. However, the mechanism by which IGFBP7 exerts this 'by-stander' effect remains to be determined. In this manuscript we describe the generation and characterization of an Igfbp7 knockout ($Igfbp7^{-/-}$) mouse, which establishes the tumor suppressor functions of IGFBP7 and unravels a novel role of IGFBP7 in regulating an anti-tumor immune response. These studies indicate that IGFBP7 inhibits cancer by pleiotropic mechanisms and might be an effective therapeutic for HCC and other cancers.

3.2 Results:

3.2.1 Generation of Igfbp7$^{-/-}$ mouse:

The Sarkar lab generated Igfbp7$^{-/-}$ mice on C57BL/6J background by targeting promoter region and exon 1 of mouse Igfbp7 gene by Cre-loxP strategy (Figure 3.1A-B). The authenticity of Igfbp7$^{-/-}$ mice was confirmed by Q-RT-PCR, Western blot analysis and immunohistochemistry (IHC) (Figure 3.2A-C). IHC staining of the Igfbp7$^{+/+}$ liver showed more IGFBP7 staining in interstitial cells when compared to hepatocytes (Fig. 3.3). QRT- PCR analysis demonstrated significantly more Igfbp7 mRNA expression in stellate cells and macrophages compared to hepatocytes (Fig. 3.4). In liver and spleen, macrophage marker F4/80 positive cells showed positive staining for IGFBP7 further indicating that parenchymal cells are the major source of IGFBP7 (Fig. 3.3). The levels of other IGFBPs and IGF-1 and IGF-2 mRNAs were similar in Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ mice (Fig. 3.5).

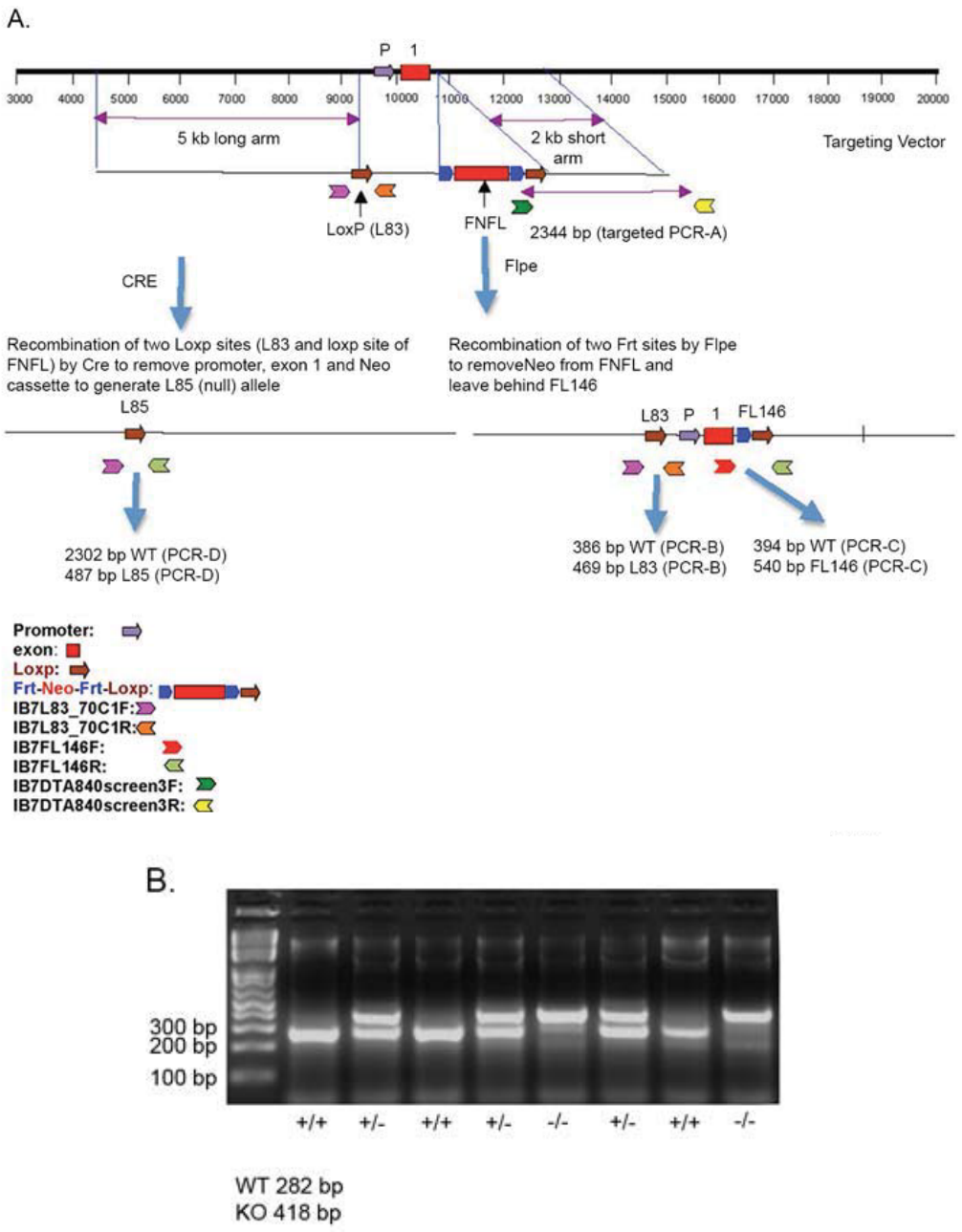


Figure 3.1: A. The schematic representation of the targeting vector used to delete promoter, exon1 and part of intron 1 of Igfbp7 gene.. Mice carrying heterozygous Igfbp7-null allele Igfbp7+/- were intercrossed to generate homozygous Igfbp7-/- mice. Pups were weaned at 4 weeks and tail DNA was obtained to genotype the animals. **B.** Genotyping by PCR using tail DNA as template in 4 weeks old mice.

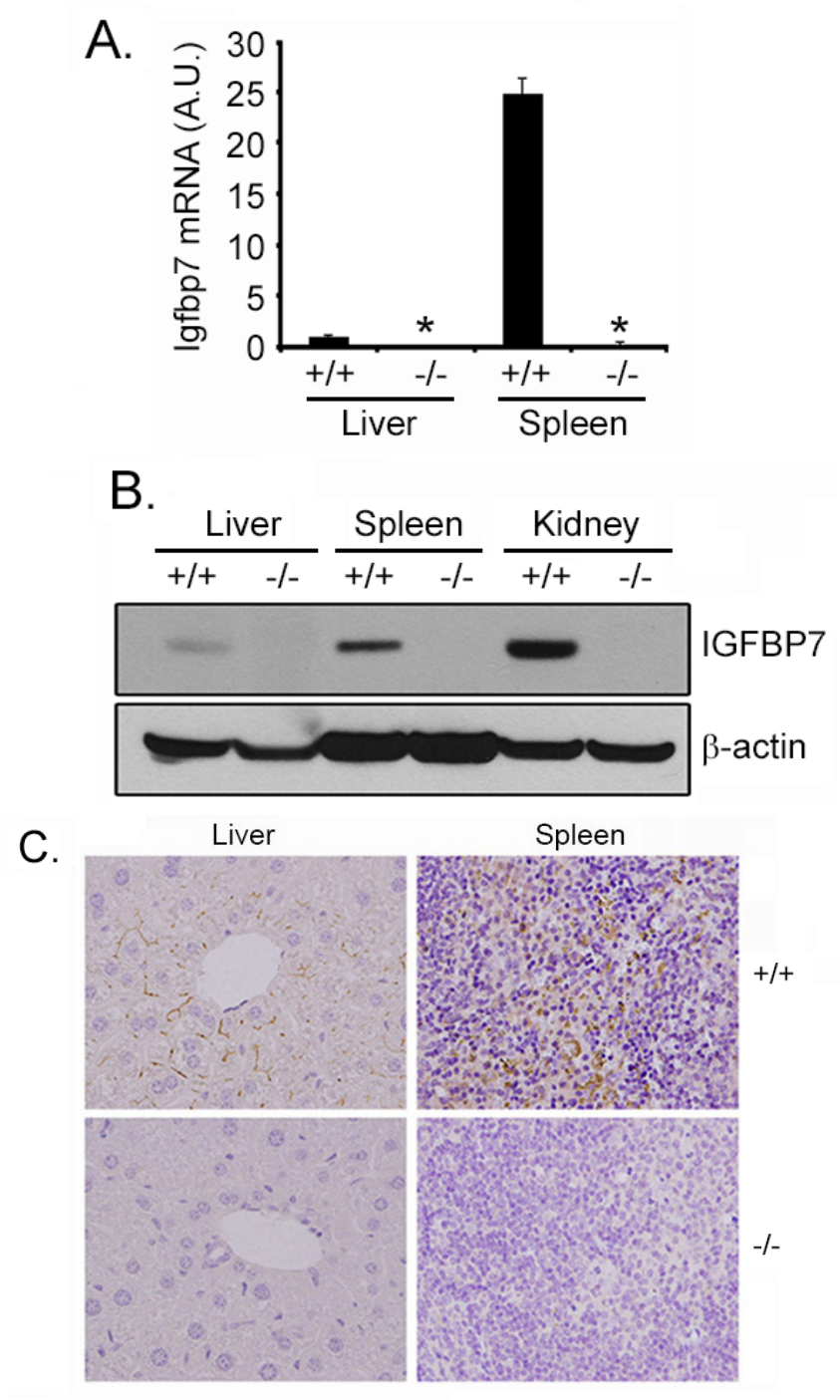


Figure3.2: A. Igfbp7 mRNA levels in livers and spleens of Igfbp7+/+ and Igfbp7-/- mice. **B.** IGFBP7 protein levels in the indicated organs. β-actin: loading control. **C.** Immunohistochemistry for IGFBP7 in sections of liver and spleen of adult mice.

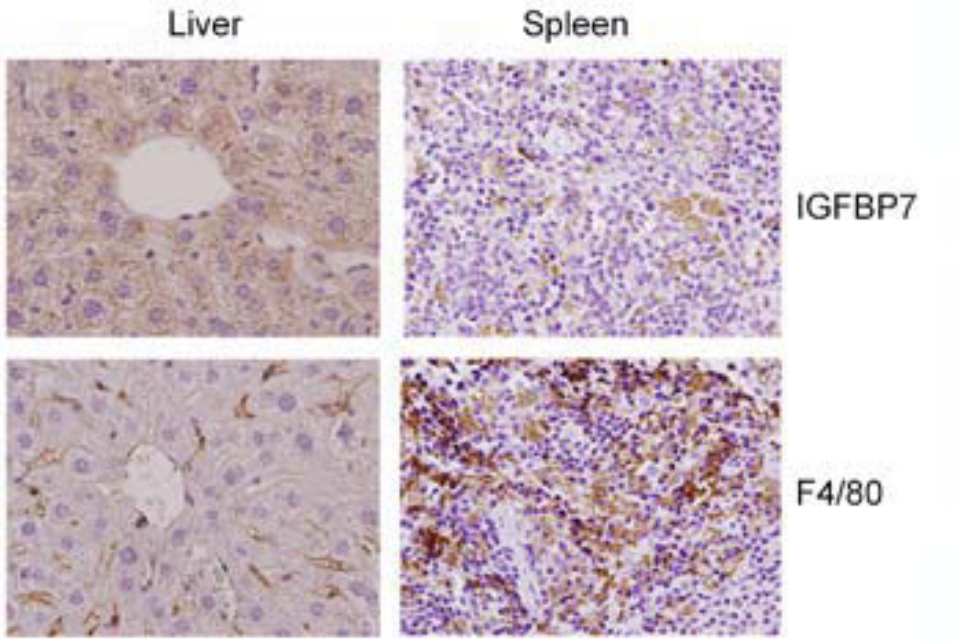


Figure3.3: Immunohistochemistry for IGFBP7 and F4/80 in serial sections of liver and spleen of Igfbp7+/+ mice.

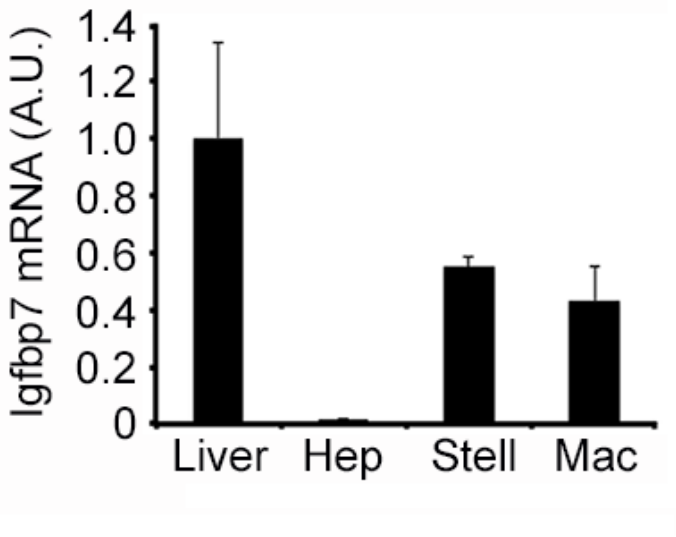


Figure3.4: Igfbp7 mRNA levels in total liver and in hepatocytes (Hep), stellate cells (Stell) and peritoneal macrophages (Mac).

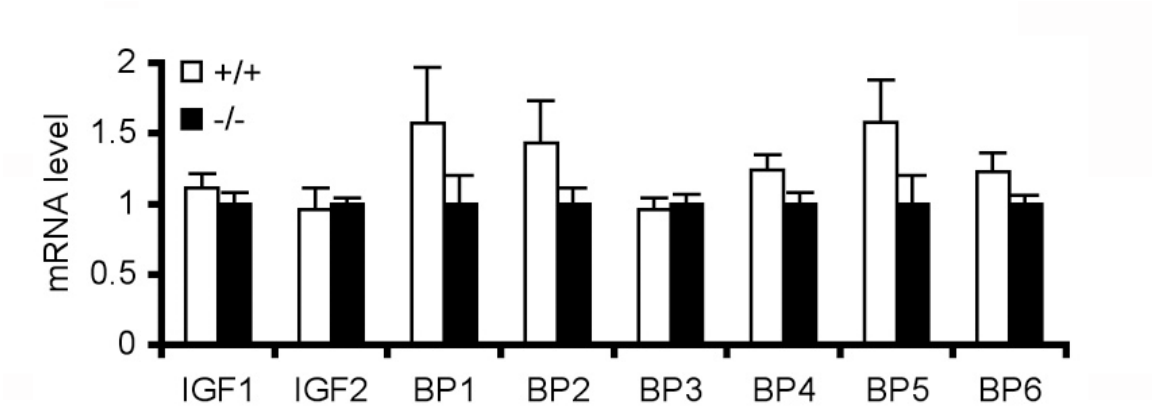


Figure 3.5: mRNA expression levels from RNA-sequencing analysis of IGF1, IGF2, IGFBP1(BP1). IGFBP2(BP2), IGFBP3(BP3), IGFBP4(BP4), IGFBP5(BP5) and IGFBP6(BP6).

3.2.2 Loss of Igfbp7 increases spontaneous tumorigenesis without affecting normal development:

Igfbp7$^{-/-}$ mice were viable and fertile having normal litter size. Histological analysis of internal organs from 8 weeks old mice did not show any discernable difference between Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ mice indicating that Igfbp7 may not play a role in normal development (Fig. 3.6). However, when monitored for 24 months, Igfbp7$^{-/-}$ mice (3 out of 10) developed spontaneous tumors in liver and lung while no tumors were detected in Igfbp7$^{+/+}$ mice (n = 7) (Fig. 3.7). The tumors in the liver showed complete loss of hepatic architecture indicating HCC (Fig. 3.7).

At this age, splenic architecture was distorted in Igfbp7$^{-/-}$ mice compared to Igfbp7$^{+/+}$. Additionally, increased infiltration of immune cells in the tissue microenvironment of liver, lung and kidney was observed in Igfbp7-/- mice suggesting increased inflammation (Fig. 3.7). In 24 months old liver and lung, increase in Il6 mRNA levels (marker of inflammation) was detected in both liver and lung tumors, but increase in α-feto protein (Afp) mRNA levels (marker of HCC) was observed only in liver tumors in Igfbp7$^{-/-}$ mice indicating that the lung tumors are primary tumors and not metastatic lesions from the liver (Fig 3.8). Increased staining for glutamine synthetase (GS, a marker for HCC) and increased infiltration of macrophages (determined by F4/80 staining) was observed in 24 months old Igfbp7-/- liver sections compared to Igfbp7+/+ liver sections (Fig. 3.9A and B). Staining of lung tumors for F4/80 identified macrophages in between large tumor cells and the tumors showed strong positive staining for the proliferation marker PCNA and increased expression of angiogenesis marker Vegf in Igfbp7-/- but not in Igfbp7+/+ (Fig. 3.10 A, B and C).

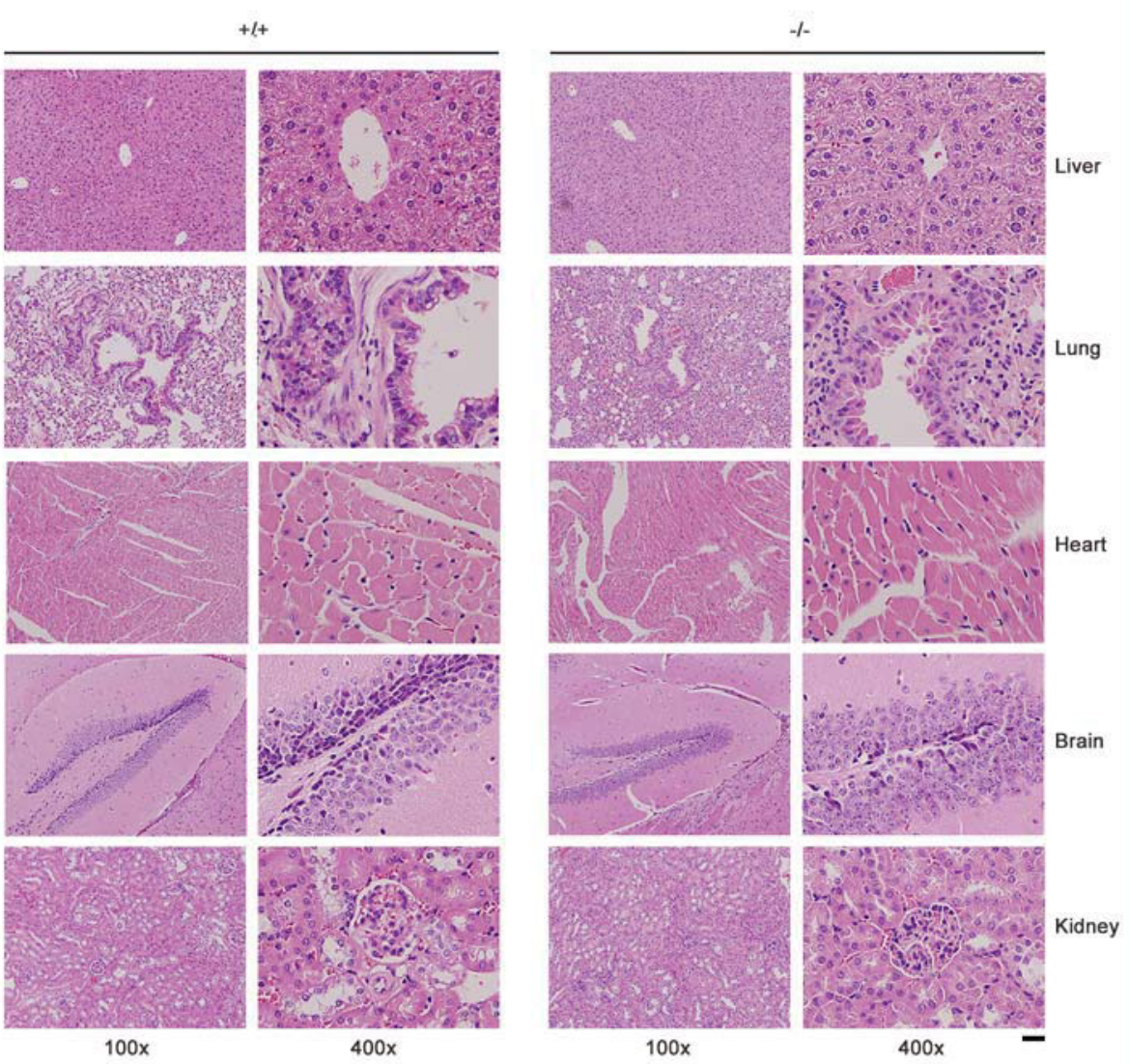


Figure 3.6: H&E staining of the sections of the indicated organs of Igfbp7+/+ and Igfbp7-/- mice at 8 wks of age. Scale bar: 20 μm.

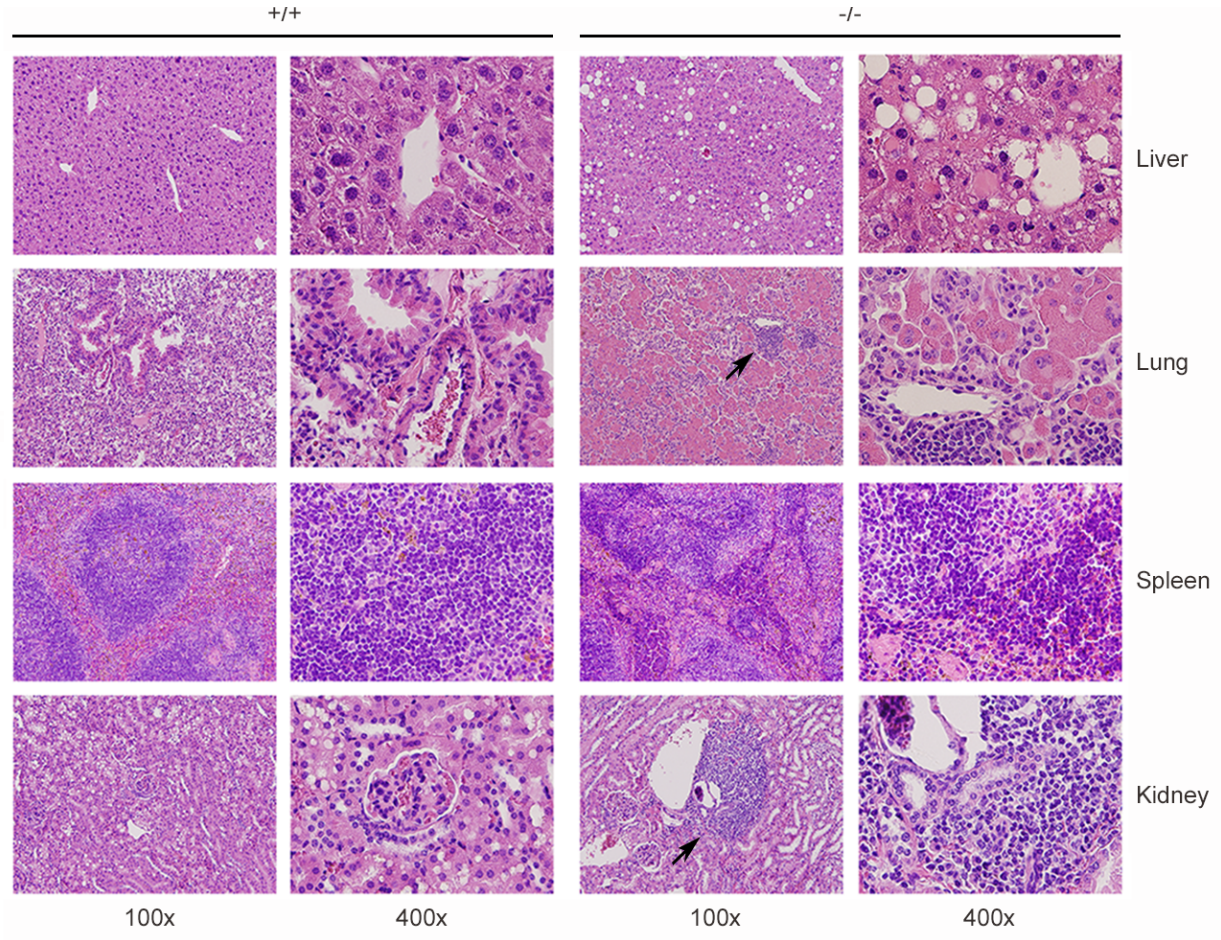


Figure 3.7: H&E staining of sections of the indicated organs in 24 months old mice. Arrows indicate lymphocytic infiltration.

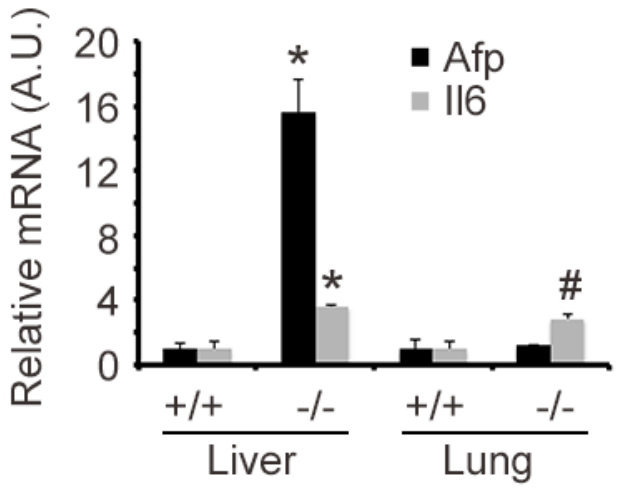


Figure 3.8: Afp and Il6 mRNA levels in livers and lungs of 24 months old Igfbp7+/+ and Igfbp7-/- mice. For graphs, data represent mean ± SD. *: $p<0.01$; #: $p<0.05$. A.U.: arbitrary unit. For all analysis 3 mice per group were used.

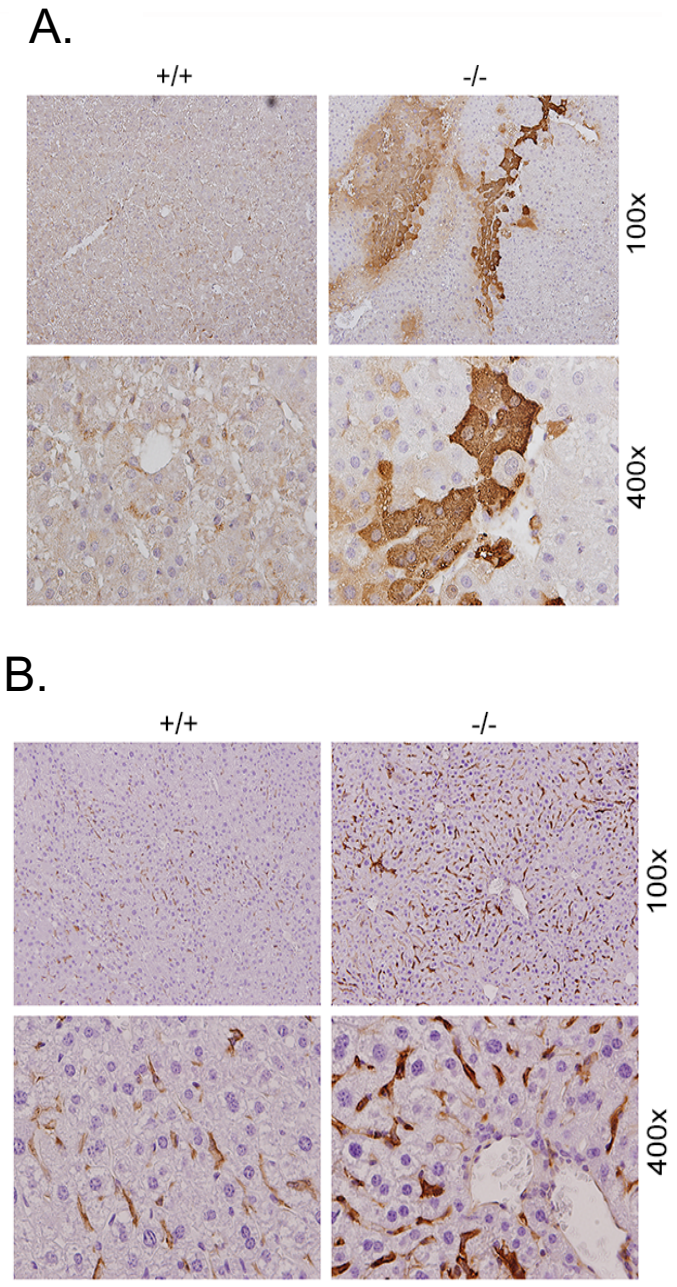


Figure 3.9: A. Glutamine synthase (GS) staining of liver tissue sections of 24 months old mice. **B.** F4/80 staining of liver tissue sections of 24 months old mice. For all analyses at least 3 mice or more were used per group.

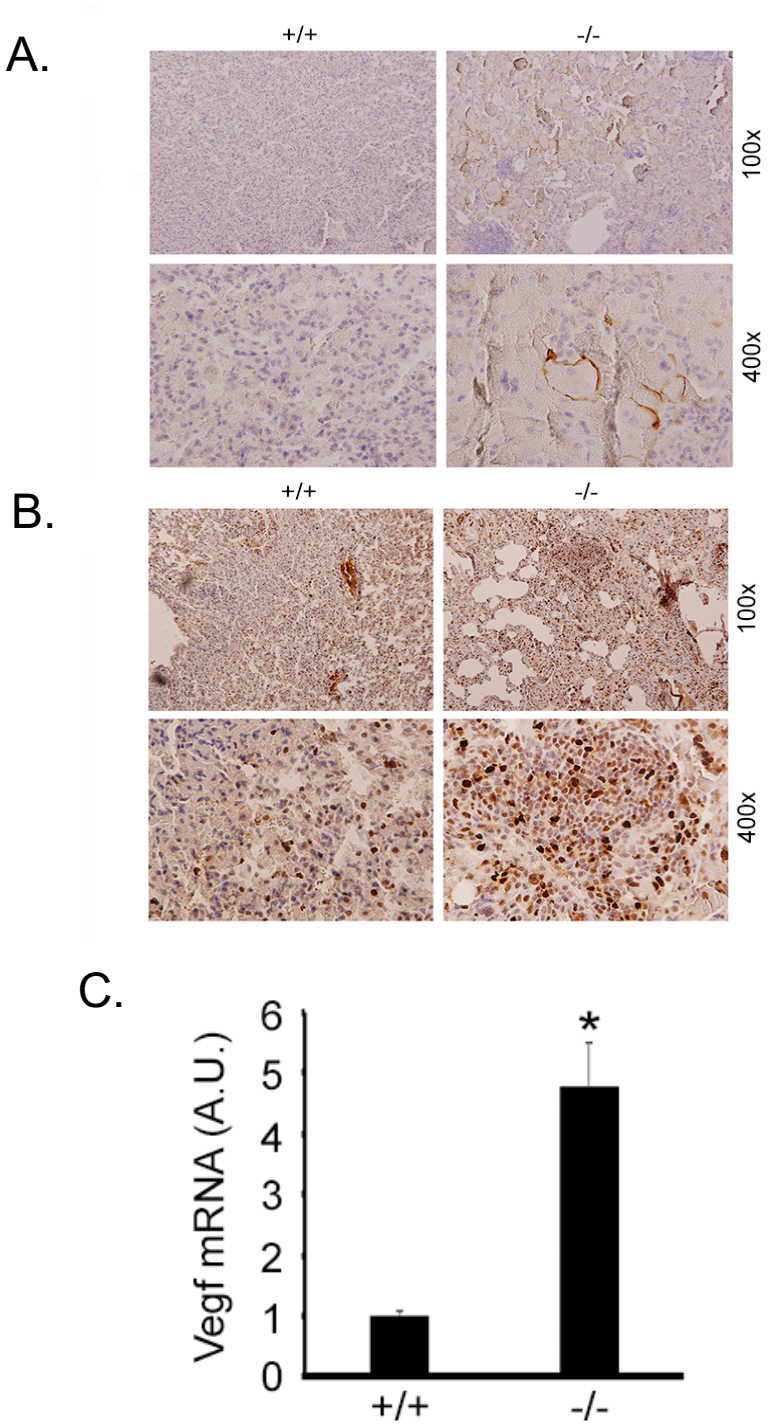


Figure 3.10: A. F4/80 staining of lung tissue from 24 months old mice. **B.** PCNA staining of lung tissue from 24 months old mice. **C.** Vegf mRNA levels in 24 months old mice (RNA isolated from the liver). For all analyses at least 3 mice or more were used per group.

3.2.3 Loss of Igfbp7 increases proliferation and prevents senescence:

We next checked the effect of genetic deletion of Igfbp7 on cell proliferation and senescence. Igfbp7$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed increased proliferation as measured by cell number and colony formation assays when compared to Igfbp7$^{+/+}$ (Fig. 3.11). Similarly, Igfbp7$^{-/-}$ hepatocytes showed increased proliferation by increased cell number and increased BrdU incorporation upon IGF-1 treatment versus Igfbp7$^{+/+}$ (Fig. 3.12).

Mouse hepatocytes do not proliferate in vitro and start undergoing senescence by 96 h. At day 8 of culture approximately 80% of Igfbp7$^{+/+}$ hepatocytes showed senescence versus only around 30% of Igfbp7$^{-/-}$ hepatocytes (Fig. 3.13A). As a corollary, γ-H2AX positive nuclei were significantly reduced in Igfbp7-/- MEFs after 12 hours of serum starvation (Fig. 3.13 B).

To check the effect of Igfbp7 deletion on cell cycle progression, MEFs were synchronized in serum free media for 24 hours following which they were released in complete growth media and subjected to cell cycle analysis. At 18 and 36 h post-release there was a significant decrease in cells in G1 phase and a corresponding increase in cells in G2/M phase in Igfbp7-/- MEFs compared to Igfbp7+/+ MEFs indicating that Igfbp7-/- MEFs cycle faster than their wild type counterparts (Fig. 3.14).

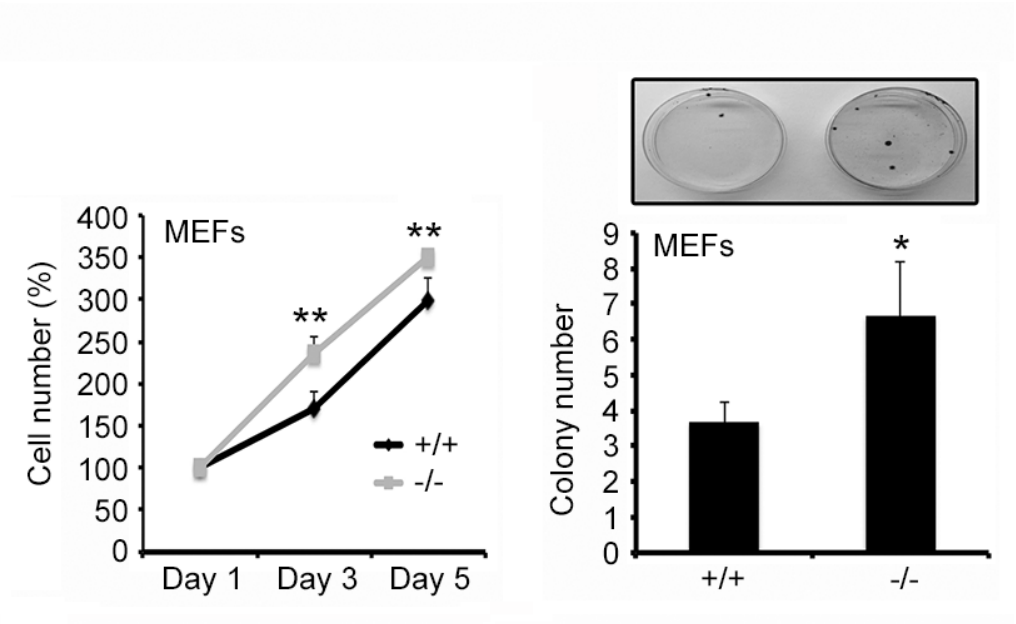


Figure 3.11: Cell proliferation by cell number (left) and colony formation assays (right) in mouse embryonic fibroblasts (MEFs). For all analyses at least 3 mice or more were used per group; data represent mean ± SD. *: $p<0.01$.

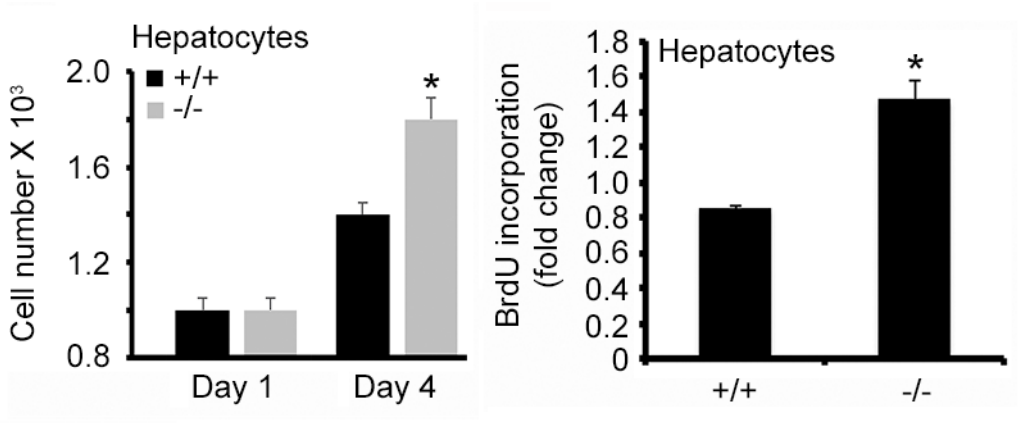
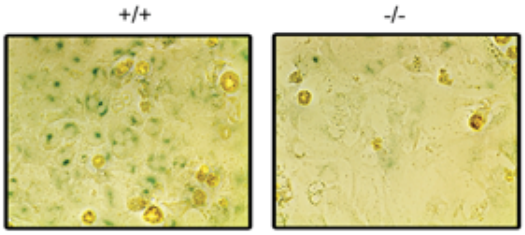
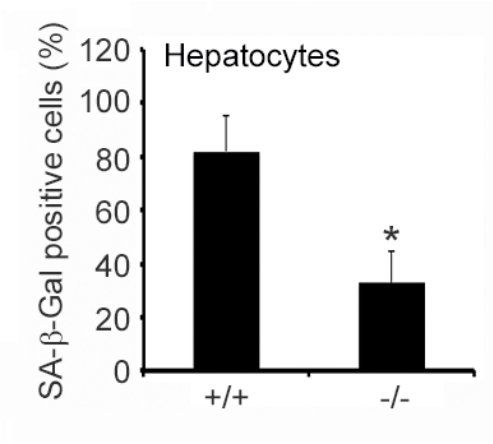


Figure 3.12: Cell proliferation by cell number (left) and IGF-1-stimulated BrdU incorporation assays (right) in hepatocytes. Hepatocytes were cultured in insulin-free medium for 12 hours prior to treatment with IGF-1 (20ng/mL). For all analyses at least 3 mice or more were used per group; data represent mean ± SD. *: $p<0.01$.

A.





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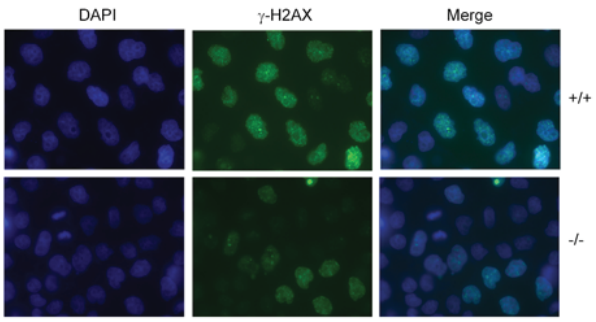


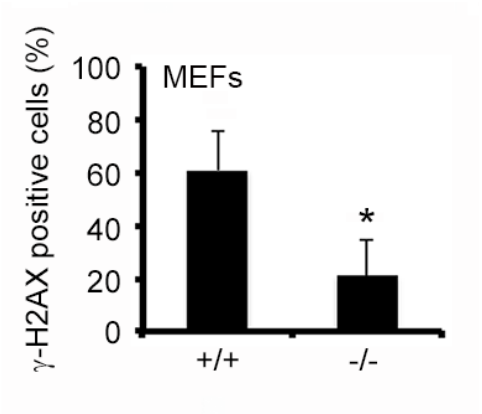


Figure 3.13: A. Senescence-associated β-galactosidase (SA-β-Gal)-positive hepatocytes at day 8 of culture. **B.** γ-H2AX positive MEFs after 12 h of serum starvation. For all analyses at least 3 mice were used; data represent mean ± SD. *: $p<0.01$.

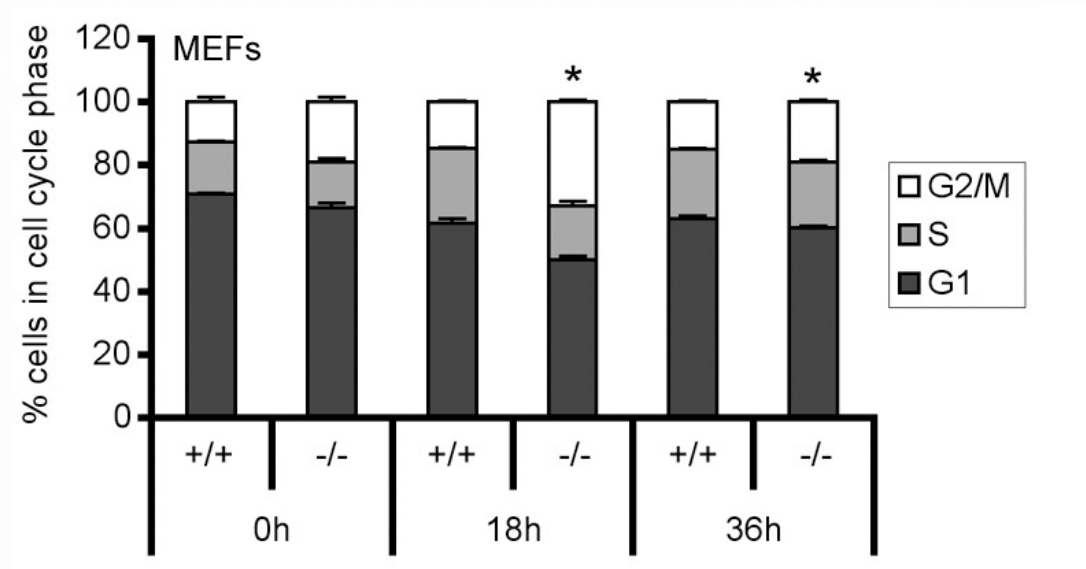


Figure 3.14: Cell cycle analysis of MEFs at 18 and 36 h after release from serum-free medium. For all analyses at least 3 mice were used; data represent mean ± SD. *: $p<0.01$.

3.2.4 Loss of Igfbp7 activates the IGF-1 signaling pathway:

Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ hepatocytes were treated with recombinant mouse IGF-1 (20ng/ml). Constitutive activation of IGF-1R and downstream Akt and GSK3β was observed in Igfbp7$^{-/-}$ hepatocytes, and there was a further increase in IGF-1R, Akt and GSK3β activation in Igfbp7$^{-/-}$ hepatocytes, compared to Igfbp7$^{+/+}$, at 5 min of exposure to IGF-1 suggesting that Igfbp7 null hepatocytes have increased propensity for activation of the IGF-1 pathway (Fig. 3.15 A). Activation persisted until 60 min. Analysis of livers of adult mice also showed increased activation of Akt and its downstream target GSK3β further confirming constitutive activation of IGF-1 signaling in Igfbp7$^{-/-}$ mice (Fig. 3.15B).

Constitutive activation of Akt, GSK3β and ERK was also observed in Igfbp7$^{-/-}$ hepatocytes, macrophages and MEFs compared to Igfbp7$^{+/+}$ (Fig. 3.16 and 3.18). Since Igfbp7$^{-/-}$ MEFs cycle faster than Igfbp7$^{+/+}$ MEFs, we checked the levels of cyclin D1 (CCND1) and cyclin E1 (CCNE1), which facilitate G1 to S cell cycle progression. Increased levels of CCND1 and CCNE1 was observed in Igfbp7$^{-/-}$ MEFs versus Igfbp7$^{+/+}$ thus explaining the accelerated cell cycle progression in the former (Fig. 3.16 and 3.18). We next checked how constitutive activation of IGF-1 signaling pathway modulates response of Igfbp7$^{-/-}$ MEFs to OSI-906, a dual kinase inhibitor of IGF-1R and insulin receptor (INSR). Igfbp7$^{-/-}$ MEFs were more sensitive to growth inhibition by OSI-906 compared to Igfbp7$^{+/+}$ suggesting that Igfbp7$^{-/-}$ MEFs might have preferential addiction to IGF signaling (Fig. 3.17). Overall, these results indicate that activation of the IGF-1 signaling pathway contributes to increased proliferation upon Igfbp7 deletion.

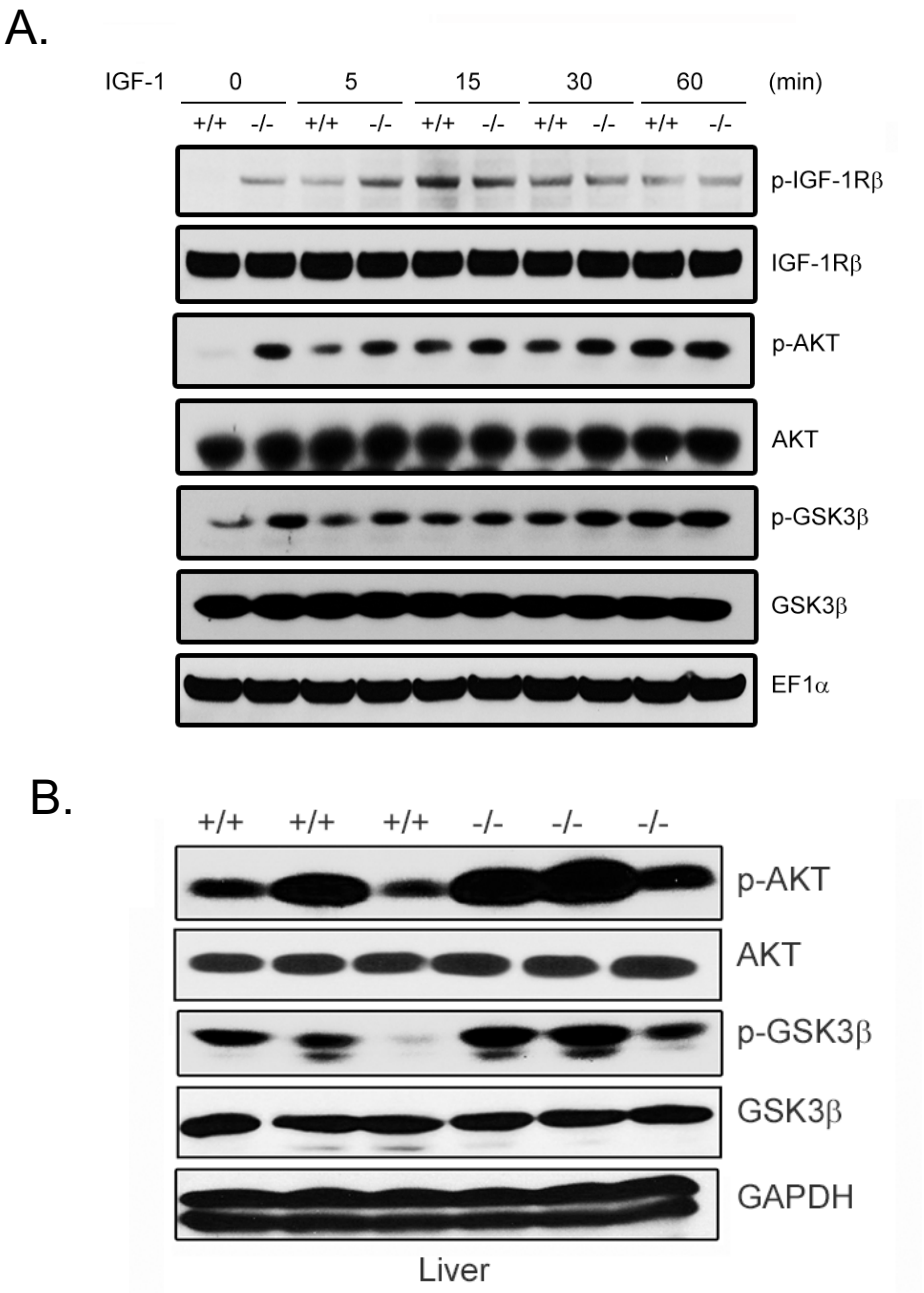


Figure 3.15: A. Western blot analyses for the indicated proteins were performed. Hepatocytes were cultured in insulin-free medium for 12 hours prior to treatment with IGF-1 (20ng/mL) **B.** Western blot analysis for the indicated proteins in liver lysates of three independent Igfbp7+/+ and Igfbp7-/- mice.

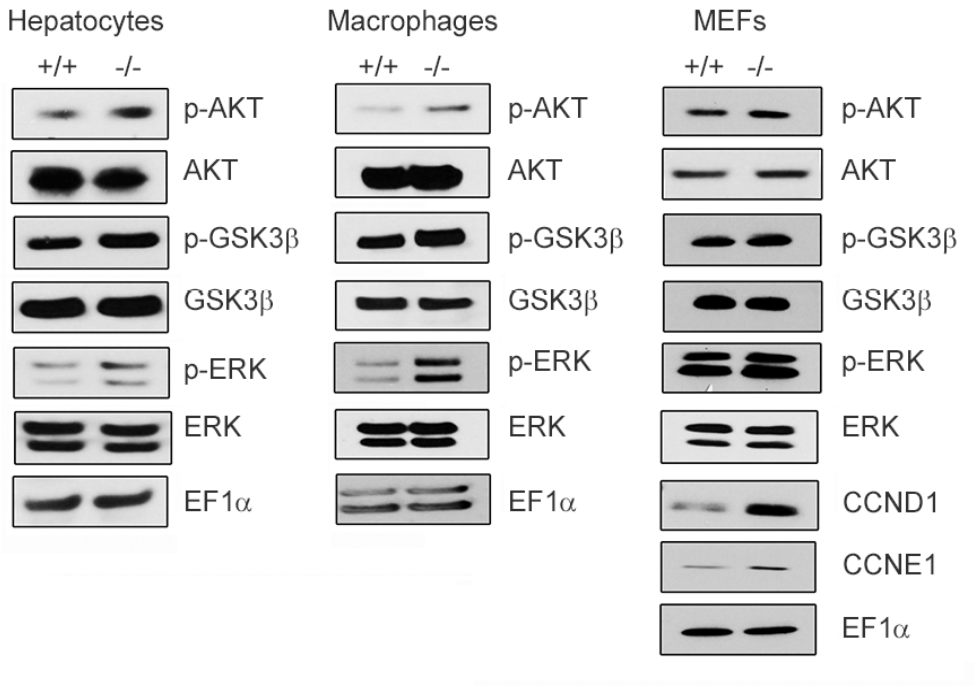


Figure 3.16: Western blot analysis for the indicated proteins in the indicated cells isolated from adult mice. For each cell type a representative EF1α blot is shown as loading control.

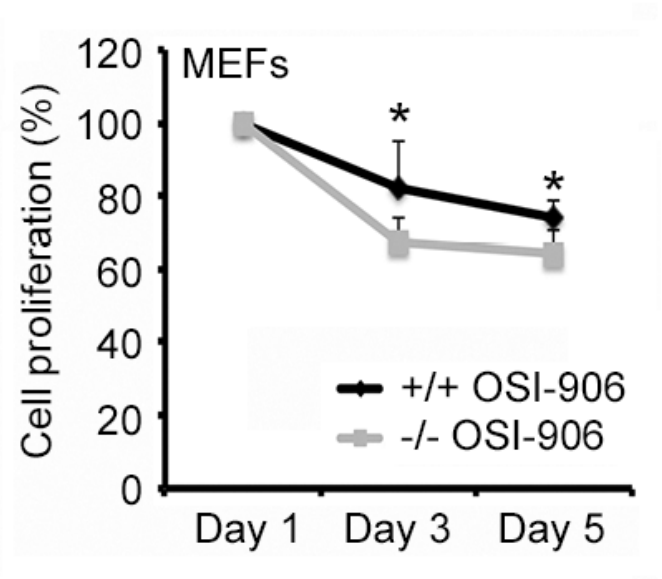


Figure 3.17: Cell proliferation analysis by MTT assay in MEFs upon treatment with OSI-906 (4 μmol/L). 3 independent Igfbp7+/+ and Igfbp7-/- mice were used; data represent mean ± SD. *: $p<0.01$.

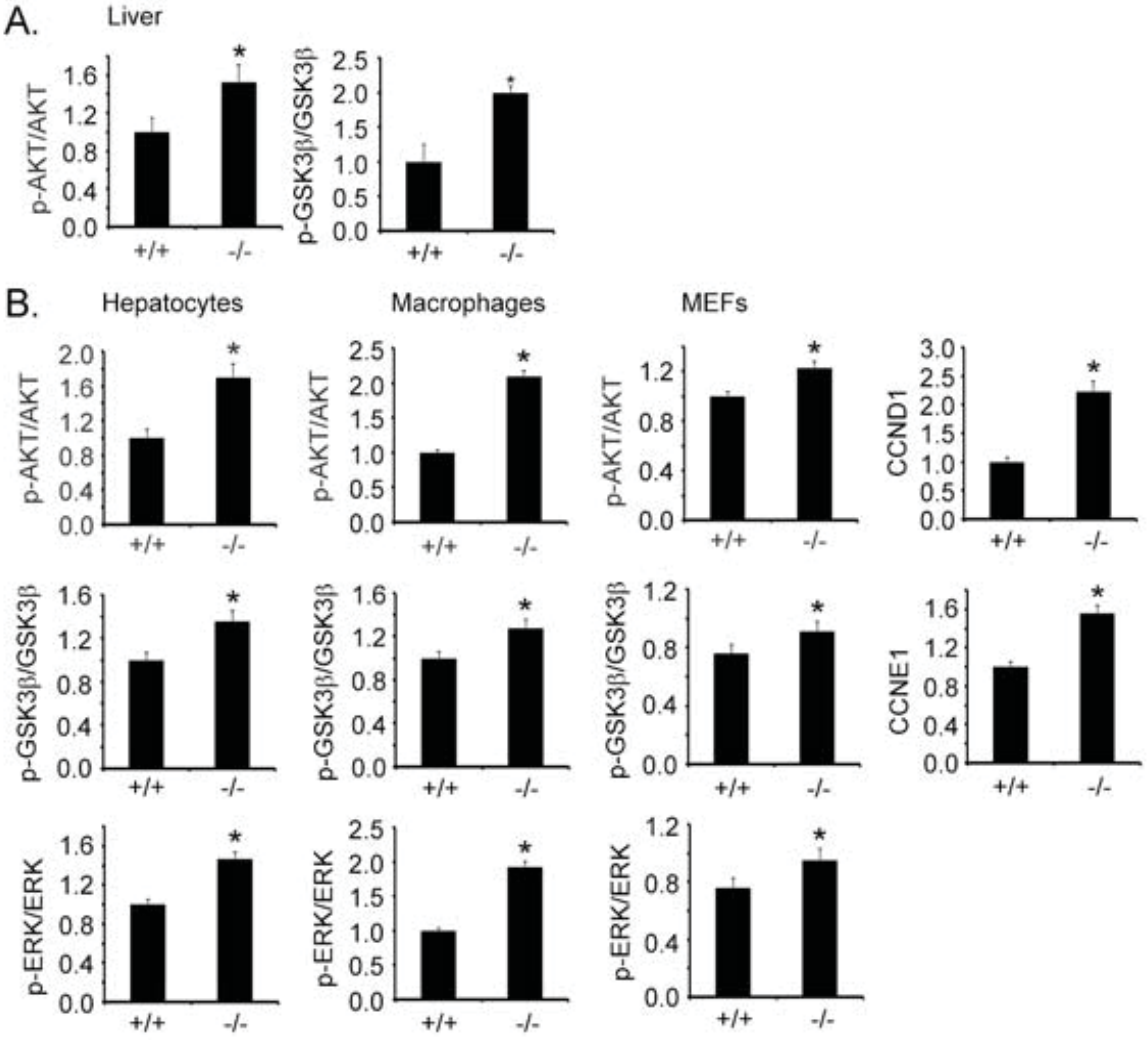


Figure 3.18: **A.** Densitometric analysis of p- AKT/AKT and p-GSK3β/GSK3β ratios in the livers of Igfbp7+/+ and Igfbp7-/- mice. **B.** Densitometric analysis of p-AKT/AKT, p-GSK3β//GSK3β and p-ERK/ERK ratios in Igfbp7+/+ and Igfbp7-/- hepatocytes, macrophages and MEFS, and densitometric analysis of CCND1 and CCNE1 levels in Igfbp7+/+ and Igfbp7-/- MEFs. Data represent mean ± SD of three independent experiments. *: $p<0.01$.

3.2.5 Loss of Igfbp7 results in markedly accelerated DEN-induced HCC:

Since aged Igfbp7$^{-/-}$ mice develop spontaneous HCC, we further analyzed HCC development upon exposure to the hepatocarcinogen N-nitrosodiethylamine (DEN), a well-established HCC model [172, 173]. HCC was induced in Igfbp7$^{+/+}$, +/- and -/- littermates by a single i.p. injection of DEN (10 μg/gm) when the mice were two weeks of age. At 32 weeks, Igfbp7$^{-/-}$ mice demonstrated a marked increase in both number and size of hepatic nodules when compared to the other genotypes (Fig. 3.19 A-B). This increase in hepatic nodules contributed to increased liver to body weight ratio in Igfbp7$^{-/-}$ mice (Fig. 3.19C). Liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and serum total protein were significantly increased in Igfbp7$^{-/-}$ mice versus +/+ and +/- mice (Fig. 3.20 A and B).

Histological analysis of the liver showed extensive abnormality in liver architecture with increased infiltration of immune cells and abnormal blood vessel formation (Fig.3.21). Increased staining for AFP, proliferating cell nuclear antigen (PCNA; proliferation marker), c-MYC, CD31 (angiogenesis marker) and F4/80 (macrophage marker) was observed in Igfbp7-/- liver sections when compared to Igfbp7$^{+/+}$ indicating increased proliferation, angiogenesis, inflammation and development of frank HCC (Fig. 3.21). Increased staining for glutamine synthetase was also observed in DEN-treated Igfbp7-/- livers compared to Igfbp7+/+ (Fig. 3.22).

To exclude the possibility that the increased HCC development in Igfbp7-/- mice is because of increased sensitivity to DEN we analyzed liver functions 48 hours after injection of DEN. Both Igfbp7$^{+/+}$ and -/- littermates showed similar levels of changes in AST, ALT and Alkaline Phosphatase (ALP), a marker of acute liver damage, suggesting that littermates of both genotypes respond similarly to DEN-induced liver injury (Fig.3.23A). DEN needs to be metabolically activated by CYP2E1 in zone 3 hepatocytes. Cyp2e1 mRNA levels were similar in Igfbp7+/+ and -/- livers (Fig. 3.23B) suggesting that altered metabolism of DEN does not underlie differential tumorigenic response.

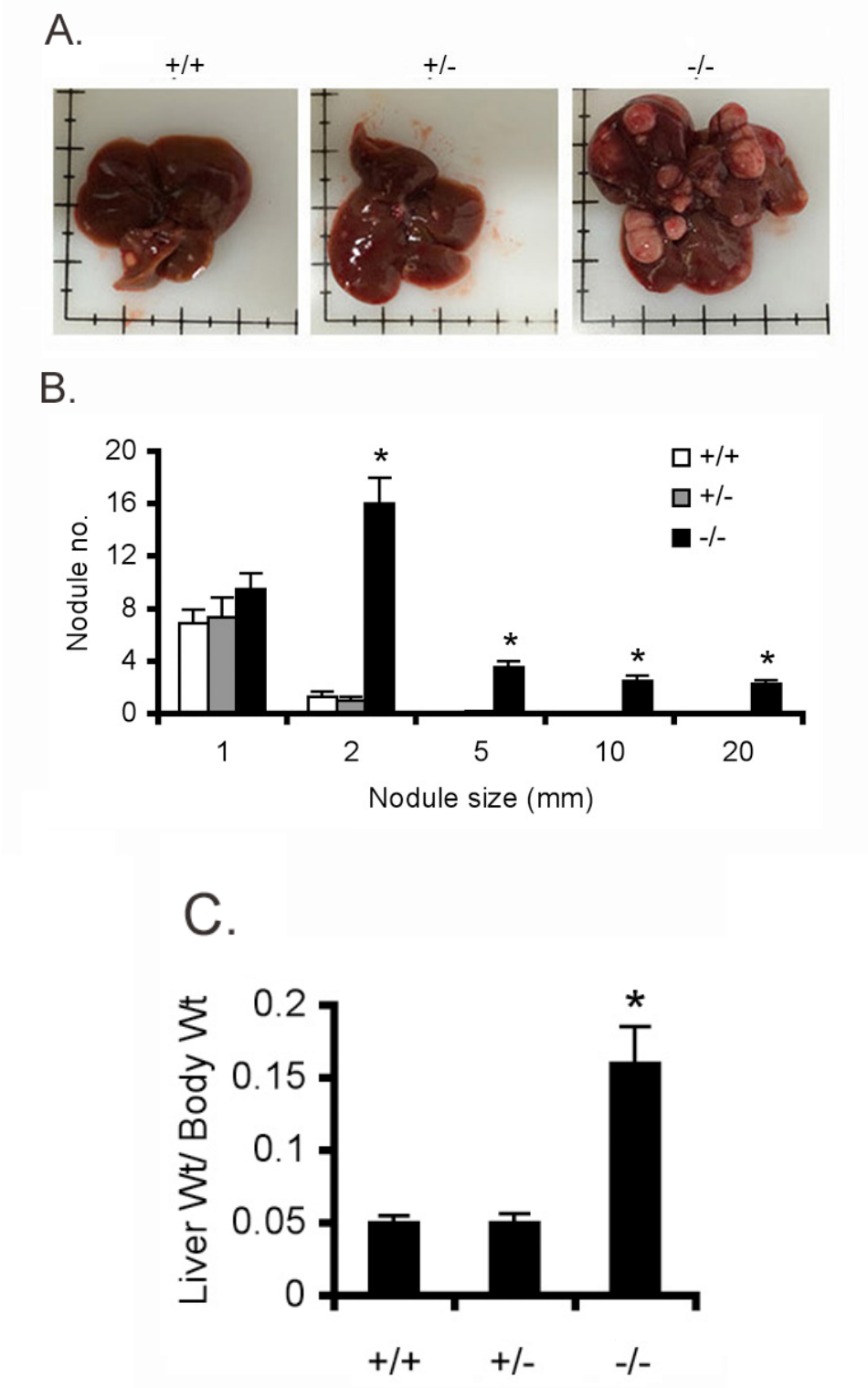


Figure 3.19: **A.** Photograph of DEN-treated mouse livers of the indicated genotypes. **B.** Number and sizes of liver nodules. **C.** Liver weight to body weight ratio of DEN-injected mice (15 mice for +/+, 12 mice for +/- and 11 mice for -/-). Data represent mean ± SD.

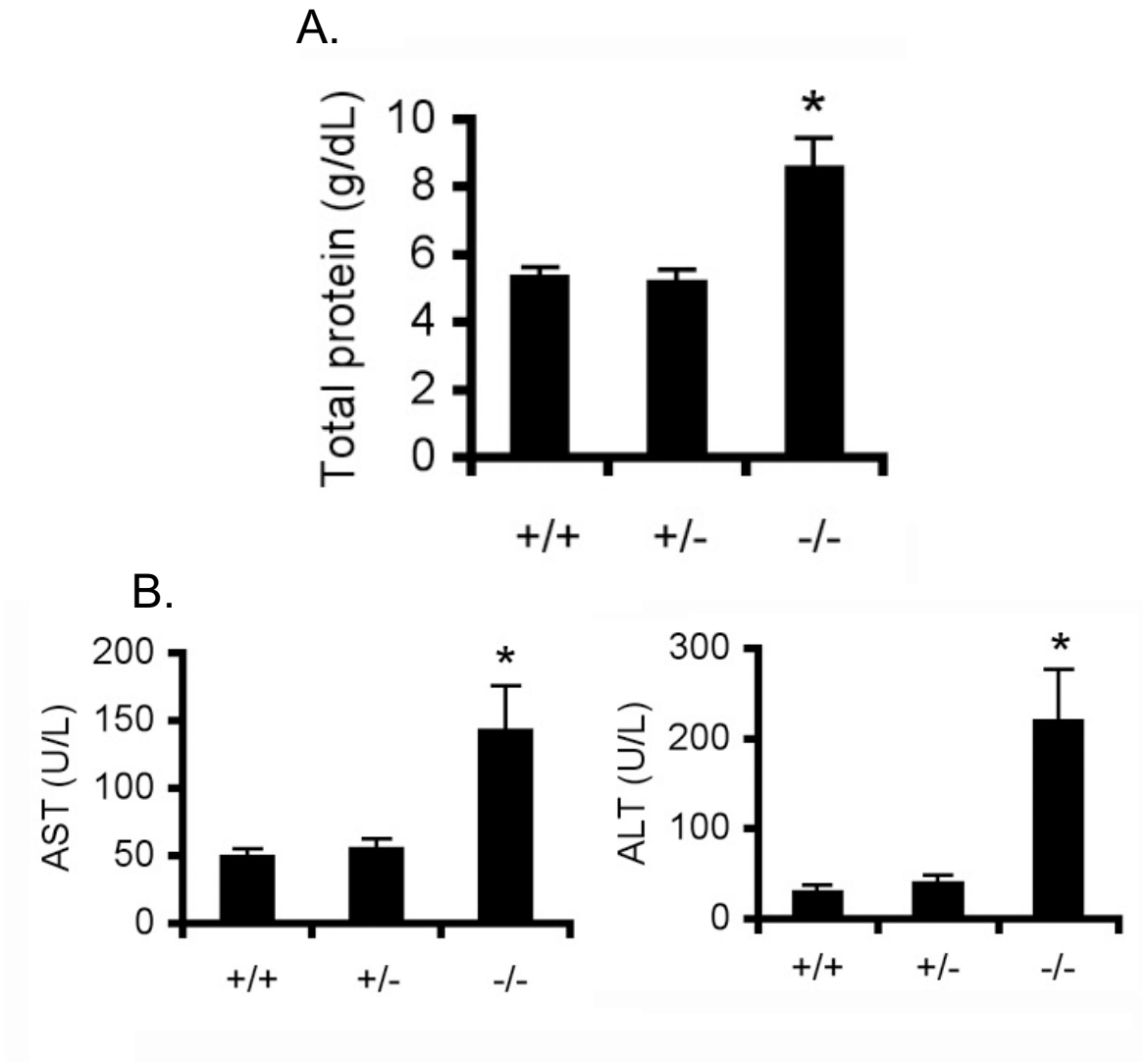


Figure 3.20: **A.** Total protein and **B.** Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) in DEN-injected mouse sera. Data represent mean ± SD.

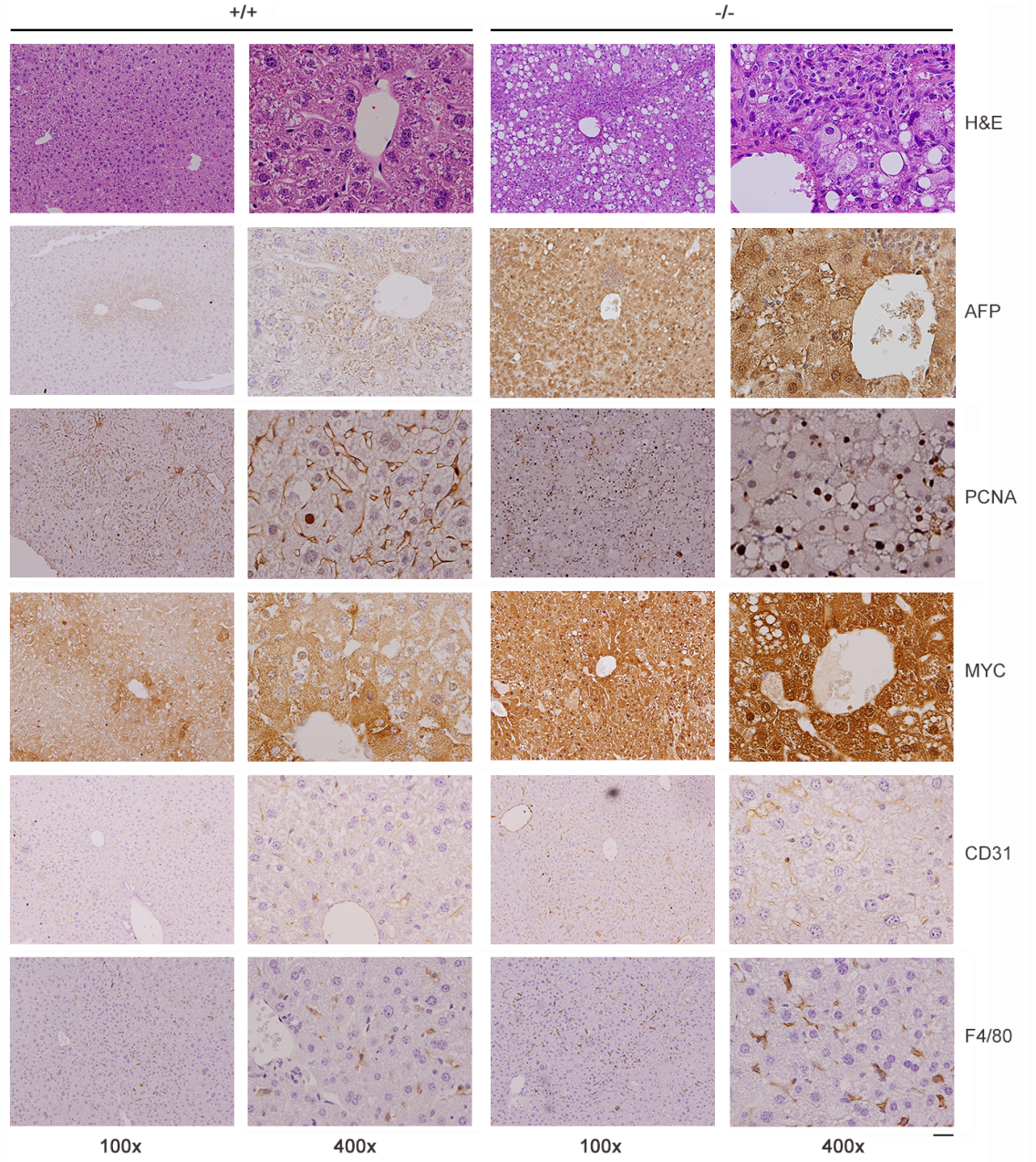


Figure 3.21: H&E and immunostaining for the indicated proteins in liver sections. Scale bar: 20 μm.

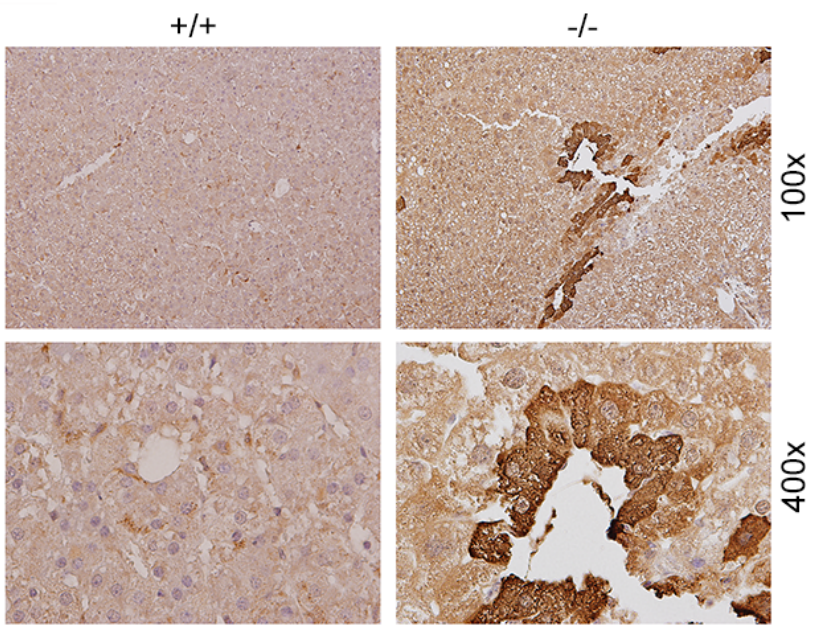


Figure 3.22: Glutamine synthase staining of liver sections from 32 weeks DEN injected mice.

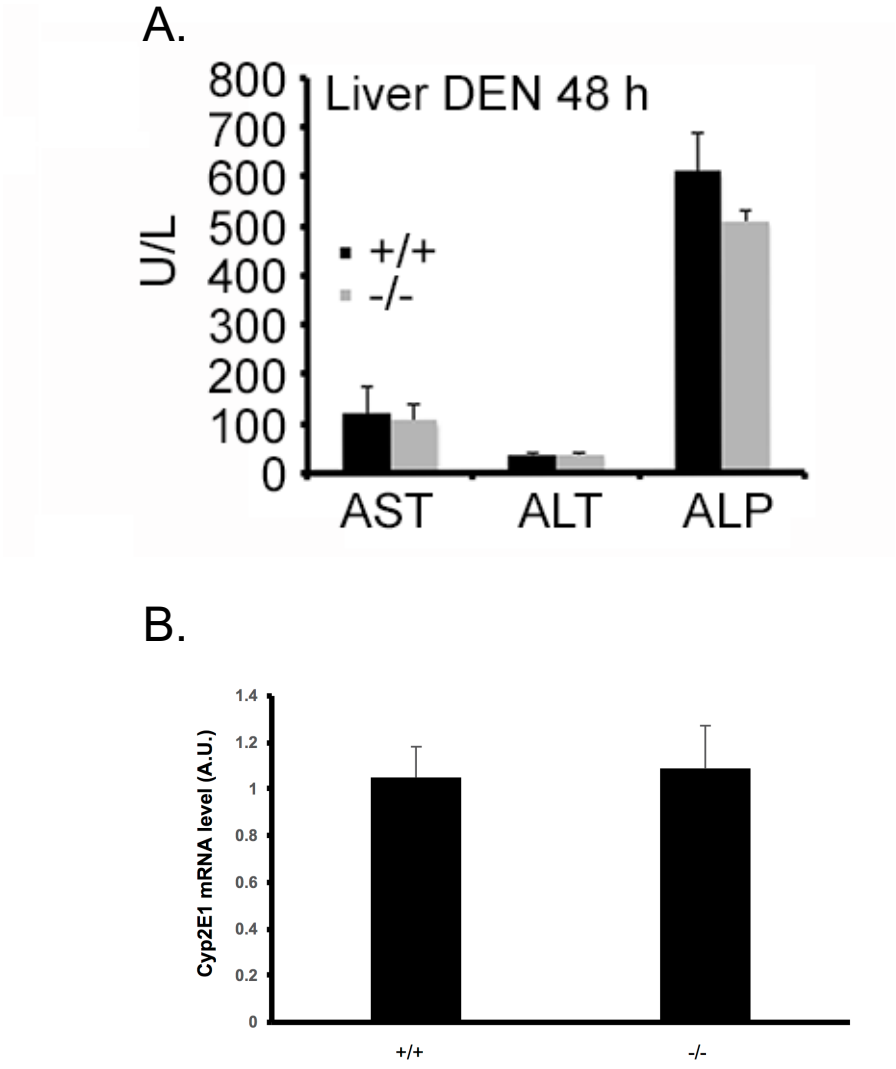


Figure 3.23: A. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels in mouse sera 48 h after DEN injection. Data represent mean ± SD (n = 3 per group). **B**. Cyp2E1 levels from liver RNAs.

3.2.6 Igfbp7 deletion creates an inflammatory and immunosuppressive tumor microenvironment:

DEN-induced HCC is an inflammatory-type of HCC in which damaged hepatocytes release cytokines, mainly IL-1β, which stimulate Kupffer cells to release IL-6 and TNFα by activating NF-κB signaling [174-176]. In this context, we analyzed immune cell profile in Igfbp7$^{+/+}$ and -/- mice by fluorescence-activated cell sorting (FACS). Examination of 8 weeks old naïve liver and spleen showed no significant difference in CD8^{+} and CD4^{+} T cells, natural killer (NK) cells, B cells, dendritic cells (DCs), splenic and liver macrophages, and myeloid-derived suppressor cells (MDSCs), indicating that IGFBP7 may not be required for immune cell development (Fig. 3.24A-B). However, upon examining the livers of DEN-treated mice at 20 weeks of age, a time-point before the development of overt hepatic nodules but having microscopic tumors as evidenced by increased AFP and c-Myc expression (Fig. 3.26), we found a significant increase in Kupffer cells (CD11b^{low}/F4/80^{high}), infiltrating macrophages (CD11b^{high}/F4/80^{high}) and CD11b^{+} monocytic MDSCs (Ly6G-/Ly6C+) in Igfbp7-/- livers compared to +/+ livers (Fig. 3.25A).

These results suggest that Igfbp7-/- mice have an inflammatory and immunosuppressive tumor microenvironment. M-MDSCs mediate immune suppressive function through L-arginine depletion by increasing expression of arginase-1 (ARG1) that abrogate antitumor responses by T cells [177]. Indeed, DEN-treated Igfbp7-/- livers showed substantially increased levels of ARG1 compared to Igfbp7$^{+/+}$ (Fig. 3.25B). This increased immune suppressive environment might contribute to a decrease in liver infiltrating NK cells as well as CD4^{+} and CD8^{+} T cells (Fig3.25A). The inflammatory environment in Igfbp7$^{-/-}$ cells was further checked at gene expression levels. Il6, Il1b, Tnfa and c-Myc (marker of proliferation) mRNAs showed significantly increased levels in the livers of DEN-treated Igfbp7$^{-/-}$ livers when compared to +/+

at 32 weeks (Fig. 3.27A upper panel). Igfbp7-/- hepatocytes, isolated at 24 wks after DEN treatment, and Igfbp7-/- Kupffer cells, isolated at 5 weeks after DEN treatment, also showed increased levels of Il6, Il1b and Tnfa versus Igfbp7$^{+/+}$ (Fig. 3.27A lower panel).

Primary hepatocytes treated in vitro with DEN for 24 h showed significantly increased levels of Il1b and Tnfa, but not Il6, in Igfbp7$^{-/-}$ compared to Igfbp7$^{+/+}$ indicating that Igfbp7$^{-/-}$ hepatocytes respond more to DEN-treatment in terms of generating cytokines leading to a robust activation of Kupffer cells generating Il6 (Fig. 27B, top). We treated macrophages with IL-1β (10 ng/ml) and measured Il6 and TNFa mRNA levels. Basal Il6 and TNFa levels were significantly higher in Igfbp7$^{-/-}$ macrophages versus +/+ (Fig. 27B, bottom). IL-1β treatment induced Il6 and TNFa mRNA expression in both Igfbp7$^{+/+}$ and -/- macrophages. However, the magnitude of induction was significantly more in Igfbp7$^{-/-}$ macrophages when compared to +/+. While inflammatory cytokine levels were similar in 6 weeks old Igfbp7+/+ and Igfbp7-/- livers, they were higher in 24 weeks old Igfbp7-/- livers compared to Igfbp7+/+ indicating that Igfbp7 deficiency with age creates a precancerous milieu for development of spontaneous HCC (Fig. 3.28 A and B).

It is well-established that NF-κB is the pivotal regulator of inflammation-driven HCC and other cancers (25,27). Under basal condition, p50/p65 NF-κB resides in the cytoplasm and upon stimulation translocates to the nucleus. In Igfbp7$^{+/+}$ hepatocytes, p65 NF-κB was located exclusively in the cytoplasm, while some nuclear staining of p65 was detected in Igfbp7$^{-/-}$ hepatocytes (Fig. 3.29A). Upon stimulation with lipopolysaccharide (LPS) p65 translocated to the nucleus in both cells. However, the magnitude of translocation was more robust in Igfbp7$^{-/-}$ hepatocytes compared to Igfbp7$^{+/+}$. To quantify these changes, we measured NF-κB luciferase reporter activity in Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ primary hepatocytes. Both basal and LPS-induced

luciferase activity was significantly increased in Igfbp7$^{-/-}$ hepatocytes when compared to Igfbp7$^{+/+}$ (Fig. 29B). Igfbp7$^{-/-}$ hepatocytes and bone marrow-derived macrophages showed increased levels of phosphorylated p65 upon LPS treatment further confirming that Igfbp7 deletion results in activation of NF-κB (Fig. 3.29C).

We checked whether Igfbp7 status in the macrophages affects tumorigenesis by HCC cells. For this purpose, we injected mouse HCC cells Dihxy [142] either alone or with Igfbp7$^{+/+}$ or Igfbp7$^{-/-}$ bone marrow-derived macrophages at a tumor: macrophage ratio of 10:1 or 20:1 s.c. in NSG mice and monitored tumor development. Co-injection of Igfbp7$^{-/-}$ macrophages significantly stimulated tumorigenesis by Dihxy cells compared to Igfbp7$^{+/+}$ cells indicating that Igfbp7 loss intrinsically changes macrophage properties rendering them more tumor-promoting (Fig. 3.30A).

We cultured bone marrow-derived macrophages either in serum-free medium (control) or in conditioned media (CM) from Dihxy cells and analyzed expression of Il6 (marker for M1 macrophage) and Arg1 (marker for M2 macrophage) (Fig. 3.30B). Dihxy CM induced Il6 and Arg1 expression in both Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ macrophages. However, this induction was more robust in Igfbp7$^{-/-}$ macrophages, especially for Arg1, indicating that Igfbp7$^{-/-}$ macrophages have a propensity for polarization to M2 subtype, which promotes angiogenesis and tissue remodeling as well as immunosuppression [178-180].

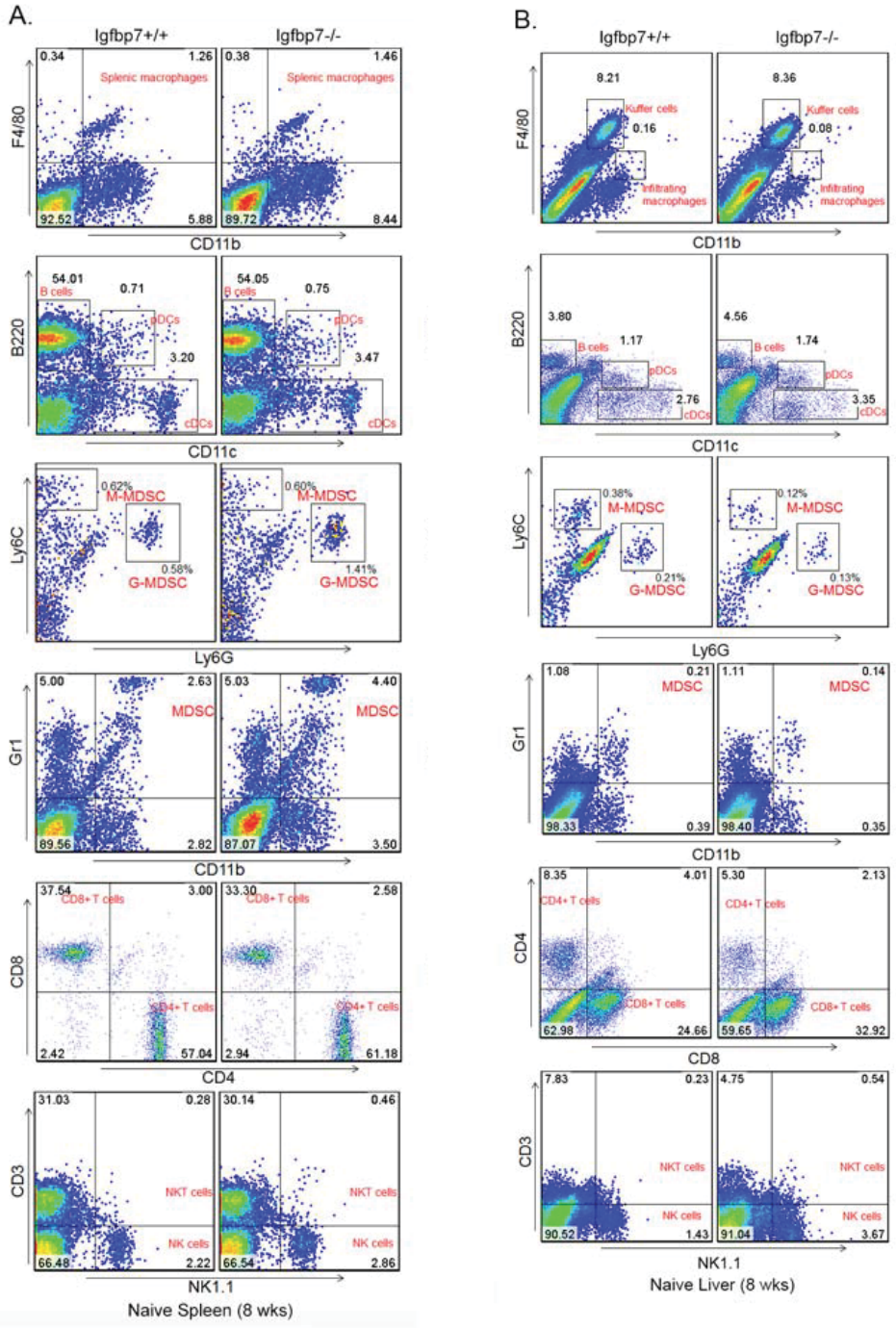


Figure 3.24: A. Splenic immune profile of 8 weeks old naïve mice determined by FACS analysis. **B.** Liver immune profile of 8 weeks old naïve mice determined by FACS analysis. 3 mice were used per group.

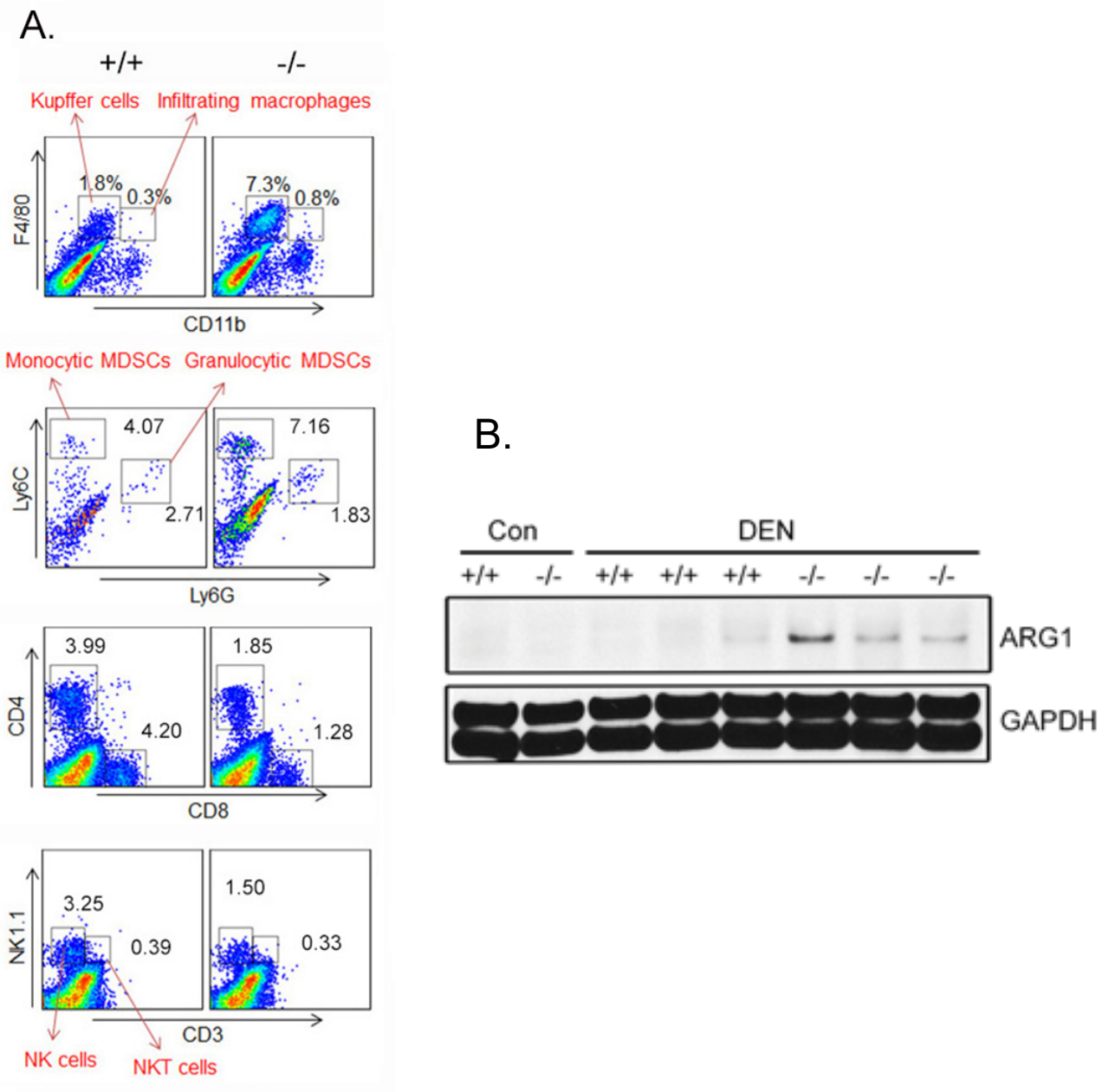


Figure 3.25: A. Immune profile of DEN-treated Igfbp7+/+ and Igfbp7-/- mice at 20 weeks. **B.** Western blot analysis for arginase-1 (ARG1) in the livers of naïve Igfbp7+/+ and Igfbp7- /- mice or three independent DEN-treated Igfbp7+/+ and Igfbp7-/- mice.

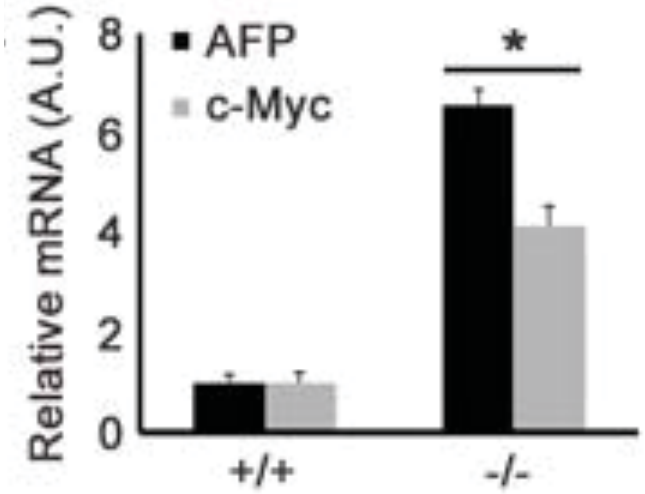


Figure 3.26:. AFP and c-Myc mRNA expression levels in Igfbp7+/+ and Igfbp7-/- livers at 20 wks after DEN treatment analyzed by Q-RT-PCR. Data represent mean ± SD. *: $p<0.01$. 3 mice were used per group.

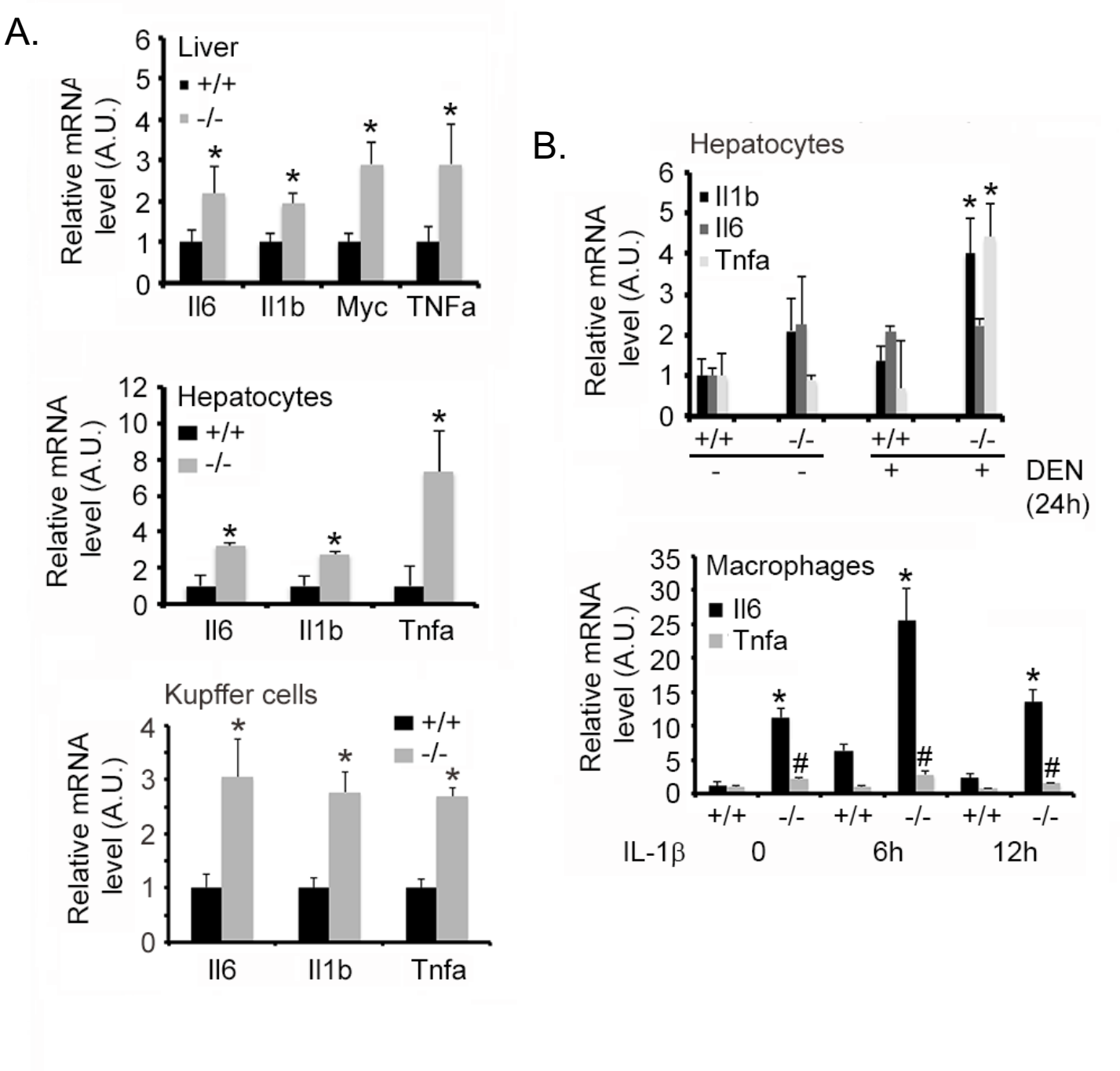


Figure 3.27: A. Levels of the indicated mRNAs in the livers of DEN-injected mice at 32 wks (top), in hepatocytes isolated from DEN-injected mice at 24 wks (middle), and in Kupffer cells isolated from DEN-injected mice at 5 wks (bottom). **B.** Levels of the indicated mRNAs in hepatocytes in vitro treated with DEN for 24 h (top). Levels of the indicated mRNAs in macrophages treated or not with IL-1β (10 ng/ml) for 6 or 12h. data represent mean ± SD. *: $p<0.04$. For all analyses at least 3 mice or more were used per group.

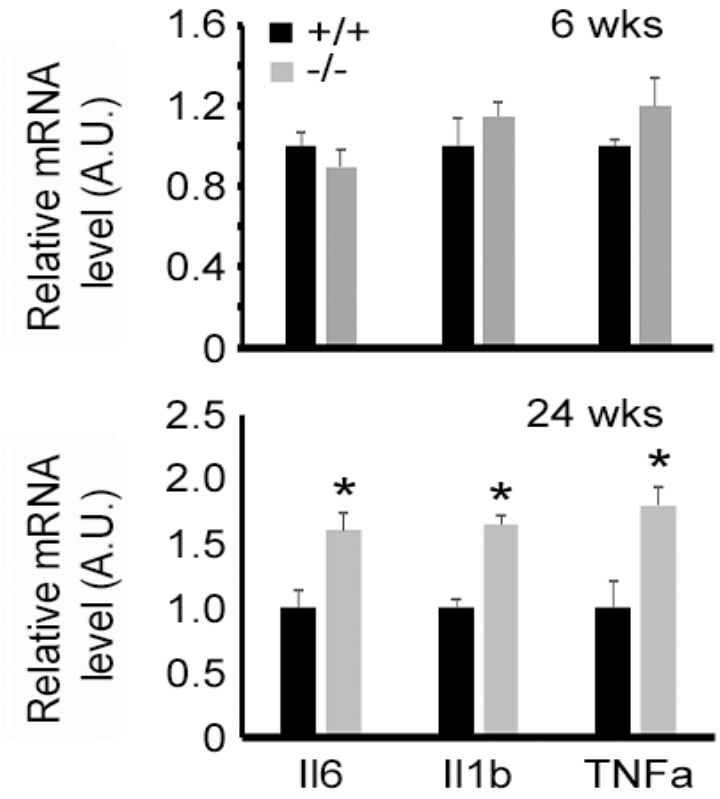


Figure 3.28:. Il6, Il1b and TNFa mRNA expression levels in Igfbp7+/+ and Igfbp7-/- livers at 6 and 24 weeks. Data represent mean ± SD. *: $p<0.01$. 3 mice were used per group.

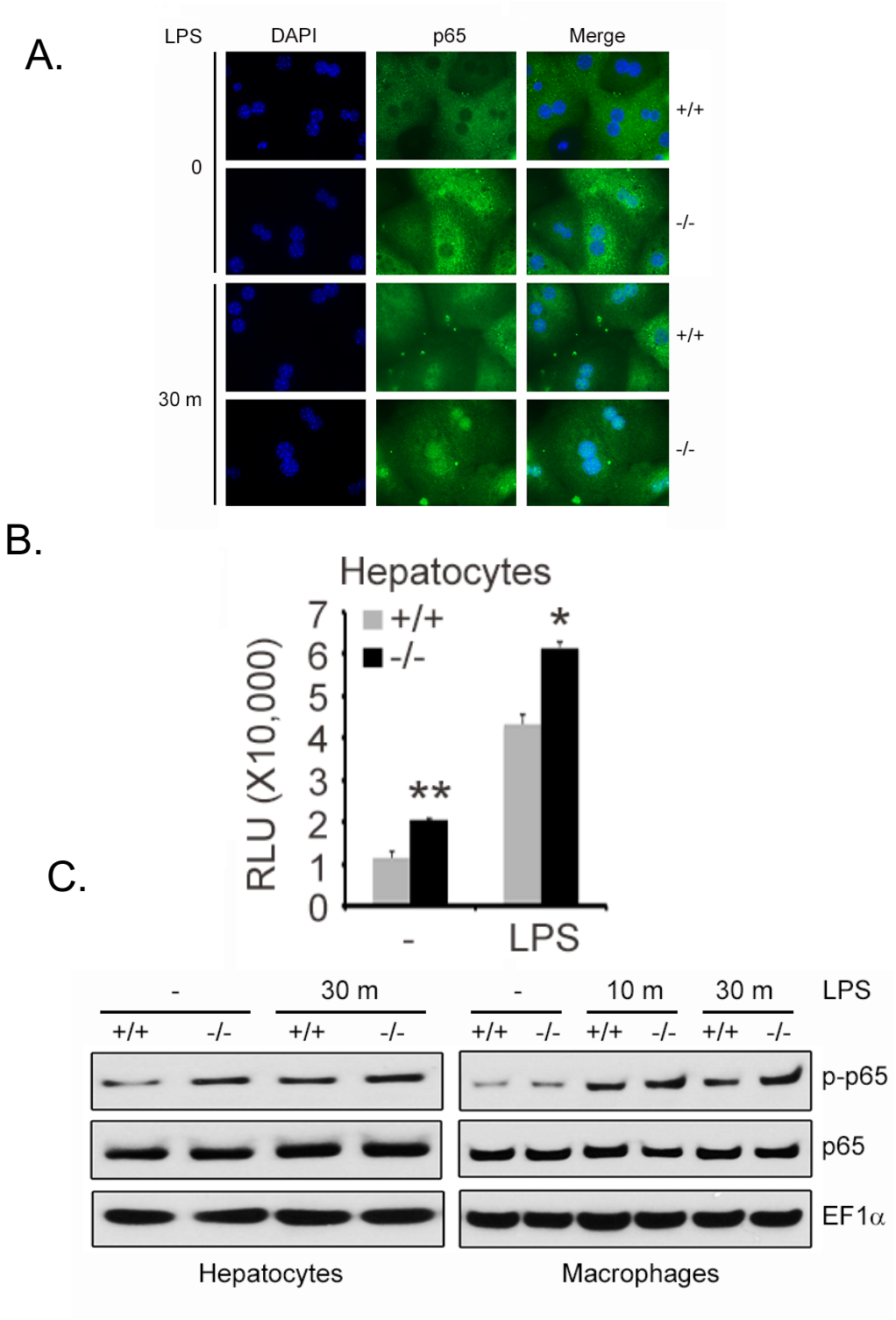


Figure 3.29: A. Immunofluorescence for p65 NF-κB in hepatocytes treated with LPS (500 ng/mL) for 30 min. **B.** NF-κB luciferase reporter activity was measured in Igfbp7+/+ and Igfbp7-/- hepatocytes. Firefly luciferase activity was normalized by Renilla luciferase activity. The activity of empty pGL3-basic vector was considered as 1. R.L.U.: Relative luciferase units. **C.** Western blot analysis for the indicated proteins in LPS-treated hepatocytes and bone marrow-derived macrophages. For graphs, data represent mean ± SD. *: $p<0.01$; **: $p<0.05$; #: $p<0.04$. For all analyses at least 3 mice or more were used per group. A.U.: arbitrary unit.

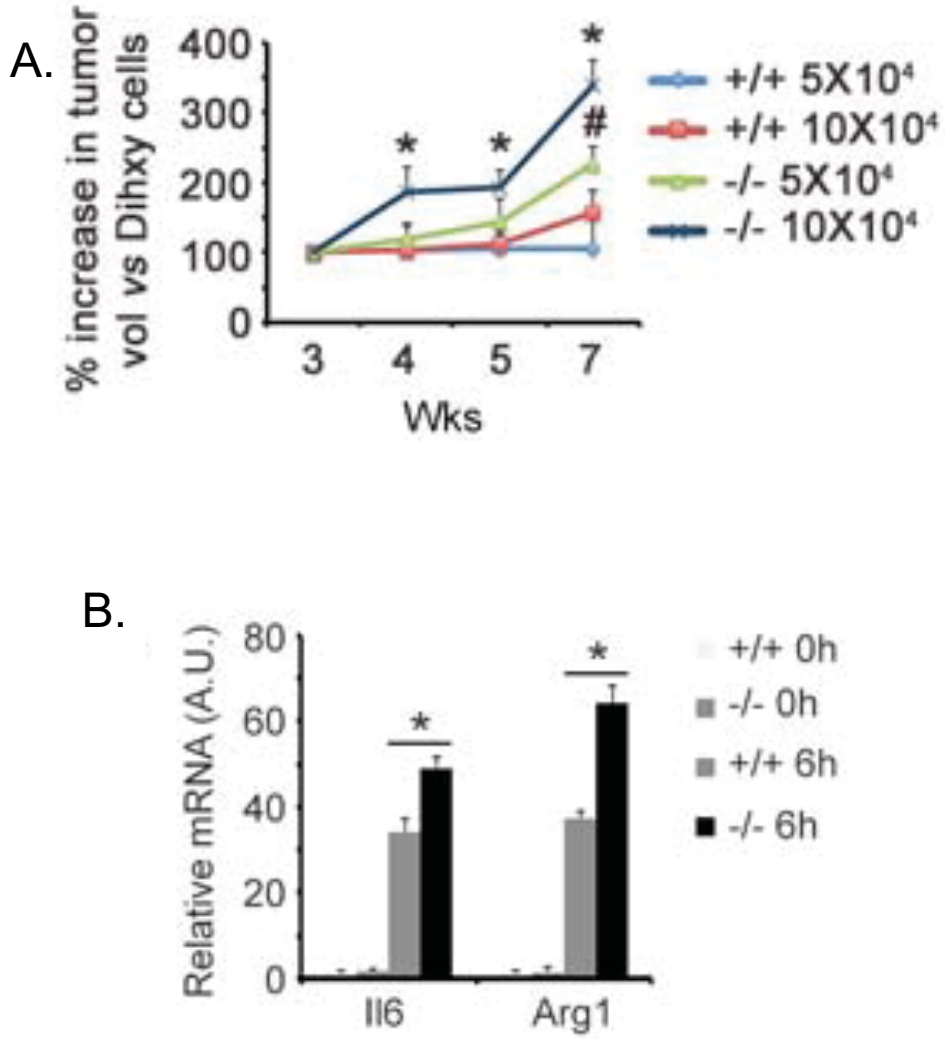


Figure 3.30: A. Dihxy cells were injected s.c. either alone or with Igfbp7+/+ or Igfbp7-/- bone marrow-derived macrophages at a tumor:macrophage ratio of 10:1 or 20:1 s.c. in NSG mice and tumor development was monitored. Tumor volume is represented as percentage increase when compared to Dihxy cells alone. Data represent mean ± SD. *: $p<0.01$. For each group 5 mice were used. **B.** Bone marrow-derived macrophages were cultured either in serum free media (Con) or conditioned media from Dihxy cells for 6 h and the mRNA levels of Il6 and Arg1 were measured by Taqman QRT- PCR. Data represent mean ± SD of three independent experiments. *: $p<0.01$

3.2.7 RNA-sequencing (RNA-Seq) identifies inhibition of immune surveillance in Igfbp7$^{-/-}$ mice:

To obtain insights into the gene expression changes facilitating HCC in Igfb7-/- mice we performed RNA-Seq using total RNA from naïve liver samples from 8 weeks old Igfbp7+/+ and -/- mice (n=3). Genes showing absolute fold-change of >1.5, FDR of <0.1 and p-value of <0.05 were selected. 473 genes showed differential changes, out of which 209 were upregulated and 264 were downregulated in Igfbp7-/- livers when compared to +/+. We performed biological processes and pathway analysis using the software DAVID and Ingenuity.

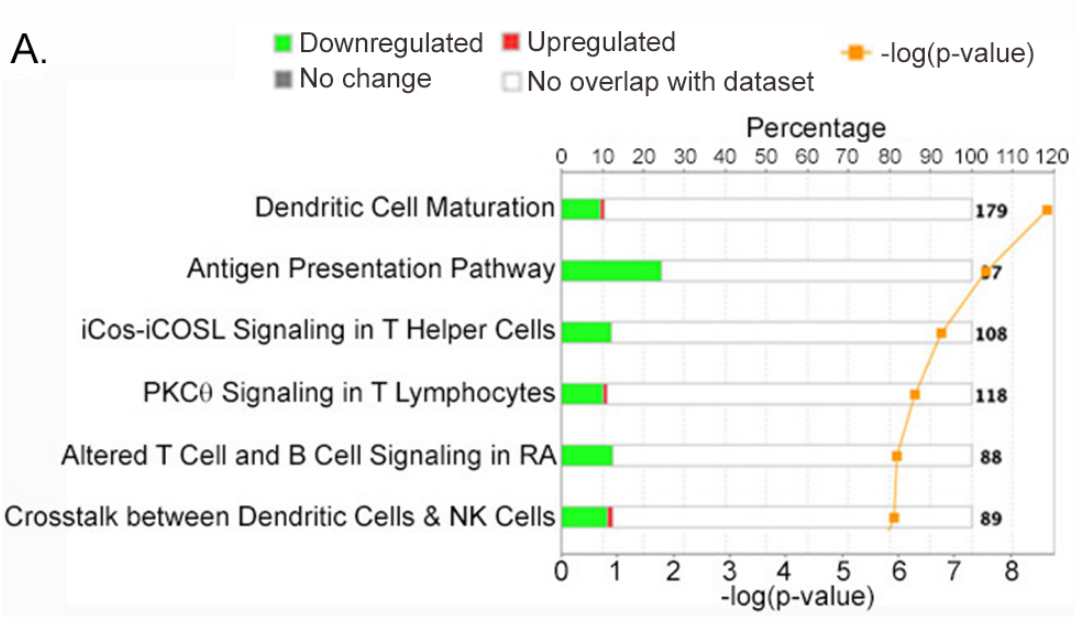
Surprisingly, the topmost biological pathways identified to be inhibited in Igfbp7$^{-/-}$ livers are all associated with immune surveillance, such as antigen presentation or dendritic cell (DC) maturation (Fig. 3.31A and Table 1). The genes regulating antigen presentation that are downregulated in Igfbp7$^{-/-}$ liver include MHC class I and II genes, proteasome components LMP2 and LMP7 that process the antigenic peptides, Transporter associated with Antigen Processing 1 (Tap1) that transports the processed peptide to the ER for assembly with MHC molecules, and co-stimulators CD80 and CD86.

Downregulation of Tap1 mRNA in Igfbp7-/- liver was confirmed by Taqman Q-RT-PCR (Fig. 3.31B). Mature dendritic cells (DCs) present high cell surface expression of CD80 and CD86 [181]. Downregulation of CD86 and CD80 in Igfbp7$^{-/-}$ CD11b^{+}CD11c^{+} bone marrow-derived DCs (BMDCs) were confirmed by flow cytometry (Fig. 3.31C). Mean fluorescence intensity (MFI) for CD86 was 138 and 155, while MFI for CD80 was 240 and 165, respectively for Igfbp7$^{+/+}$ and Igfbp7$^{-/-}$ DCs.

Since there was a discrepancy between percentage of cell surface expression versus MFI for CD86 we confirmed these observations by biological assays. We measured antigen presentation capacity of BMDCs from 6 weeks old Igfbp7$^{+/+}$ and - /- littermates. DCs, loaded with a tumor antigen gp100 peptide, was used to stimulate T cells from pmel-17 mice, a transgenic mouse expressing rearranged T cell receptor (TCR) recognizing gp100. T cell activation by Igfbp7$^{-/-}$ DCs was significantly blunted when compared to that by Igfbp7$^{+/+}$ DCs as evidenced by decreased proliferation of T cells by [^{3}H]-thymidine incorporation assay and decreased production of IL-12 and IL-2 in the conditioned media (Fig 3.32A and B).

The inhibition in antigen presentation by Igfbp7$^{-/-}$ DCs could be rescued by addition of recombinant IGFBP7 protein (rIGFBP7) or treatment with OSI-906 (Fig 3.33). In Igfbp7$^{-/-}$ DCs, co-treatment of OSI-906 and rIGFBP7 did not further augment T-cell proliferation or IFN-γ production when compared to rIGFBP7 treatment alone although a significant increase in IL-2 production was observed. These findings suggest a possible IGF1R-independent function of IGFBP7. Treatment of Igfbp7$^{+/+}$ DCs with rIGFBP7 did not modulate T-cell proliferation or IFN-γ or IL-2 production in antigen presentation assay suggesting a lack of activation of IGF signaling in these cells which may not be affected by rIGFBP7 or OSI-906 (Fig. 6.34). Antigen presentation is positively regulated by IFN-γ that functions by stimulating the JAK-STAT1 pathway. Upon LPS treatment a substantial inhibition in STAT1 activation was observed in Igfbp7$^{-/-}$ DCs versus +/+ DCs indicating that IFN-γ signaling pathway is interfered in Igfbp7$^{-/-}$ DCs (Fig. 3.35).

To check potential role of Igfbp7 in regulating NK cell function splenocytes from Igfbp7+/+ and -/- mice were cultured in the presence of NK cell activating cytokines (IL-2 and IL-15) for 48 h and Granzyme B and IFN-γ production from NK cells (NK1.1+CD3-) was assayed by flow cytometry. No significant difference in NK cell activation was observed between Igfbp7+/+ and -/- indicating that Igfbp7 does not affect intrinsic functions of NK cells (Fig. 3.36).


A.
Downregulated
Upregulated
-log(p-value)
No change
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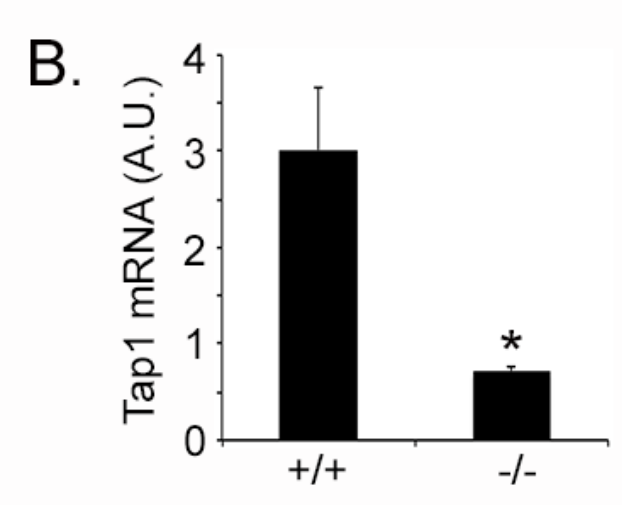
Percentage
0 10 20 30 40 50 60 70 80 90 100 110 120
Dendritic Cell Maturation
Antigen Presentation Pathway
iCos-iCOSL Signaling in T Helper Cells
PKCθ Signaling in T Lymphocytes
Altered T Cell and B Cell Signaling in RA
Crosstalk between Dendritic Cells & NK Cells
179
7
108
118
88
89
0 1 2 3 4 5 6 7 8
-log(p-value)


B.
Tap1 mRNA (A.U.)
4
3
2
1
0
*
+/+
-/-

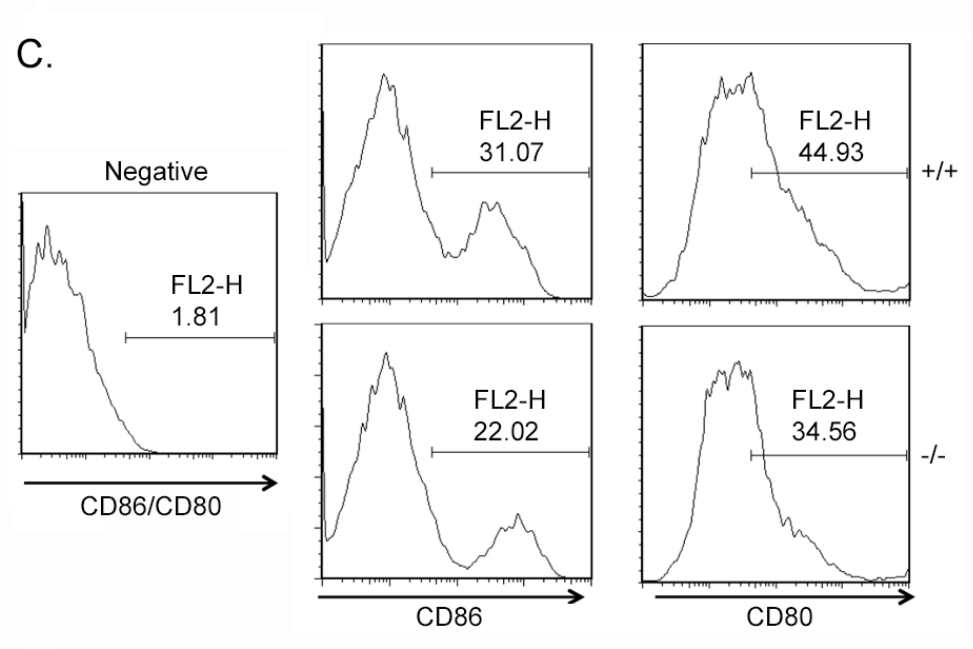


Figure 3.31: Inhibition of immunosurveillance in Igfbp7-/- mice. A. Top biological pathways downregulated in naive Igfbp7-/- livers. B. Tap1 mRNA levels in total liver. C. Levels of CD86 and CD80 in bone marrow-derived dendritic cells (BMDCs).

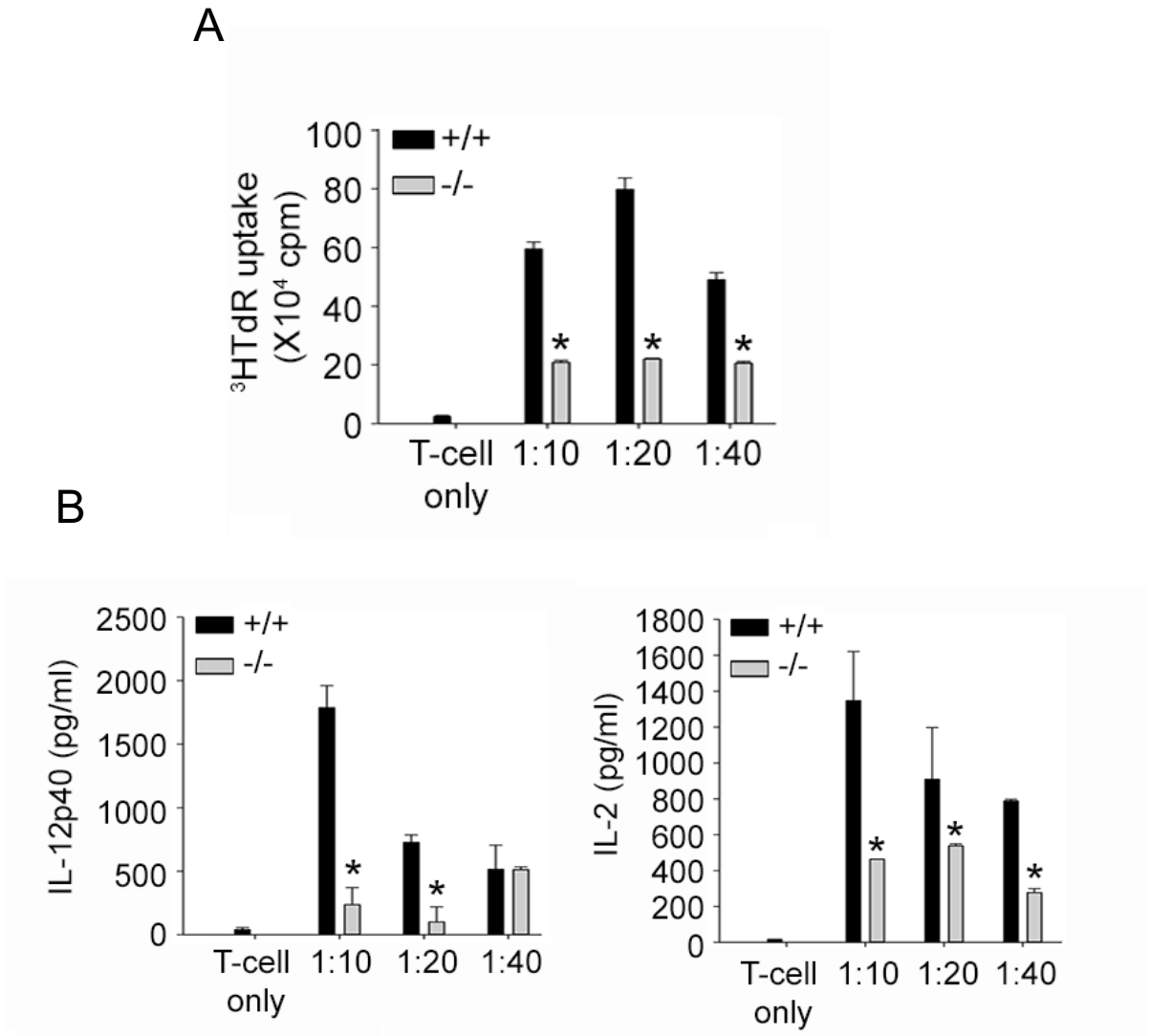


Figure 3.32: Donor pmel-17 T cells were co cultured with BMDCs pulsed with gp100 at the indicated molar ratios (DC:TC) for 60 h with 2 h pre-treatment with LPS (500 ng/mL) for priming. 3H-thymidine (3HTdR) incorporation was measured (**A**). IL-12p40 (middle) and IL-2 (**B**) levels were measured in the media by ELISA.

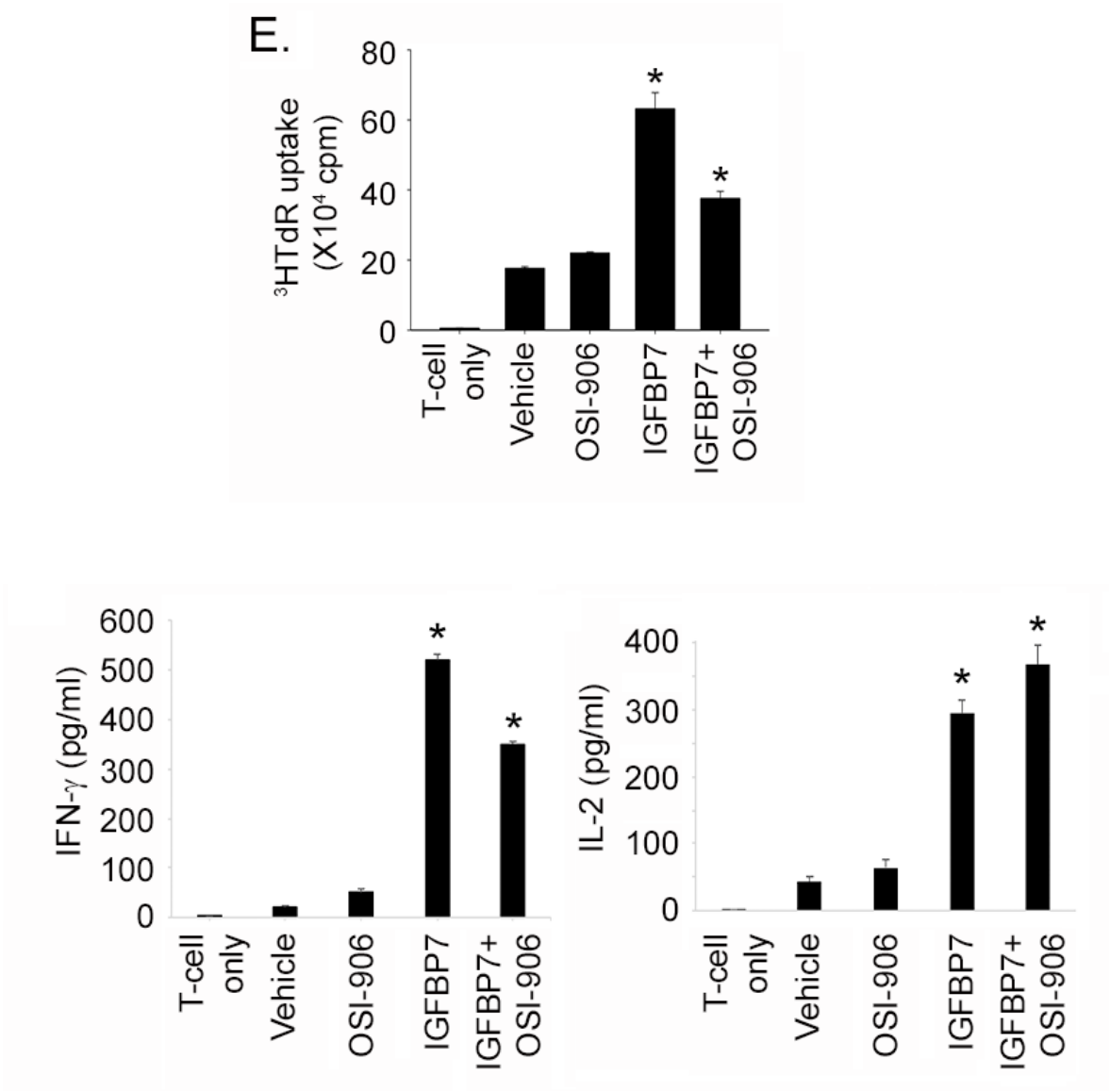


Figure 3.33: gp100-loaded Igfbp7-/- BMDCs were treated or not with rIGFBP7 protein for 3 h followed by OSI-906 (4 μmol/L) for 2 h and LPS (500 ng/mL) for 2 h. The cells were washed and co-cultured with pmel-17 T cells for 60 h. 3Hthymidine (3HTdR) incorporation was measured (left). IFN-γ (middle) and IL-2 (right) levels were measured in the media by ELISA.

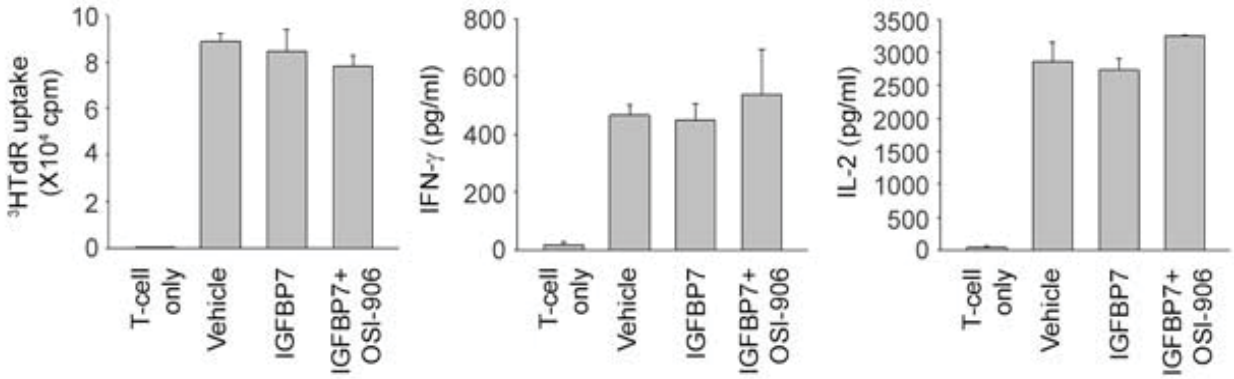


Figure 3.34: gp100-loaded Igfbp7+/+ BMDCs were treated or not with rIGFBP7 protein for 3 h followed by OSI-906 (4 μmol/L) for 2 h and LPS (500 ng/mL) for 2 h. The cells were washed and co-cultured with pmel-17 T cells for 60 h. 3Hthymidine (3HTdR) incorporation was measured (left). IFN-γ (middle) and IL-2 (right) levels were measured in the media by ELISA.

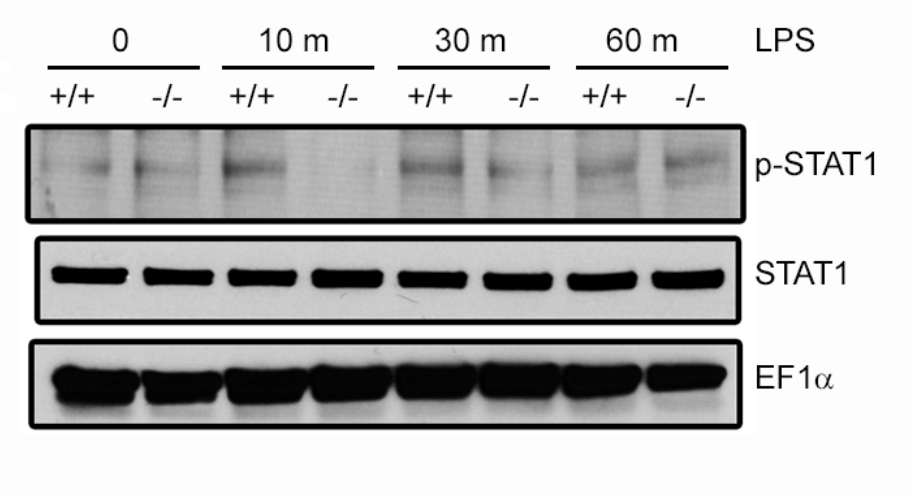


Figure 3.35: BMDCs were treated with LPS (500 ng/mL) for the indicated time points and Western blot analysis was performed for the indicated proteins. For graphs, data represent mean ± SD. *: $p<0.01$.

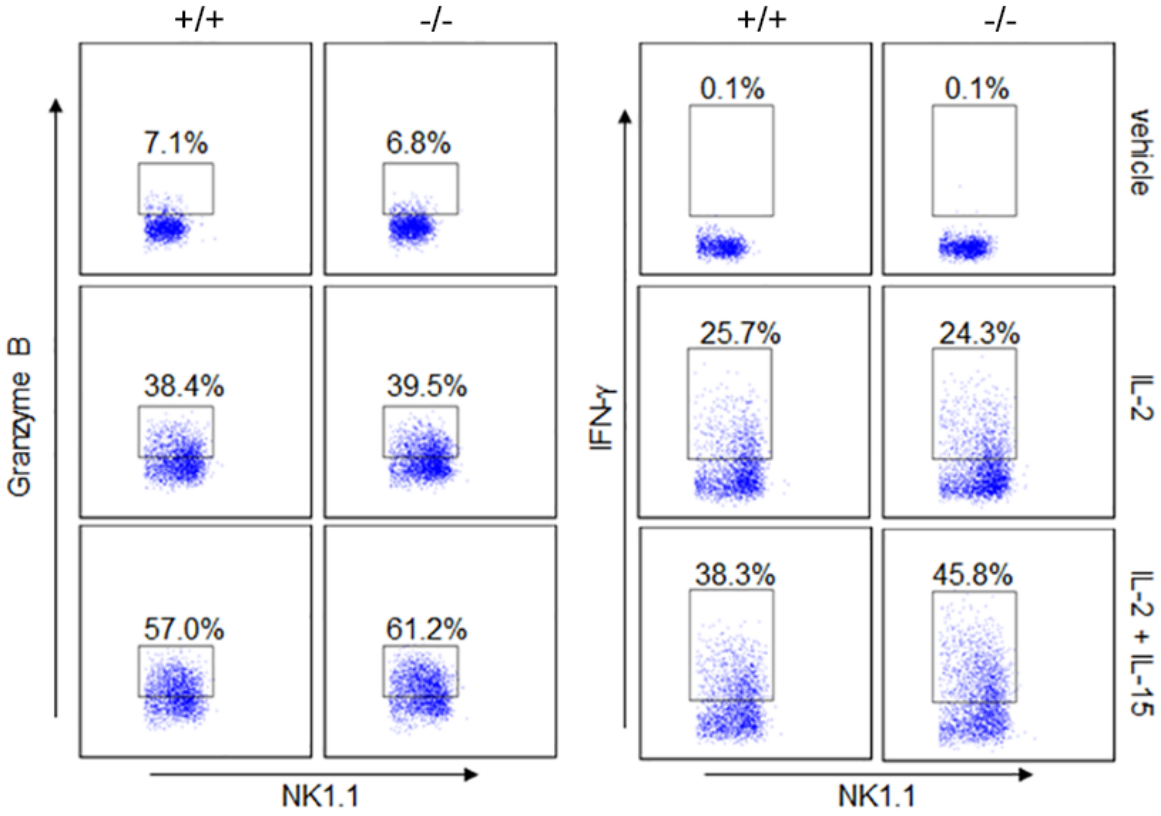


Figure 3.36: IGFBP7 deficiency does not affect NK cell activation. Splenocytes from IGFBP7$^{+/+}$ or IGFBP7$^{-/-}$ mice were cultured in the presence of NK cell activating cytokines for 48 h. Granzyme B and IFN-γ production from NK cells (NK1.1+CD3-).

Table 1: Downregulation of genes of antigen presentation pathway in Igfbp7-/- livers

Type of Gene	Gene name	Fold decrease in Igfbp7-/- liver
MHC class I	H2-D1	0.66
	H2-Q6	0.58
MHC class II	H2-Aa	0.44
	H2-Ab1	0.41
	H2-Dma	0.44
	H2-Dmb1	0.39
	H2-Eb1	0.42
Proteasome	Psmb8 (LMP7)	0.63
	Psmb9 (LMP2)	0.58
Transporters	Tap1	0.61
Complement	C1qa	0.65
	C1qb	0.59
	C1qc	0.60
Co-stimulators	Cd80 (B7.1)	0.51
	Cd86 (B7.2)	0.66

3.2.8 Overexpression of Igfbp7 decreases tumor growth by activating an anti-tumor immune response:

As yet all studies analyzing the effect of IGFBP7, either recombinant protein, stable overexpression or via an adenovirus, have been performed in athymic nude mice thereby not detecting immunomodulatory properties of IGFBP7 [103]. To examine the impact of IGFBP7 on tumor immune environment, we established stable pooled clone of mouse HCC Hepa1-6 cells overexpressing mIGFBP7 (BP7-OE).

Overexpression of secreted mouse IGFBP7 in BP7-OE clones was confirmed by Western blot analysis in the conditioned media and by Q-RT-PCR in the cells (Fig. 3.37A). Subcutaneous xenografts from BP7-OE cells grew significantly slowly in syngeneic C57L mice, from which Hepa1-6 cells were established, compared to the control tumors (Fig. 3.37B). BP7-OE tumors showed decreased activation of Akt and ERK and marked decrease in CD-31 staining confirming inhibition of IGF-1 signaling and angiogenesis (Fig. 3.37C and Fig. 3.40A).

Interestingly, BP7-OE tumors presented with marked tumor infiltration of IFN-γ-producing $CD8^+$ or $CD4^+$ T cells (Fig. 3.38A), marked elevation of cytokine IL-12 (Fig 3.38B), which is crucial for antitumor Th1 immunity, moderate elevation of IFN-γ (Figure 3.38B) and decrease in MDSCs in the tumors (Fig. 3.38A). These tumors also showed significant upregulation of Tap1 (Fig. 3.38B).

Collectively these findings in BP7-OE tumors reflect reverse findings of what is observed in Igfbp7$^{-/-}$ mice. To further confirm the role of induction of anti-tumor immunity, we established BP7-OE tumors in C57L mice and depleted CD8^{+} and CD4^{+} cells with neutralizing antibody. The depletion of CD8^{+} or CD4^{+} cells was confirmed by FACS analysis of the tumor (Fig. 3.40B). Depletion of CD8^{+} and CD4^{+} cells significantly rescued the growth of BP7-OE tumors, supporting a major role of immune activation in mediating tumor suppressor function of IGFBP7 (Fig. 3.39).

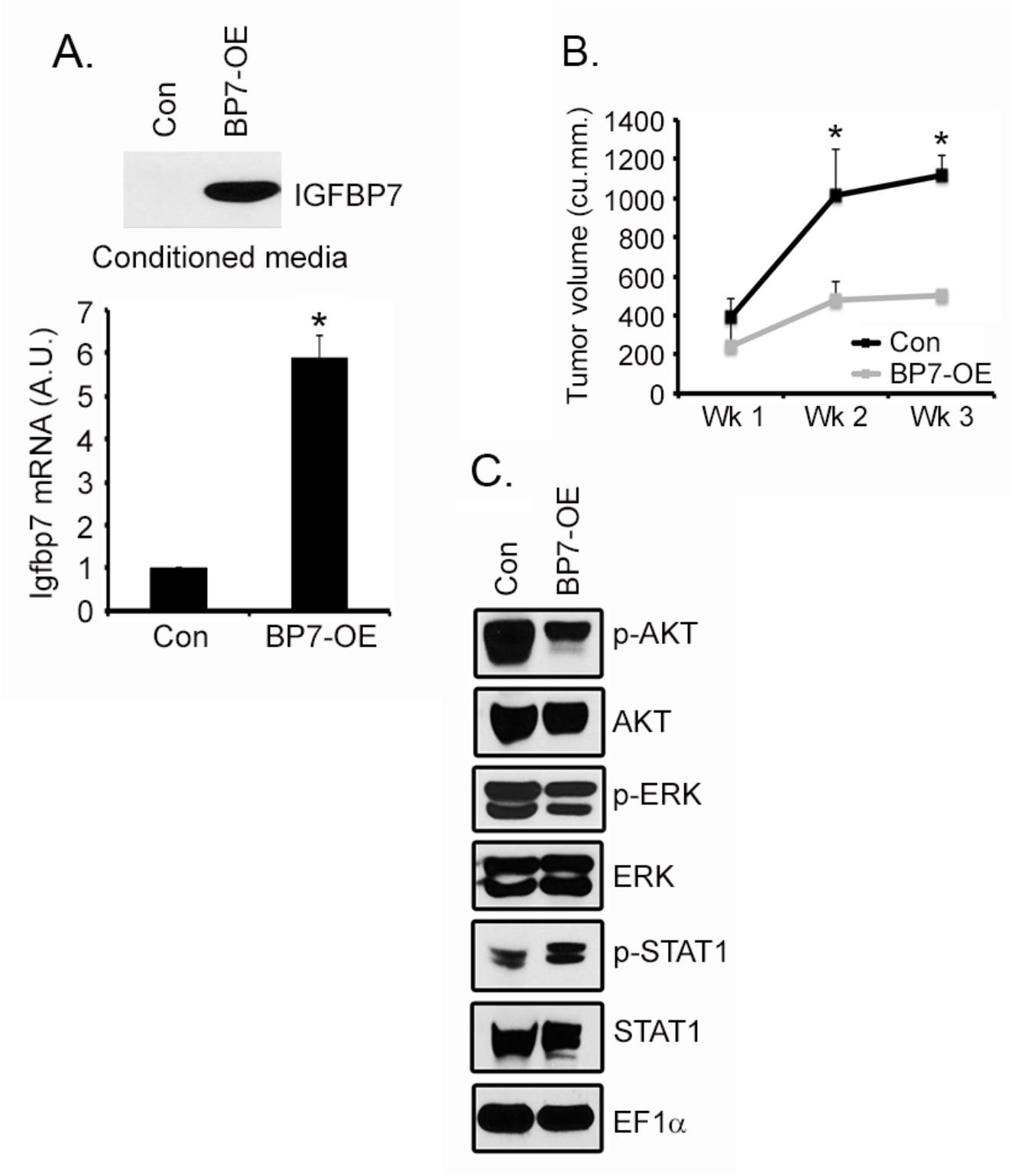
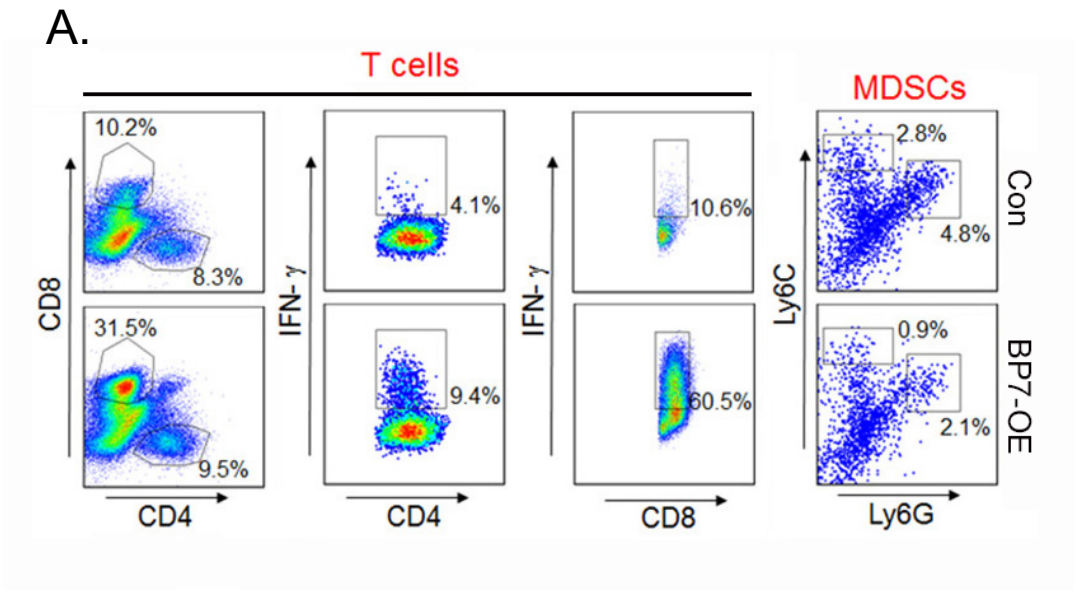


Figure 3.37: A. IGFBP7 protein levels in the conditioned media (top) and Igfbp7 mRNA levels in the cells (bottom) of control and IGFBP7-overexpressing clone (BP7-OE) of Hepa1-6 cells. **B.** The indicated clones (1X106 cells) were injected s.c. in C57L mice (n=5 per group) and tumor volume was measured. **C.** Levels of the indicated proteins in tumor lysates at the end of the study.



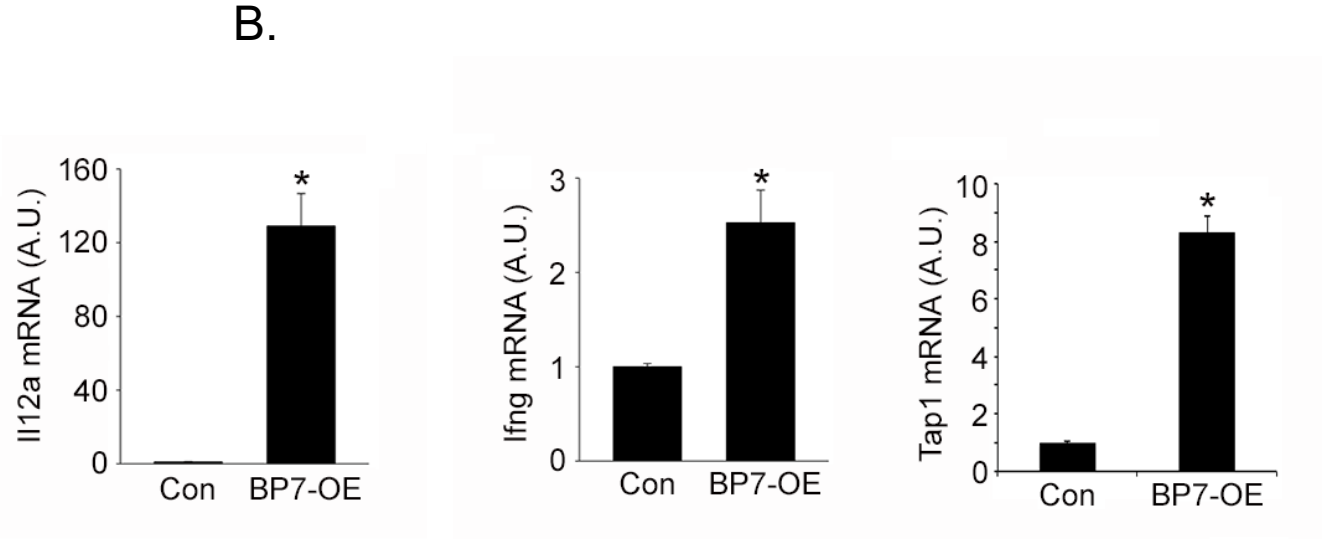


Figure 3.38: A. IFN-γ-producing CD8+ or CD4+ T cells and MDSCs in the tumors at the end of the study. **B.** Il12a (left), IFN-γ (middle) and Tap1 (right) mRNA levels in the tumors at the end of the study.

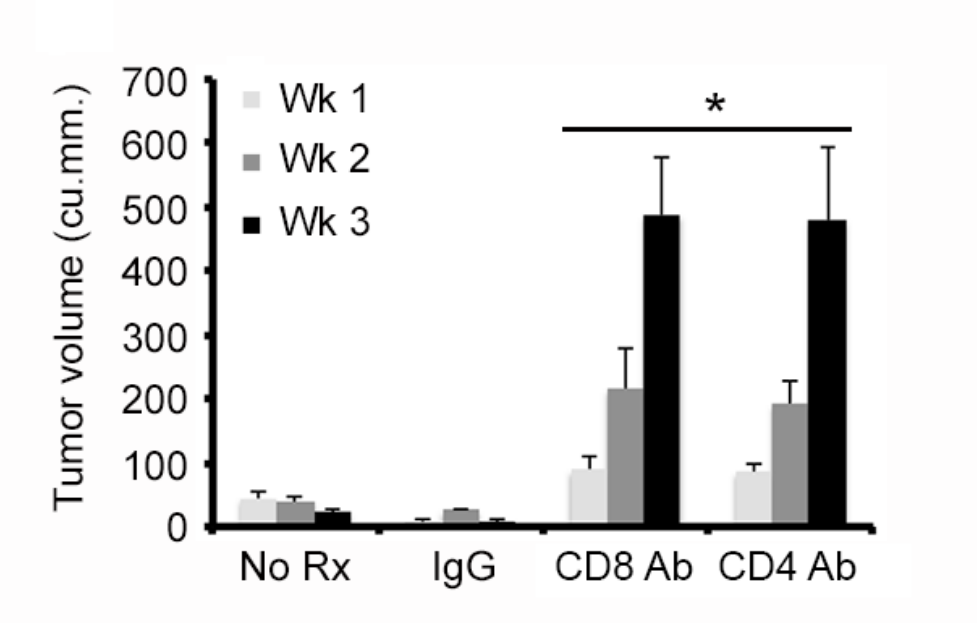
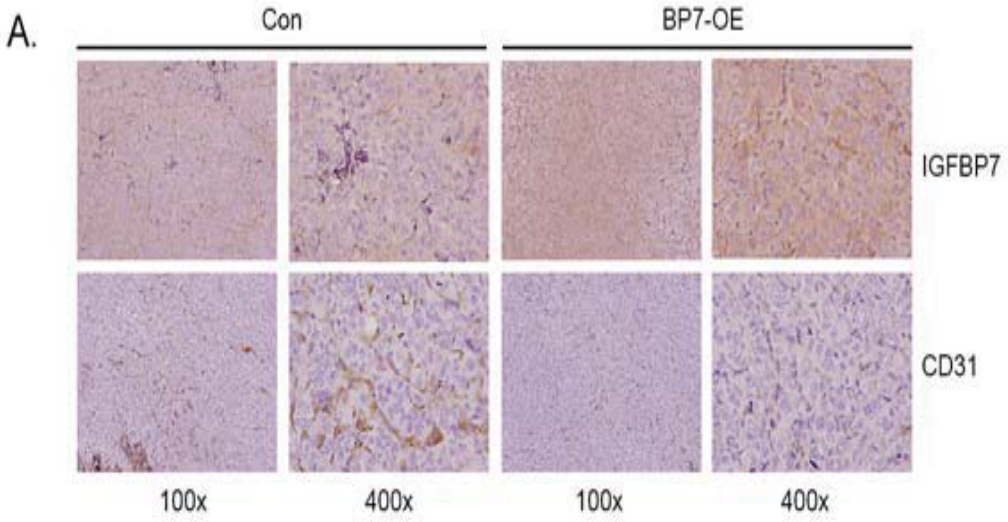
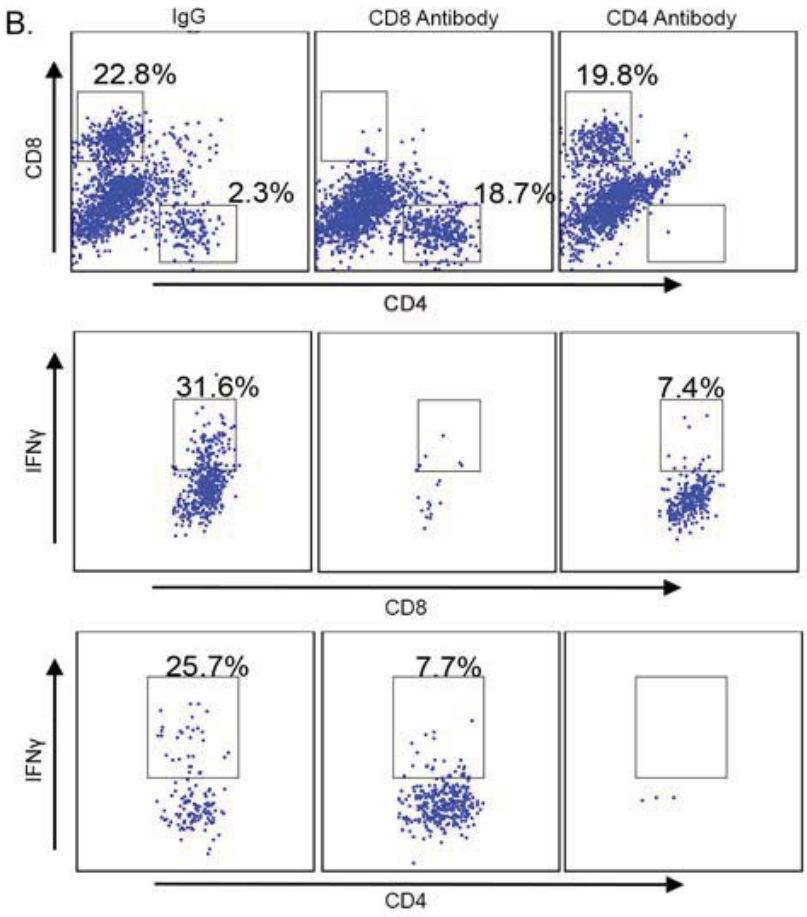


Figure 3.39: BP7-OE cells were injected s.c. in C57L mice (n=5 per group) and treated with Control IgG and CD8+ or CD4+ neutralizing antibodies (200 μg i.p.). Tumor volume was measured. For all graphs, the data represent mean ± SD; *: $p<0.01$.


A.
Con
BP7-OE
IGFBP7
CD31
100x
400x
100x
400x


B.
IgG
CD8 Antibody
CD4 Antibody
22.8%
2.3%
18.7%
19.8%
CD8
CD4
31.6%
7.4%
IFNγ
CD8
25.7%
7.7%
IFNγ
CD4

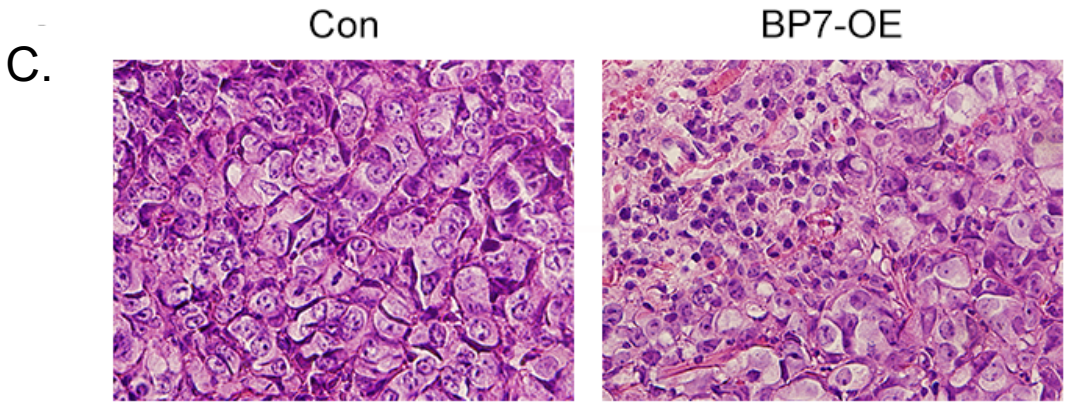


Figure 3.40: A. Immunohistochemistry for the indicated proteins in Control and BP7-OE tumor sections at the end of the study. **B.** FACS analysis of tumors for $CD8^+$ and $CD4^+$ cells following treatment with $CD8^+$ and $CD4^+$ neutralizing antibodies. **C.** H&E staining of the tumor sections.

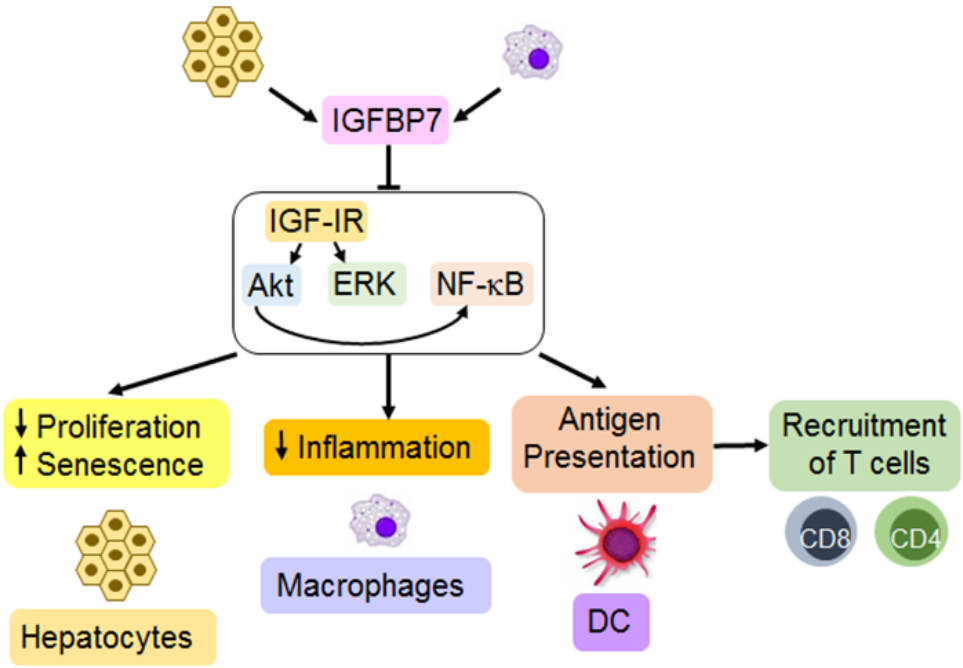


Figure 3.41: Schematic representation of the molecular mechanism by which IGFBP7 suppresses tumors. IGFBP7, secreted from hepatocytes, inhibits IGF and NF-κB signaling in macrophages preventing inflammation. IGFBP7, secreted from the microenvironment cells, inhibits IGF and NF-κB signaling in hepatocytes thus impeding proliferation and inducing senescence. IGFBP7 promotes antigen presentation by dendritic cells resulting in recruitment of CD4+ and CD8+ cells. These effects collectively protect from HCC. In addition to the paracrine effects depicted in this diagram there might be autocrine effects regulating the same end-points.

3.3 Discussion:

Previous studies in multiple tumor models, including HCC, have identified IGFBP7 as a potential tumor suppressor [99, 103, 170]. However, an in vivo model is necessary to conclusively establish the tumor suppressor function of IGFBP7 and the present studies fulfill this need. We document that Igfbp7$^{-/-}$ mice develop spontaneous tumors in multiple organs, although not at 100% penetrance, and present with a pro-inflammatory milieu that might provide a fertile ground for sustenance and progression of cancers once cells become transformed following a mutagenic event. Indeed, hepatocarcinogenesis following DEN treatment is markedly accelerated in Igfbp7$^{-/-}$ mice compared to +/+ and +/- mice thereby establishing IGFBP7 as a bona fide tumor suppressor according to the two-hit hypothesis in which both alleles need to be mutated to manifest the tumorigenic phenotype.

The loss of Igfbp7 resulted in constitutive activation of IGF pathway in parenchymal cells, such as hepatocytes and MEFs, which translated into increased proliferation, accelerated cell cycle progression and inhibition of senescence. Activation of IGF-1 pathway protects from stress-induced premature senescence, IGFBP7 is induced in senescent cells and loss of IGFBP7 mediates escape from oncogene-induced senescence [74, 99, 182].

In DEN-induced HCC model, DEN causes DNA damage, induces reactive oxygen species (ROS) and ultimately apoptosis in the hepatocytes [175]. However, there is also compensatory proliferation to mitigate this effect. Upon activation of survival pathways, such as IGF-1, the damaged (and mutated) hepatocytes do not undergo apoptosis and continues to survive and proliferate resulting in expansion of mutated, transformed cells. Induction of senescence in pre-malignant cells and clearing of these senescent cells by immune system serves as a mechanism of inhibition of HCC [183].

Thus deleting Igfbp7 in hepatocytes confers both proliferative and survival advantage and protection from senescence (Fig. 7.1G). On the other hand, we observed that the expression of IGFBP7 in macrophages is significantly more than that in hepatocytes suggesting an important role of IGFBP7 in modulating liver microenvironment. Igfbp7$^{-/-}$ macrophages show activation of NF-κB and increased expression of pro-inflammatory cytokines thereby establishing a chronic inflammatory environment in which senescence-resistant transformed hepatocytes might thrive. Activated Akt, following activation of IGF-1R, phosphorylates IKK leading to activation of NF-κB pathway [184].

Thus activation of IGF signaling might contribute to both proliferative and pro-inflammatory phenotypes (Fig. 7.1G). Cross-talk between hepatocytes and macrophages is fundamental in HCC development. Macrophages require secreted factors, such as IL-1β, released from damaged hepatocytes, for activation, while damaged hepatocytes require macrophage released factors, such as IL-6, for survival and proliferation and NF-κB plays an important role in regulating this cross-talk [146, 176, 185]. The tumorigenic effects of NF-κB in HCC are highly dependent on cell type. For instance, hepatocytes specific knockout of IKKβ promoted DEN-induced HCC via increased ROS and hepatocyte compensatory proliferation, while double knockout of IKKβ in the macrophage and hepatocyte reduced DEN-induced HCC [175].

In Igfbp7$^{-/-}$ mice NF-κB is activated in both hepatocytes and macrophages thereby creating a situation that is mirror image of IKKβ double knockout mice. The simultaneous activation of NF-κB in our model explains the concomitant increase in inflammatory cytokines, such as IL-6, IL1-β and TNFα, and the subsequent increase in Kupffer cell population in DEN-induced HCC. One surprising finding deduced from our model is the ability of IGFBP7 to modulate the antigen presentation machinery and thereby an anti-tumor immune response.

We document that deletion of Igfbp7 resulted in downregulation of an IFN-γ-regulated cluster of genes regulating antigen presentation. Indeed we observe decreased activation of IFN-γ signaling in Igfbp7$^{-/-}$ BMDCs which functionally translated into decreased antigen presentation and decreased infiltration of CD8^{+} and CD4^{+} T cells and NK cells in DEN-induced tumor. Treatment of Igfbp7$^{-/-}$ BMDCs with rIGFBP7 protein restored antigen presentation capacity in BMDCs documenting a key role of IGFBP7 in proper functioning of antigen presenting cells. As a corollary, overexpression of Igfbp7 in mouse HCC cells inhibited tumorigenesis in syngeneic mice with robust infiltration of CD8^{+} and CD4^{+} T cells and depletion of CD8^{+} and CD4^{+} T cells facilitated tumor growth. While the immunomodulatory effects of IGF-1 are less understood, several lines of evidence supports the potential involvement of IGF signaling in regulating tumor immunogenicity and immune cell phenotype or function. Silencing IGF-1 expression in Hepa1-6 cells increased its immunogenicity and decreased tumorigenicity by upregulation of MHC class I molecules and mobilization of T cells [186].

In T cells IGF-1 treatment results in decreased surface expression of IFN-γR2 and inhibits IFN-γ-STAT1 signaling [187]. Additionally, dendritic cells (DCs), upon treatment with IGF-1, showed a defect in maturation following LPS stimulation, and produced immune suppressive cytokines such as IL-10 [188]. Indeed we observe an increase in arginase-1 and MDSCs in DEN-induced HCC in Igfbp7$^{-/-}$ mice and a decrease in MDSCs in BP7-OE tumors. Thus, Igfbp7 deletion creates an immunosuppressive environment preventing clearance of transformed hepatocytes by the immune system (Fig. 3.41).

In summary, we document that IGFBP7 can not only inhibit cancer cells but also modulate tumor microenvironment and this dual effect might have lasting effect in inhibiting both primary tumors and distant metastasis. Even though HCC has an immunosuppressive milieu, immune targeted therapies are beginning to demonstrate significant objective responses in clinical trials. Targeted delivery of rIGFBP7 protein might be an effective therapeutic for HCC and other cancers.

3.4 Summary and future directions:

In the US, the incidence and mortality rates of hepatocellular carcinoma (HCC) are alarmingly increasing since no effective therapy is available for the advanced disease. Activation of IGF signaling is a major oncogenic event in diverse cancers, including HCC. Insulin-like growth factor binding protein-7 (IGFBP7) inhibits IGF signaling by binding to IGF-1 receptor (IGF-1R) [78] and functions as a potential tumor suppressor for hepatocellular carcinoma (HCC) [103]. We identified loss of heterozygosity in the IGFBP7 4q12 in liver cancer in more than 26% of 50 HCC patients studied compared to 9 control patients. This was in parallel with loss of IGFBP7 in invasive HCC from a tissue array of 109 HCC patients. This loss was associated with poorer prognosis and correlated with disease stages [103]. IGFBP7 abrogates HCC by inducing cancer-specific senescence and apoptosis and inhibiting angiogenesis [103]. The role of IGFBP7 as tumor suppressor was also reported in a number of cancers such as gastric, lung, thyroid, breast, and glioblastoma multiform (GBM). The reported loss was through genomic deletions or promoter hypermethylation in these cancers [189-193].

In this study we document that Igfbp7 knockout (Igfbp7-/-) mouse shows constitutive activation of IGF signaling, presents with proinflammatory and immunosuppressive microenvironment, and develops spontaneous tumors in lungs and liver and markedly accelerated carcinogen-induced HCC. Loss of Igfbp7 resulted in increased proliferation and decreased senescence in hepatocytes and mouse embryonic fibroblasts that could be blocked by an IGF-1 receptor inhibitor. A significant inhibition of genes regulating immune surveillance was observed in Igfbp7-/- livers which was associated with marked inhibition in antigen cross presentation by Igfbp7-/- dendritic cells. IGFBP7 overexpression inhibited growth of HCC cells

in syngeneic immune competent mice, which could be abolished by depletion of CD4+ or CD8+ T lymphocytes. Our studies unravel modulation of immune response as a novel component of pleiotropic mechanisms by which IGFBP7 suppresses HCC. Activation of IGF pathway in T-cells reduced the expression of IFGNR2 and as a result reduced the signaling of IFN-γ [187]. Additionally, a separate study indicated that IL-4-induced activation of Akt is JAK3-dependent, which is enhanced by release of IGF-1 that was found to be necessary for full adoption of the M2 phenotype. Blocking IGF-1 activity abolished the ability of IL-4 to induce activation of Akt to upregulate expression of some M2-associated genes such as ARG-1 [194]. These studies indicated the involvement of IGF signaling in immune modulation. In our study we observed deregulation in signaling of IFN-γ and ARG-1 and an immune modulation phenotype favoring the propagation of an inflammatory immune response as seen by levels of IL-6, IL-1β and TNF-α. However, as HCC progresses this response shifts towards an anti-inflammatory immune response as seen by accumulation of anti-inflammatory immune response such as MDSCs and increased ARG-1 expression.

In our models of HCC, we know that the loss of IGFBP7 in cancer cells abolishes tumor development and is necessary for progression of the disease. However, in the context of a real patient microenvironment, the surrounding stroma still express IGFBP-7. Why then IGFBP-7 coming from supporting stroma does not induce senescence in developing cancer cells? Are there secreted proteolytic enzymes in the microenvironment that could degrade IGFBP7? What are they? We know that IGFBP7 has a matriptase cleaving site, and we know that the N-terminal portion of IGFBP7 engages the IGF-1R and blocks its activation. In such way, can this proteolytic enzyme affects the tumor suppressive function of IGFBP7 at the extracellular space?

Levels of matriptase was observed to be upregulated in HCC [195]. Cell specific knockout or double knockouts in hepatocyte or supporting stroma or temporal cell specific knockouts of IGFBP7 would help to further analyze at what point or cells type loss of IGFBP7 accelerates the progression of HCC. Further analysis on the role of IGFBP7 in the function of individual immune cells would help to characterize the cell specific role of IGFBP7. Previous studies attempted to use IGFBP-7 as a therapeutic approach. The Sarkar lab used adenovirus mediated gene delivery [116]. As discussed earlier in chapter 1, IGFBP7 gene delivery induced growth arrest and apoptosis in HCC. Others have reported similar success in melanoma and breast cancers. IGFBP7 has IGF dependent and unknown IGF independent functions (mainly through the C-terminal domain). What are the IGF independent functions of IGFBP7? And how using only C-terminal of IGFBP7 was capable of inducing apoptosis? [78]. Could IGFBP7 recombinant protein be a good candidate for HCC therapy? To address all these questions, we need to test the efficacy of different IGFBP7 constructs (N-terminal versus C-terminal constructs) compared to a full wild type IGFBP7 protein? The area of cancer therapeutics and immune targeted therapeutics are hot area of research and further studies are required to support inclusion of IGFBP7 in the therapeutic approaches.

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