THERAPEUTIC APPROACHES TO THE PREVENTION OF LIVER FIBROSIS AND CANCER PROGRESSION

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By

Muammer Merve Aydın

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We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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ABSTRACT

THERAPEUTIC APPROACHES TO THE PREVENTION OF LIVER FIBROSIS AND CANCER PROGRESSION

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Ph.D. in Molecular Biology and Genetics

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August, 2015

In our previous studies on liver regeneration, we demonstrated that following partial hepatectomy (PH) FLT3 contributes cellular proliferation that provides a basis for liver regeneration. Moreover, we were able to suggest a potential role for FLT3 in hepatocarcinogenesis for the first time. Therefore, we further investigated the effect of FLT3 inhibition on the invasiveness and aggressiveness of hepatocarcinogenesis. Our findings were parallel to our previous results supporting the contribution of FLT3 in hepatocarcinogenesis. Thus, we are presenting FLT3 as a novel candidate for the diagnosis and treatment of HCC. We also focused on liver fibrosis since it is the initial wound healing response generated by the liver against damaging insults. Liver fibrosis is a reversible process, but if its progression is not prevented it might turn into cirrhosis and end up with HCC. Toll-like receptors (TLRs) have been reported to contribute to this fibrotic response generated in the liver resulting from the activating effects of various danger ligands. We show that using suppressive oligodeoxynucleotide (ODN) A151 might control TLR dependent immune activation that takes place after the induction of liver fibrosis. Our results show that suppressive ODN A151 administration has a negative effect on αSMA expression and collagen accumulation, which are the major events taking place during liver fibrogenesis. Additionally, this suppressive effect of suppressive ODN A151 was revealed to be systemic. Splenocytes of suppressive ODN A151 administered mice showed different

cytokine secretion patterns and antigen presenting cell (APC) function after being stimulated with various TLR ligands. These findings suggested us that using suppressive ODN might be a rational and novel approach to control the liver fibrogenesis and even prevent its progression into cirrhosis reducing the number of liver transplantations needed by the patients. Finally, we focused on HSPs, some of which are also known to activate TLR signaling. Additionally, HSP27 has a role in actin cytoskeleton organization and controlling cellular motility, which are among the events that take place in liver fibrogenesis. Therefore, for the first time we present preliminary data on the potential role of HSP27 in liver fibrosis and quercetin treatment as a therapeutic approach due to its HSP27 and αSMA expression changing effects.

Keywords: Liver, liver cancer, hepatocellular carcinoma, liver fibrosis, FLT3, TLRs, suppressive ODN A151, HSPs, HSP27, quercetin.

ÖZET

KARACİĞER FİBROZİSİ VE KANSERİNİN İLERLEMESİNİ ENGELLEYİCİ TERAPÖTİK YAKLAŞIMLAR

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Karaciğer yenilenmesi ile ilgili önceki çalışmalarımızda, kısmi hepatektomi (PH) sonrasında FLT3'ün karaciğer yenilenmesinin temelini oluşturan hücresel çoğalmaya katkı sağladığını göstermiş bulunmaktayız. Buna ek olarak, ilk kez FLT3'ün hepatokarsinojeneziste potansiyel bir rol oynadığını önerebilmiştik. Bu nedenle, FLT3 inhibisyonunun hepatokarsinojenezisin invazyon ve agresif özellikleri üzerindeki etkisini detaylı olarak araştırmış bulunmaktayız. Bulgularımız FLT3'ün hepatokarsinojenezise katkısı olduğunu destekleyen daha önceki bulgularımızla uyumludur. Bu bağlamda, FLT3'ü hepatosellüler karsinomun (HCC) teşhis ve tedavisi için yeni bir aday olduğunu sunmaktayız. Ayrıca, zarar verici etkenlere karşı karaciğer tarafından oluşturulan ilk yara iyileştirme yanıtı olması nedeniyle karaciğer fibrozisi üzerine de yoğunlaştık. Karaciğer fibrozisi geri döndürülebilen bir süreçtir ancak ilerleyişi önlenmezse siroza dönüşebilmekte ve hatta HCC ile sonlanabilmektedir. Toll-benzeri algaçların (TLR) karaciğerde farklı tehlike ligandlarının aktifleştirici etkileri ile oluşturulan bu fibrotik yanıta katkıda bulunduğu gösterilmiştir. Baskılayıcı A151 oligodeoksinükleotid (ODN) kullanımının TLR'ye bağlı karaciğer fibrozisinin indüksiyonu sonrasında ortaya çıkan immün aktivasyonunu kontrol edebildiğini göstermiş bulunmaktayız. Sonuçlarımız baskılayıcı A151 ODN kullanımının karaciğer fibrojenezi esnasında ortaya çıkan temel olaylar olan αSMA ifadesinim artışı ve

kolajen birikimi üzerine negatif etkisi olduğunu göstermektedir. Ek olarak, A151 ODN'nin bu baskılayıcı etkisinin sistemik olduğu da ortaya konulmuştur. Baskılayıcı A151 ODN uygulanmış farelere ait splenositler farklı TLR ligandlarıyla uyarıldıktan sonra değişen sitokin salgı paternleri ve antijen sunucu hücre (APC) fonksiyonları göstermiştir. Bu bulgu bize baskılayıcı ODN kullanımının karaciğer fibrojenezini kontrol etmek ve hatta siroza ilerleyişi engelleyerek hastalar tarafından ihtiyaç duyulan karaciğer nakil sayısını azaltmak için mantıklı ve yeni bir yaklaşım olabileceğini önermiştir. Son olarak, bazı üyelerinin TLR sinyalizasyonunu da aktifleştirdiği bilinen ısı şok proteinleri (HSP) üzerine yoğunlaşmış bulunmaktayız. Ek olarak, HSP27'nin karaciğer fibrozisinde meydana gelen olaylardan olan aktin hücre iskeleti organizasyonu ve hücresel motilitenin kontrolünde rol oynadığı bilinmektedir. Böylelikle, ilk defa HSP27'nin karaciğer fibrojenezindeki potansiyel rolüyle ve HSP27 ve αSMA ifadesi değiştirici etkilerinden dolayı quercetin tedavisinin terapötik bir yaklaşım oluşuyla alakalı veriler sunulmuştur.

Anahtar sözcükler: Karaciğer, karaciğer kanseri, hepatosellüler karsinom, karaciğer fibrozisi, FLT3, TLR, baskılayıcı ODN A151, HSP, HSP27, quercetin.

To my precious family…

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Abbreviations

ALL	Acute lymphoblic leukemia
ALR	AIM2-like receptor
ALT	Alanine transaminase
AML	Acute myeloid leukemia
AP-1	Activator protein-1
APC	Antigen presenting cell
APS	Ammonium persulfate
AST	Aspartate aminotransaminase
ATP	Adenosine triphosphate
BDL	Bile duct ligation
bp	Base pairs
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLL	Chronic lymphocytic leukemia
CLR	C-type lectin receptor

CML	Chronic myeloid leukemia
CpG	Unmethylated cytosine-guaniosine motifs
CT	Computed tomography
CTGF	Connective tissue growth factor
CV	Central vein
DAMP	Damage associated molecular pattern
DC	Dendritic cell
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxynucleotide
dsRNA	Double stranded ribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FLK2	Fetal liver kinase-2
FLT3	FMS-like tyrosine kinase 3
FLT3L	FMS-like tyrosine kinase 3 ligand
FN	Fibronectin
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein-2
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HIP	HSP70 interacting protein
HOP	Heat shock organizing protein
HRP	Horseradish peroxidase
HSC	Hepatic stellate cell
HSF1	Heat shock factor-1
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IκK	Inhbitor of κB kinase

IL	Interleukin
ip	Intraperitoneal
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor
ITD	Internal tandem duplication
JMD	Juxtamembrane domain
JNK	c-Jun N-terminal kinase
KC	Kupffer cell
kDa	Kilodalton
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MET	Mesenchymal-epithelial transition
MHC	Major Histocompatibility Complex
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NEAA	Non-essential amino acid

NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nod-like receptor
OD	Optical density
ODN	Oligodeoxynucleotide
OS	Overall survival
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD	Poorly differentiated
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PGN	Peptidoglycan
PH	Partial hepatectomy
PI3K	Phosphatidylinositol 3 kinase
pIC	Polyriboinosinic polyribocytidylic acid
PLC-γ	Phospholipase C-γ
PNPP	Para-nitrophenyl phosphate
PRR	Pattern Recognition Receptor

RIP-1	Receptor interacting protein-1
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RTK	Receptor tyrosine kinase
sc	Subcutaneous
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC	Sinusoidal endothelial cell
SHIP	Src-homology 2 containing inositol phosphatase
SHP-2	Src-homology 2 containing protein tyrosine phosphate
shRNA	Small hairpin ribonucleic acid
sHSP	Small heat shock protein
SNP	Single nucleotide polymorphism
ssRNA	Single stranded ribonucleic acid

STAT	Signal transducer and activator of transcription
TACE	Transarterial chemoembolization
TAE	Tris Acetate EDTA
TAK1	Transforming growth factor-β activated kinase-1
TBS	Tris Buffered Saline
TGF-β	Transforming growth factor-β
Th1	T helper 1
Th2	T helper 2
TIRAP	Toll/IL1 receptor-associated protein
TKD	Tyrosine kinase domain
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-α
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTP	Time to progression
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

WD	Well differentiated
αSMA	Alpha smooth muscle actin

Chapter 1

1 Introduction

1.1 Liver

1.1.1 Liver Development

Being the largest organ in the body, the liver undertakes a wide range of important metabolic functions for the survival of the living organism. Among these functions, blood glucose level regulation, serum protein and clotting factor production, and metabolism of all dietary compounds are highly critical. Moreover, it is also responsible for the synthesis of bile, and biotransformation of the products generated by the metabolism and xenobiotics [1].

Initial signs of the liver organogenesis are seen in the 4^{th} week of the embryogenesis. A structure called the liver bud or hepatic diverticulum is observed, which develops from the ventral foregut endoderm. Later on, different parts of the liver bud develop into different structures; the cranial part becomes the liver and intrahepatic biliary tree, and the caudal part becomes the gallbladder and extrahepatic biliary tree. Moreover, the primitive cells, also known as hepatoblasts, in this bud develop into hepatocytes and cholangiocytes [2, 3]. Hepatic plates are formed as a result of the migration of growing hepatoblasts and separated by the mesenchymal cells forming the sinusoids. On the other hand, bile ducts are generated as a result of various interactions of periportal hepatoblasts with the neighboring cells [4].

1.1.2 Liver Anatomy

Liver is a relatively homogenous organ compared to the other complex organs. A lobule is the basic unit of the liver, which is a hexagonal structure that is generated by the lining of sinusoidal capillaries the hepatocyte plates. These sinusoidal capillaries diverge towards the central efferent vein. There is a portal triad of vessels at each corner of the hexagonal liver lobule containing a portal vein, hepatic artery, which supply blood, and the bile duct. Most of the liver volume is made of hepatocytes, approximately 78%, but still they need to be in contact with the other cell types found in the liver; Kupffer cells (KCs) (resident macrophages), pit cells (natural killer cells), hepatic stellate cells (HSCs), cholangiocytes, endothelial cells, and sinusoidal endothelial cells (SECs) [5, 6].

HSCs, KCs, and SECs are the cell types that line the hepatic sinusoid. Liver SECs have an important role in the diffusion of substances between the hepatocyte surface and blood. KCs are the liver resident macrophages and show a high capacity for endocytosis and phagocytosis. They are responsible for the secretion of the mediators of inflammatory response, such as NO, CO, TNF-α, and other cytokines. Thus, KCs have the major role in the control of inflammation ongoing in the liver, eventually in the innate immune response. Moreover, KCs are responsible for the secretion of enzymes and cytokines that participate in extracellular matrix (ECM) remodeling.

HSCs possess branched cytoplasmic processes that are functional in the embracing of endothelial cells and lining the sinusoids. They are also characterized by the presence of intracytoplasmic fat droplets. HSCs have a role in vitamin A storage, regulation of sinusoid contractility, and ECM organization in the normal liver. A damage to the liver results in the activation of quiescent HSCs transforming into myofibroblast-like cells that have a significant role in the generation of fibrotic response. Pit cells are liver associated large granular lymphocytes, such as the natural killer (NK) cells. In addition to the pit cells, there is another type of lymphocytes in the liver that are gamma delta T cells, and alpha beta T cells [7].

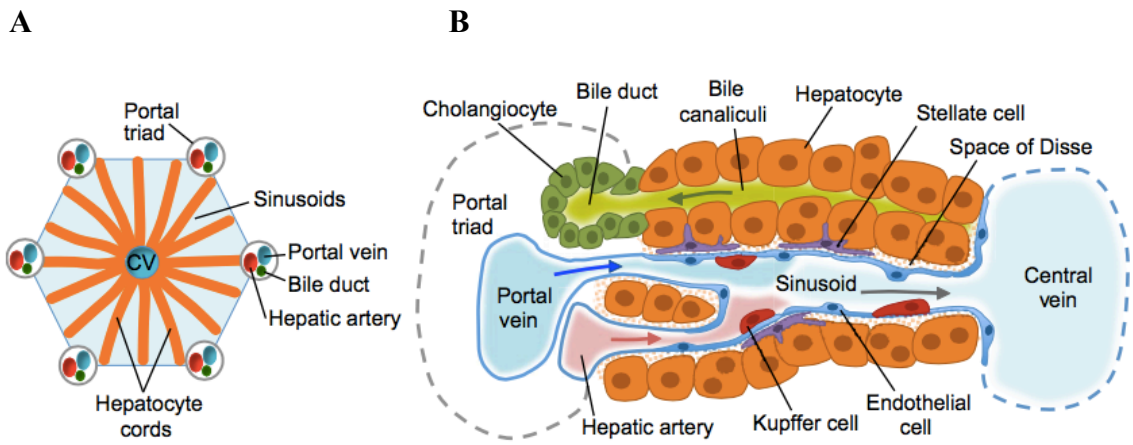


Figure 1.1. Organization and cell types of the liver

(A) Liver consists of many lobules, each of which is made of a central vein (CV). Hepatocytes line up from CV to portal triads of portal vein, bile duct, and hepatic artery. (B) Each lobule contains many sinusoids, in which KCs are found. HSCs are found in the space of Disse and cholangiocytes line the bile duct. *Adapted from* [8].

1.1.3 Epithelial-Mesenchymal Transition

Epithelial cells are polarized, adherent cells that form layers by attaching each other. On the other hand, mesenchymal cells are non-polarized and able to move individually due to absence of intercellular connections. Epithelial-mesenchymal transition (EMT) is a process that takes place when the cells start losing their epithelial features and becoming mesenchymal-like. The reverse of this process is known as mesenchymal-epithelial transition (MET). Both of these processes are based on the changes in cell shape and adhesiveness of the cells [9, 10]. Normal epithelial expression, cytoskeletal organization that determines the epithelial polarity, and the presence of proteins that participate in the cell-cell and cell-matrix contacts are lost during EMT. On the other hand, migratory and invasive characteristics that require cytoskeletal rearrangements are gained during this process [9].

Basically, EMT/MET take place in development/embryogenesis, tissue regeneration/wound healing/fibrosis, and neoplasia. EMT is categorized in three different types; type 1, type 2, and type 3. Type 1 EMT takes place during

implantation, embryogenesis, and the development of organs without causing fibrosis. Fibrosis is the main result of type 2 EMT, which is normally associated with inflammation. If the tissue injury continues and causes a prolonged inflammation, type 2 EMT generates fibroblastic cells that cause organ destruction. Finally, type 3 EMT takes place as a result of genetic and epigenetic changes in cancer cells. This type of EMT results in the invasion of tumor cells, eventually causing metastasis [11, 12].

1.1.3.1 Epithelial-Mesenchymal Transition in the Liver

EMT/MET take place in the liver, like many other organs. According to the previous findings, hepatocytes, HSCs, and cholangiocytes are the potential cell types in the liver that are capable of undergoing EMT/MET especially in the presence of liver injury, and fibrosis. But still, due to the lack of a convincing technology to show that EMT/MET process certainly happens in the regenerating liver, for the time being this idea is assumed to be uncertain to some extent [10].

1.1.4 Liver Regeneration

Injury and repair are the major events that take place in all mammalian organ systems. Three basic processes initiate following injury; inflammation, new tissue formation, and tissue remodeling [13]. Liver regeneration is an intense event that is based on the rapid replication of hepatocytes to rebuild the actual liver [14].

Previous studies showed that hepatocytes have the ability to differentiate into cholangiocytes after biliary injury [15]. However, under normal physiological circumstances hepatocytes regenerate in order to replace the aged ones [16]. Hepatocyte growth factor (HGF), interleukin-6 (IL-6), and tumor necrosis factor (TNF) are well known for their roles in liver regeneration through the activation of KCs resulting in the initiation of hepatocyte regeneration. Briefly, TNF and its type I receptor association results in the activation of nuclear kappa B (NF-κB) in KCs. This

leads to IL-6 release from KCs allowing it to bind to its receptor activating STAT3 pathway, which ends up with the initiation of hepatocyte regeneration [17].

Complete remodeling of the liver is highly possible following acute injuries with the help of oval stem cells, hepatocyte progenitor cells, and bone marrow derived stem cells [18]. However, this remodeling process may not be as effective in the case of chronic liver diseases, such as liver fibrosis and cirrhosis.

1.1.5 Liver Fibrosis

Liver fibrosis can be simply defined as a wound healing response generated following an insult to the liver leading to an injury. If not prevented, liver fibrosis progresses into cirrhosis that might end up with liver cancer and liver failure [19].

Activation of HSCs constitutes the basis of liver fibrosis. HSCs normally reside in the quiescent state, but they become activated following liver injury. In the activated state, they proliferate and produce ECM components [20]. In addition to HSCs, many studies supported the potential contribution of bone marrow-derived cells and myofibroblasts to liver fibrosis. Previous studies claim that bone marrow derived cells are able to migrate to the liver during progression and regression of liver fibrosis [21, 22]. Moreover, recent studies started to focus more on the idea that EMT has significant effects on liver fibrosis progression [19].

1.1.5.1 Causes of Liver Fibrosis

There are various causes of HSC activation that results in myofibroblastic phenotype formation leading to chronic liver injury. Toxin exposure, viral hepatitis, autoimmune disorders, alcoholic and non-alcoholic steatohepatitis are the major factors resulting in liver injury [23]. HSC activation takes place in two steps, initiation and perpetuation. If the injury settles down, then these two steps are followed by another step known as resolution [24]. Initiation step takes place just after the injury, which mainly covers all

changes in gene expression and cell phenotype. This helps cells to be able to respond cytokines and other signals. Following activation, perpetuation step starts as an outcome of these signals that lead to fibrosis. At the perpetuation step, there is an increase in profibrogenic, proinflammatory and promitogenic factor release. As a final step, resolution is based on major cellular events leading to HSC quiescence, apoptosis, or senescence [25, 26]. Cellular and molecular changes in the phenotype of HSCs upon liver injury and during resolution of fibrosis are depicted in Figure 1.2.

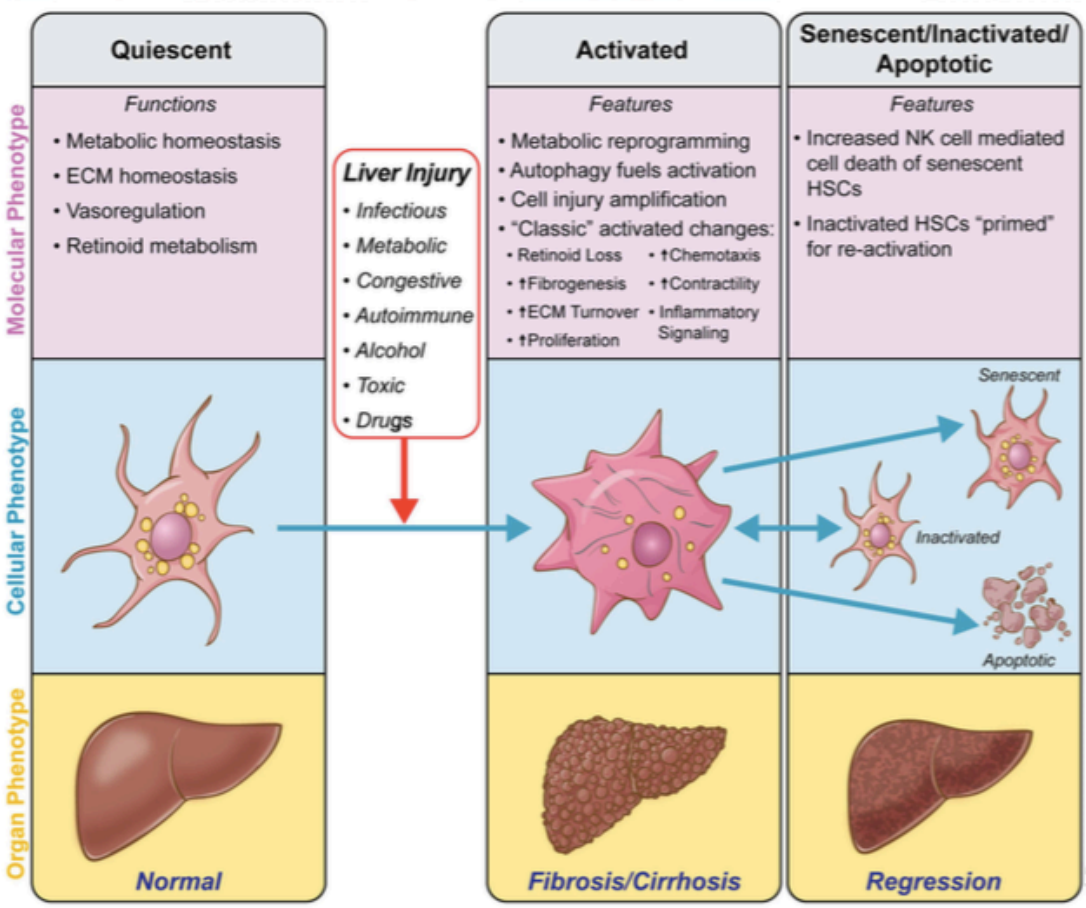


Figure 1.2. Both molecular and cellular phenotype changes in HSCs following liver injury

Liver injury caused by different factors results in the activation of HSCs and phenotypic changes in their molecular phenotypes as well as cellular phenotypes. Activation of HSCs promotes liver fibrosis and cirrhosis. Progression of this fibrotic response might be prevented by the inactivation, apoptosis or senescence of activated HSCs. Regression of liver fibrosis may take place depending on the state of HSCs; senescent HSCs may be cleared by NK cells, whereas inactivated HSCs stay primed for another injury. *Adapted from* [27].

1.1.5.2 Signaling Pathways Involved in Liver Fibrosis

It is well known that major fibrogenic and HSCs proliferative stimulators are transforming growth factor β (TGF-β) and platelet derived growth factor (PDGF). However, growing evidence suggests that there are other pathways and regulators, such as connective tissue growth factor (CTGF), underlying liver fibrosis and different cell types are affected differently by them [28, 29]. In the last decade, another important step was taken towards the connection between innate immune response and liver fibrosis. A study demonstrated that activation of TLR4 by its ligand, lipopolysaccharide (LPS), increases the activity of TGF-β1 resulting in hepatic scar formation [30]. Additionally, severity of liver fibrosis progression was shown to differ among hepatitis C patients that carry varied TLR4 polymorphisms [31, 32]. In addition to these, HSC activation was demonstrated to be influenced by ECM breakdown as a result of matrix metalloproteinase (MMP) activity [33].

Interaction of HSCs with other cell types is another way of HSCs to contribute to liver fibrogenesis. Recent studies reported that apoptotic hepatocytes release DNA, which in turn results in the activation of HSCs through TLR9 stimulation [34]. NK cells on the other hand, were found to enhance liver fibrosis by increasing TGF-β levels as a result of tumor necrosis factor related apoptosis inducing ligand (TRAIL) associated hepatocyte apoptosis [35]. Additionally, lymphocytes were shown to influence HSC activation in a direct manner without any cytokine release [36].

1.1.5.3 Experimental Liver Fibrosis Models

There are different experimental models to study mechanisms underlying liver fibrosis. CCl_4 and bile duct ligation (BDL) are the most commonly used models in liver fibrosis studies. CCl_4 model is mainly based on administration of CCl_4 to the model animals for a certain time period, whereas BDL is a surgical method generated by the ligation of the bile duct as the name implies. Both of these models were reported to be almost identical in terms of gene expression patterns. However, culture activated HSCs showed partial similarity to HSCs activated with these models [37].

1.1.5.4 Therapeutic Approaches for Liver Fibrosis

Even though there has been a great advance in liver fibrosis treatment, it is still hard to claim that there are certain therapeutic approaches to solve this problem. In order to find a way of treating liver fibrosis, studies are trying to focus on shared fibrotic pathways among different organs. Since the contribution of TGF-β pathway to liver fibrosis is well established, studies are mainly based on the selective inhibition of TGF-β activity in the fibrotic site. IL-4 and IL-13 are the other targets for liver fibrosis treatment. Additionally, there are ongoing studies on targeting miRNAs to regulate progression of liver fibrosis due to their tissue remodeling activities in various organs. Clinical trials for fibrotic diseases are not thought to be very successful for now, but accumulating evidence is expected to improve the effectiveness of these trials in the upcoming years [38].

1.1.6 Cirrhosis

Cirrhosis is becoming a more serious health problem especially in the developed countries, being the cause of the most of liver transplantation procedures. Abnormal alcohol consumption and hepatitis C virus infection are the most prevalent causes of the cirrhosis. Other than these, hepatitis B virus infection is also becoming an important cause for this disease [39].

There are various events happening for the transition of chronic liver diseases into cirrhosis. Inflammation and HSC activation leading to fibrosis, angiogenesis, and parenchymal lesion formation results in cirrhosis. Activation of HSCs cause sinusoidal remodeling by the accumulation of ECM elements, and the angiogenesis results in the formation of intrahepatic shunts [40, 41]. In addition to these, portal pressure increases in the cirrhotic liver as a consequence of increase in the hepatic resistance to blood flow [42].

1.1.6.1 Diagnosis of Cirrhosis

Diagnosis of chronic liver diseases is not simple until the development of cirrhosis with clinical symptoms. The most commonly seen symptoms of cirrhosis are accumulation of abdominal ascites, variceal bleeding, sepsis, hepatic encephalopathy, and jaundice. There are various imaging techniques for the diagnosis of cirrhotic liver with nodular and irregular structure, such as magnetic resonance imaging (MRI), ultrasonography, and computed tomography (CT). However, for some cases a liver biopsy is needed for the confirmation of the diagnosis [43]. On the other hand, there are non-invasive methods for the diagnosis of early stage cirrhosis. There are known biomarkers for the detection of advanced fibrosis, which are measured either directly or indirectly from serum [44].

1.1.6.2 Therapeutic Approaches for Cirrhosis

Even though it is harder to treat cirrhosis at the late stages, there are possible treatment options for the early stages. Population screening by performing detailed blood tests and analyzing non-invasive fibrosis markers might be effective for the prevention of chronic liver diseases [45, 46]. In fact, lifestyle change is the most applicable way of prevention because of less side effects and cost. It was shown that metabolic syndrome and cirrhosis are highly associated [47]. Moreover, diabetes was found to be an additional risk factor for HCC formation [48]. On the other hand, alcohol intake should be ceased, because moderate use is enough to increase the risk of cirrhosis in certain cases of liver diseases, especially for chronic hepatitis C and alcoholic steatohepatitis cases, which might end up with HCC [49, 50]. Additionally, cigarette smoking was showed to have a driving effect for the progression of fibrosis in non-alcoholic steatohepatitis, chronic hepatitis C, and primary biliary cirrhosis [51].

There are known potential drugs for the treatment of cirrhosis depending on the cause and stage. However, some cases such as HCC with cirrhosis do not respond drug treatment. Thus, those cases possibly need liver transplantation as a therapeutic option [42].

1.1.7 Hepatocellular Carcinoma

HCC, being one of the leading causes of worldwide cancer related deaths, is generally related to cirrhosis in more than 80% of the cases. Once cirrhosis develops, current chemopreventive options are not much effective to prevent the formation of cancer in the liver [52]. The pathogenesis of this deadly disease is highly complex at the molecular level. Unfortunately, most of the established treatment strategies are not conclusive in terms of getting rid of the tumors except for the cases diagnosed at the early stages, even though there have been so many clinical and molecular studies ongoing [53].

1.1.7.1 Causes of Hepatocellular Carcinoma

HCC development and progression is not a single step event, it goes over multiple processes to form instead. Before anything else, a chronic insult like alcohol consumption, hepatitis B virus (HBV), hepatitis C virus (HCV) infection, must be present for the induction of injury. This happens through the generation of endoplasmic reticulum (ER) stress, DNA damage, reactive oxygen species (ROS), and hepatocyte necrosis. Following an insult, a hepatic response by the liver is generated involving different cell types. Liver injury promotes the activation of HSCs and macrophages, which in turn generate ECM components and growth factors. This results in the initiation of fibrosis and endothelial cell migration resulting in the deformation of the parenchyma and architecture of the liver. Thus, HCC is a complex event including different conditions, such as inflammation, angiogenesis, hypoxia, oxidative stress, and autophagy, with the contribution of different resident and non-resident cell types [54].

Starting from the early stages of HCC progression, angiogenesis is one of the most significant events contributing tumor growth. Angiogenesis is a well-regulated and complex event in HCC progression, which starts with the initial formation of the tumor. As the tumor grows, its need for nutrients and oxygen derives the formation of new vessels through the activation and proliferation of endothelial cells [55]. In the

previous studies, it was shown that vascular endothelial growth factor (VEGF) expression and HCC aggressiveness are related [56]. Binding of VEGF to vascular endothelial growth factor receptor 1 (VEGFR1) and vascular endothelial growth factor receptor 2 (VEGFR2) activates proliferative, migratory and invasive pathways in endothelial cells [57].

Inflammation and immunosuppression are well known to contribute HCC development and progression through sustained cytokine production stimulating different cell types in the liver. A dominancy in T helper 2 (Th2)-like cytokine activity over T helper 1 (Th1)-like was demonstrated to have an association with more aggressiveness and metastasis in HCC profile [58, 59]. In addition to the activities of cytokines, chemokines and their receptors were found to have roles in different stages of HCC development, especially for their angiogenic activities. On the other hand, immune response in HCC was highly regulated by growth factors like HGF, epidermal growth factor (EGF), and TGF-β. Previous studies showed that TGF-β is expressed at high levels in HCC [60], and HGF has a controlling effect on HCC cells proliferation and invasion together with fibroblast growth factor (FGF) [61, 62].

1.1.7.2 Therapeutic Approaches for Hepatocellular Carcinoma

Treatment options for HCC depend on the stage of the disease. Most of the patients with HCC are diagnosed at advanced or metastatic stages, but the current treatments are mostly limited to early stage patients [63]. On the other hand, there is a huge demand of liver transplantation but the number of available liver is considerably lower. Thus, it is highly important for linking treatment options to be developed for the patients with advanced HCC [64].

Most common treatment option for the patients with intermediate stage HCC is transarterial chemoembolization (TACE), and briefly it involves the administration of a mixture of chemotherapeutic and embolic agents into the feeding artery of the tumor [65]. TACE has been demonstrated to be effective in many cases, but still there were obstacles because of the infiltration of cancer cells [66].

Previous studies were able to establish another treatment option for advanced stage HCC patients, actually based on the use of an oral multi-kinase inhibitor known as sorafenib [67]. Sorafenib was shown to inhibit the activity of Raf serine/threonine kinases, VEGFRs, and platelet derived growth factor receptors (PDGFRs), which belong to the pathways known to be involved in the progression of HCC [68]. Even though the disease stabilization was not high with sorafenib, it was demonstrated to be the first to improve overall survival (OS) and time to progression (TTP) in advanced stage HCC patients [69]. Combination of TACE and sorafenib was proposed to be an effective strategy. Therefore, several studies are being conducted to show how effective and safe to use this combination therapy [64, 70–72].

1.2 FLT3

1.2.1 Structure of FLT3

FMS-like tyrosine kinase 3 (FLT3) belongs to type III receptor tyrosine kinase (RTK) family together with other receptors like FMS, KIT, and PDGFRs [73, 74]. First identification of *FLT3/FLK2* gene was done in mouse and shown to be on chromosome 5 encoding a 1000 amino acid tyrosine kinase with molecular weight of 135-155 kDa [75–77]. FLT3 has an extracellular domain at the amino terminus containing five immunoglobulin-like regions, a transmembrane region, an intracellular juxtamembrane domain (JMD), and two kinase domains separated by a kinase insert at the carboxyl terminus [78–80].

After the identification of FLT3 receptor, its ligand FMS-like tyrosine kinase 3 ligand (FLT3L) was characterized and it was demonstrated to have two forms; soluble and membrane bound [81–83]. FLT3L and FLT3 interaction results in the dimerization of receptor so that the auto-phosphorylation of tyrosine residues in kinase domains. This auto-phosphorylation induces the activation of downstream pathways leading the phosphorylation of target proteins [84].

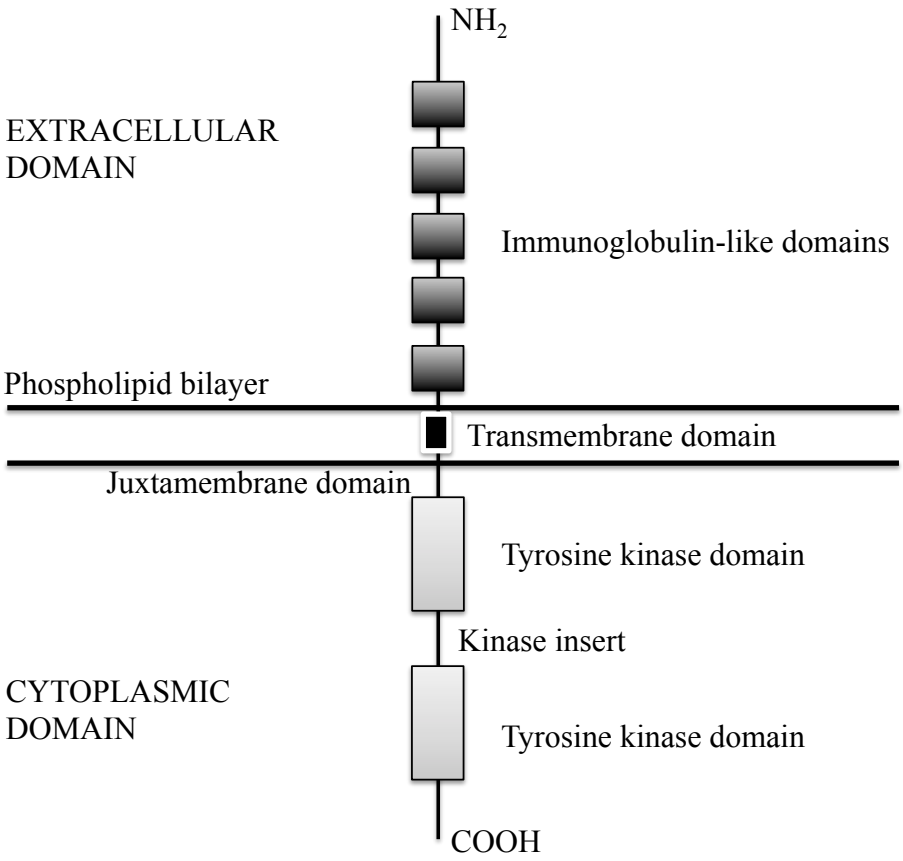


Figure 1.3. Schematic representation of FLT3 receptor and its orientation on the phospholipid bilayer

1.2.2 FLT3 Signaling

Phosphatidylinositol 3 kinase (PI3K) and Ras/Raf pathways were activated following the stimulation of FLT3 with FLT3L. This activation results in cell proliferation, differentiation inhibition, and a decrease in apoptosis based on the activities of signaling and adaptor proteins like growth factor receptor-bound protein 2 (Grb2), mitogen activated protein kinase (MAPK), signal transducer and activator of transcription 5 (STAT5), extracellular-signal regulated kinase (ERK1/2), SHC, CBL, phospholipase C-γ (PLC-γ), Src-homology 2 containing protein tyrosine phosphate (SHP-2), and Src-homology 2 containing inositol phosphatase (SHIP) [85–90].

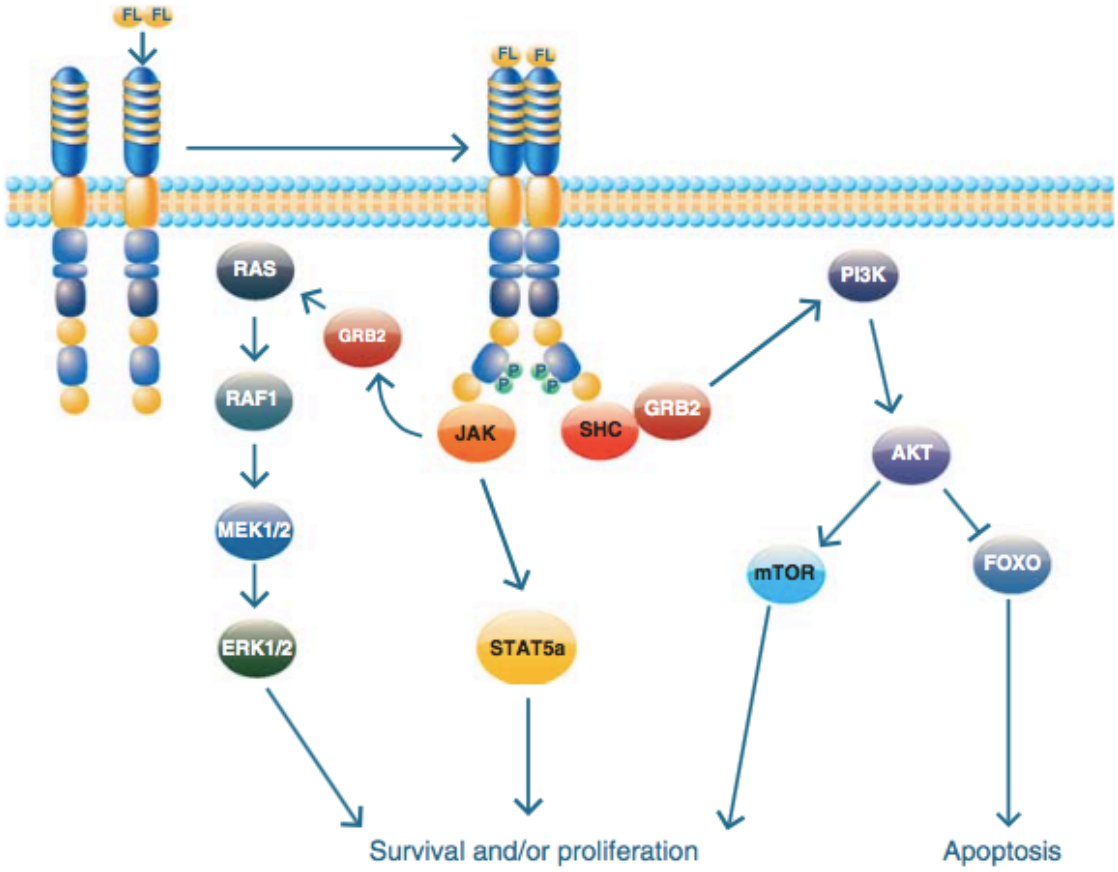


Figure 1.4. Schematic representation of FLT3 signaling and downstream pathways initiated by FLT3L
Adapted from [91].

1.2.3 Physiological and Pathophysiological Effects of FLT3

FLT3 signaling plays a pivotal role in the development of hematopoietic stem cells, NK cells, B-cell progenitors, and dendritic cell (DC) progenitors [73, 92, 93]. Previous studies demonstrated that FLT3 is expressed at very high levels in precursor B-cell in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) primary leukemia samples. Additionally, it is also expressed in some of the T-cells in ALL samples [94, 95]. It was also shown that FLT3 expression is present in chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML) blast crisis samples [95].

In a normal human bone marrow, FLT3 is expressed only in $CD34^+$ cells, but it is present on leukemia blasts independent of CD34 positivity [95]. Internal tandem duplication (ITD) and tyrosine kinase domain (TKD) point mutations are the two categories of FLT3 activating mutations in AML [80]. ITD mutations cause FLT3 receptor to be phosphorylated constitutively resulting in the activation of kinase domains, because they allow ligand-independent dimerization of FLT3 receptor [96–98]. Moreover, FLT3-ITD was demonstrated to have a proliferative effect on hematopoietic stem cells [99, 100]. On the other hand, TKD mutations are less common than ITD mutations and known to cause formation of a secondary point mutation in leukemia [80].

1.2.4 FLT3 and Liver Diseases

Oval cells are precursors for hepatocytes and bile duct cells. They are known to have a crucial role in liver regeneration, because they are activated and start to proliferate when there is a disruption in the functionality of hepatocytes [101, 102].

Oval cells have a specific protein expression pattern and FLT3 is one of those proteins [103–105]. Additionally, FLT3 was demonstrated to be a hepatic lineage surface marker [106]. Moreover, it was shown that FLT3 is activated at late stages of liver regeneration participating in the proliferation that goes on during this process [107]. In a previous study, FLT3L administration was demonstrated to improve hepatic fibrosis regression by expanding the number of DCs along the portal tract [108].

Sorafenib is a multikinase inhibitor acting against RAF kinases and RTKs such as PDGFR, VEGFR, c-Kit, Ret, and FLT3. It was proved to have an antitumor activity in different tumor types [109]. Besides, our previous studies suggested a potential role for FLT3 in hepatocellular carcinogenesis [110].

1.3 Toll-like Receptors

Pattern recognition receptors (PRRs) undertake an essential role in the initial microbe detection as a part of the innate immune response. PRRs recognize two different groups of patterns derived from different sources; pathogen associated molecular patterns (PAMPs) that are microbe specific and damage associated molecular patterns (DAMPs) that are derived from damaged cells. Following pattern recognition, PRRs are activated resulting in inflammatory cytokine and type I interferon (IFN) production, thus innate immune response induction. This response not only triggers inflammation but also causes the priming of antigen specific adaptive immune response [111, 112].

Mammalian PRRs are categorized in different groups, which are Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), and intracellular DNA sensors [113, 114].

1.3.1 Cell Surface TLRs

Human TLR family has ten members (TLR1-TLR10) whereas there are twelve members (TLR1-TLR9, TLR11-TLR13) in the mouse TLR family. Different TLRs are localized in different compartments of the cell, either on the cell membrane or intracellular compartments [115]. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are localized on the cell membrane [116]. These TLRs are known to recognize microbial membrane components like proteins, lipids, and lipoproteins. TLR4 is well known to recognize bacterial LPS. TLR2 works together with either TLR1 or TLR6 to recognize peptidoglycans (PGNs), lipoproteins, zymosan, lipotheic acid, mannan, and tGPI-mucin [116]. TLR5 is known to recognize bacterial flagellin and TLR10 works with TLR2 to recognize listeria ligands and sense influenza A virus infection [113, 117, 118].

1.3.2 Intracellular TLRs

Intracellular compartments on which TLRs are localized are ER, lysosome, endosome, and endolysosome. TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are localized on the endosomes [116, 119]. TLR3 is known to recognize viral double stranded RNA (dsRNA), small interfering RNAs, and self-RNAs from damaged cells [120–122]. TLR7 recognizes viral single stranded RNA (ssRNA) whereas human TLR8 senses viral and bacterial RNA [123, 124]. TLR9 is very well known for its recognition of bacterial and viral DNA that contain unmethylated CpG-DNA motifs [125]. TLR11 also recognize flagellin and TLR12 is similar to TLR11, which generally work together [126–128]. Finally, TLR13 senses bacterial rRNA [129–131].

1.3.3 TLR Signaling

The common pattern among all TLRs is that they are all synthesized in the ER, directed to the Golgi apparatus, and finally located on the cell surface or intracellular compartments [112]. UNC93B1 and PRAT4A are the proteins responsible for the trafficking of TLRs. While UNC93B1 controls the trafficking of intracellular TLRs from ER to endosomes, PRAT4A is mainly regulates the trafficking of TLR1, TLR2, TLR4, TLR7, and TLR9 from ER to plasma membrane and endosomes [132, 133].

TLR signaling is based on not only TLRs, but also certain adaptor molecules known as TIR domain containing adaptors like MyD88, TRIF (TIR-domain-containing adapter-inducing interferon-β), TRIF-related adaptor molecule (TRAM), or TIRAP/MAL. All TLRs use MyD88 for the signaling that results in the activation of NF-κB and MAPKs leading to inflammatory cytokine production. MyD88 is recruited to the cell surface TLRs by TIRAP, but recent studies demonstrated that TIRAP is also important for the endosomal TLR signaling [134]. A different pathway is activated utilizing IRF3, NF-κB and MAPKs for type I IFN and inflammatory cytokine production through the recruitment of TRIF to TLR3 and TLR4. Overall, there are two major TLR signaling pathways, which are MyD88-dependent and TRIF-dependent [112].

MyD88-dependent pathway starts with Myddosome complex formation as a result of MyD88 and IRAK kinase association [135]. At this step, after activating IRAK1, IRAK4 is released from MyD88 [136]. IRAK1 associates with TRAF6, which then helps the polyubiquitination of both itself and TAK1. Activation of TAK1 results in the activation of two IκK complex- NF-κB pathway and IκK complex-MAPK pathways. IκBα, known as NF-κB inhibitory protein, is degraded after phosphorylated by IκK complex. This degradation results in the translocation of NF-κB to the nucleus promoting proinflammatory gene expression. On the other hand, TAK1 also activates MAPK family members (ERK1/2, JNK, p38) leading AP-1 transcription actor family activation or mRNA stabilization [113, 116].

TRIF-dependent pathway starts with the interaction between TRAF6 and TRAF3, which is then followed by the recruitment of RIP-1 by TRAF6. This results in the activation of TAK1 complex, which in turn promotes the activation of NF-κB and MAPKs and inflammatory cytokine induction. On the other hand, TRAF3 associates with TBK1, IκKi and NEMO to phosphorylate IRF3, which then dimerizes and translocates to the nucleus resulting in type I IFN induction [113, 116].

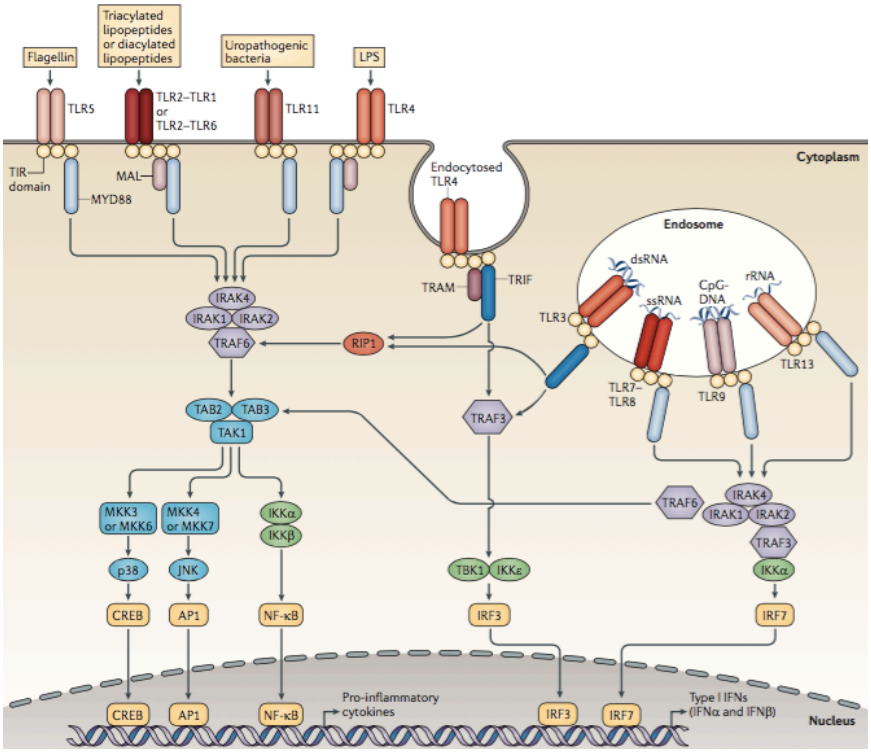


Figure 1.5. Schematic representation of mammalian TLR signaling and downstream pathways
Adapted from [137].

1.3.4 TLR Expression and Signaling in the Liver

TLR mRNA expression in the healthy liver is relatively lower compared to other organs such as spleen [138]. Hepatocytes are known to produce both secreted and membrane-bound PRRs. Primary cultured hepatocytes were shown to express all mRNAs, but respond weakly to TLR ligands *in vivo* [139, 140]. SECs are the other cells that express TLRs in the liver, which promote TNF-α, IL-6, and IFN-β production as a response to TLR ligand stimulation [141]. KCs on the other hand are very well known for their TLR4 expression ability, thus LPS responsive nature [142]. They are also known to express TLR2, TLR3 and TLR9 [143, 144]. Upon stimulation with TLR ligands, KCs were demonstrated to upregulate IFN-β, MHC-II and co-stimulatory molecule expression in addition to IFN-γ production and T cell proliferation [141]. In the liver, HSCs were also found to express TLR4 and TLR9, together with TLR2. TLR4 signaling in HSCs was demonstrated to promote chemokine and adhesion molecule expression [145]. In addition to these, intrahepatic lymphocytes, hepatic DCs, and biliary cells were also reported to express different TLRs and respond TLR ligands under various conditions [146].

1.3.5 TLR Signaling in Liver Regeneration

TLR/MyD88 signaling is known to result in inflammatory cytokine production, among which TNF-α and IL-6 are the potential role players in liver regeneration. TNF-α and IL-6 are required for the priming of hepatocytes following a liver injury [147–149]. Even though some studies claim that TLR2 and TLR4 do not contribute to proinflammatory cytokine production in liver regeneration following PH, recent findings demonstrated that high levels of LPS in portal vein results in TNF-α and IL-6 secretion in KCs helping liver to regenerate [148, 150]. In another study, TLR3, which uses TRIF as an adaptor protein instead of MyD88, was shown to have a negative effect on liver regeneration through STAT1 followed by IRF1 and p21 pathway activation [151].

1.3.6 TLR Signaling in Liver Fibrosis

Previous studies suggest that TLR4 signaling is an important role player in liver fibrosis. Experimental liver fibrosis models, either with CCl_4 or BDL, with TLR4 pathway mutations showed diminished liver fibrosis [30, 152–155]. There are different mechanisms proposed to enlighten the contribution of TLR4 signaling to liver fibrosis. One of them is through the activation of TLR4 signaling on HSCs by LPS resulting in chemokine secretion and adhesion molecule expression, which help KC and monocyte migration to the site of injury [30, 145]. Another mechanism suggested to explain the role of TLR4 signaling in liver fibrosis is through the association between TLR4 and TGF-β pathways in HSCs. Activation of TLR4 pathway downregulates Bambi expression, which is normally responsible for the inhibition of TGF-β signaling, leading to HSC activation as a result of increased TGF-β signaling [30]. Another recent mechanism relies on the inhibitory effect of TLR4 signaling on the expression of miR-29 in HSCs resulting in increased HSC activation and liver fibrosis [156]. A final mechanism to explain the role of TLR4 signaling in liver fibrosis is based on the production of fibronectin (FN) as a result of TLR4 activation, which helps LECs to migrate and initiate angiogenesis driving liver fibrosis [155].

In addition to TLR4 signaling, several studies mentioned the important contribution of TLR9 signaling to liver fibrosis. It is known that TLR9 is activated by bacterial unmethylated CpG-DNA and bacterial DNA levels increase in blood as a consequence of cirrhosis [157, 158]. This suggests that TLR9 activation by bacterial DNA has a potential effect on liver fibrosis progression. Additionally, it was also reported that TLR9 is activated by apoptotic DNA released from hepatocytes resulting in collagen and CCL2 production by HSCs [34]. Moreover, fibrotic liver originated DCs were found to be more responsive to CpG-DNA in terms of chemokine, IL-6, and TNF-α secretion [159]. In order to investigate the role of TLR3 signaling in liver fibrosis, previous studies focused on the effect of pIC treatment because of its TLR3 activating nature. They were able to show that pIC treatment diminishes liver fibrosis possibly by inducing cytotoxic activity of NK cells on HSCs [160–162]. However, this effect was abolished upon chronic ethanol consumption suggesting that cytotoxic NK

cell activity mediated by TLR3 signaling is essential in alcoholic liver diseases and liver fibrosis [163–165].

1.3.7 TLR Signaling in Cirrhosis

TLR4 signaling in cirrhotic patients was shown to decrease resulting in poor LPS responsiveness and high Gram-negative bacteria infection [166]. Moreover, cirrhosis in chronic hepatitis C patients was reported to be less in the presence of TLR4 single nucleotide polymorphisms (SNPs) [31]. Additionally, TLR7 SNPs was also found to affect the level of fibrosis in hepatitis C patients [167].

1.3.8 TLR Signaling in Hepatocellular Carcinoma

Previous studies used a chemical known as diethylnitrosamine (DEN) in order to generate an inflammation associated HCC model in rodents. DEN induced HCC was shown to involve the activities of NF-κB and JNK/AP-1, which are well known as being downstream components of TLR signaling [168–170]. TLR4 deficiency in mice was demonstrated to have a reducing effect on liver cancer induced by DEN as a result of a decrease in IL-6 and TNF-α levels in the liver [171]. TLR2 was also found to be associated with liver cancer caused by *Listeria monocytogenes* infection. Silencing TLR2 was found to result in the regression of liver tumor growth supporting the idea that TLR2 signaling assists liver cancer progression [172]. In addition to TLR4 and TLR2, TLR3 and TLR9 were shown to be associated with HCC. TLR3 was found to induce TRAIL-mediated apoptosis, whereas TLR9 activation was claimed to promote cancer cell proliferation [173, 174].

1.4 Immunosuppressive Oligodeoxynucleotides

It is well known that DNA has various effects on immune system, one of which is the generation of an extreme immune response following host DNA release into the circulation. The major causes of this effect are the repetitive motifs found in mammalian telomeric regions, which are single stranded TTAGGG hexanucleotide repeats [175–177]. Previously, it was shown that these telomeric motifs lead to a suppression in Th1 and proinflammatory cytokine, mainly IFN-γ, IL-6, IL-12, TNF-α, production by activated immune cells [175, 178, 179].

In the previous studies, synthetic oligodeoxynucleotide (ODN) bearing TTAGGG repeats were demonstrated to have a similar effect as telomeric DNA [175]. Suppressive ODN were found to have an inhibitory effect on different pathological immune hyperactivation conditions in addition to their initially identified inhibitory effect on CpG induced immune activation [180–185]. These conditions include both inflammatory and autoimmune diseases such as LPS-induced toxic shock, acute silicosis, inflammatory arthritis, ocular inflammation, lupus nephritis, and lupus erythematosus [186].

Studies on suppressive ODN suggest that they are potentially acting on inflammatory pathways involving STAT1 and STAT4. It was suggested that suppressive ODN inhibit the phosphorylation of STAT1 and STAT4 resulting in the blockage of signaling pathways crucial for inflammatory and autoimmune reactions [187].

1.5 Heat Shock Proteins

Heat shock proteins (HSPs) constitute a large family of conserved proteins that are expressed under stress conditions for the survival of cells [188]. There are four major families of mammalian HSPs, HSP60, HSP70, HSP90, and small HSPs (sHSPs), which have been classified according to their molecular weight. High molecular weight HSPs work ATP-dependently, whereas sHSPs do not need ATP [189].

The major role of HSPs is helping misfolded proteins to fold properly and prevent them to aggregate. They are also very well known to be crucial anti-apoptotic proteins since they associate with key proteins underlie the apoptotic machinery [189]. In addition to these, HSPs are responsible from either selective stabilization or degradation of specific proteins upon stress generating insults to the cell [190, 191].

Unfolded protein accumulation in the cytosol is induced by stress and refolding of these proteins requires molecular chaperones. HSP90 and HSP70 are the most important molecular chaperones and upon stress induction they dissociate from heat shock factor 1 (HSF1) in order to help refolding of unfolded proteins accumulated in the cytosol. Then, HSF1 translocates to the nucleus promoting the overexpression of all HSPs [192]. Besides HSP90 and HSP70, HSP27 is an sHSP that blocks the aggregation of unfolded proteins. As an intermediate protein, HSP40 forms a complex with HSP70 interacting protein (HIP) in order to bring unfolded protein to HSP70, which in turn transfers that protein to HSP90 with the help of heat shock organizing protein (HOP) [193, 194].

HSPs are normally responsible for the maintenance of protein homeostasis, but they are also known to be responsible for the progression of some diseases [195]. Therefore, recent studies have been targeting HSPs for the treatment of protein folding diseases, such as cardiovascular diseases, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis [196]. Moreover, they are also focus of interest in cancer studies because of their apoptosis, metastasis, and cell division driving effects [195]. In addition to their unique role in protein homeostasis, HSPs were also shown to be immunogens in the immune response generated against pathogens [197, 198]. It is believed that HSPs increase the immunogenic potential of polypeptites to which they are attached. Thus, HSPs are functional not only in the stimulation of innate immunity but also generation of specific acquired immune response [199]. Studies demonstrated that HSPs trigger innate immune system through acting on TLRs and induce acquired immunity by acting as chaperones for polypeptides to load MHC molecules [200–204].

1.5.1 Heat Shock Protein 27

HSP27 is a member of sHSP family that is expressed in various cells. Its expression is tightly regulated by environmental conditions. An increase in HSP27 protein expression was found in many different cell types just before differentiation, suggesting that it might be a potential predifferentiation marker [205]. Moreover, some studies found a high association between increased HSP27 protein expression and cancer due to its inhibitory effect on apoptotic pathways. Additionally, HSP27 expression is also present in some types of neurons at post-mitotic stages [206].

Different cellular functions of HSP27 depend on its oligomerization level. Large oligomers of HSP27 were shown to act as ATP-independent chaperones that inhibit aggregation and have antioxidant effects. On the other hand, small oligomers of HSP27 were found to be effective in actin filament stabilization [189]. It was proposed to be an actin sequester that is crucial for the maintenance of G-actin and actin filaments [207]. Another study reported that wound healing ability of cells decreased when HSP27 is blocked suggesting a role for HSP27 in cell motility regulation [208].

1.5.1.1 HSP27 and Cell Protection

In addition to its regulatory role in protein homeostasis, HSP27 is known to have direct effects on cell survival. Studies on neuronal diseases showed that HSP27 has potential activity in cell death. Pro-death enzyme inhibitory effect of HSP27 is one hypothesis about its role in cell death. HSP27 was demonstrated to act on cell death signaling not only in the presence of an interaction with apoptotic regulators. It was also suggested to target upstream regulators of apoptosome formation depending on cellular state [209, 210].

Compared to other HSPs, HSP27 has a neuronal protective effect generated in response to apoptotic stimuli. Even though HSP27 is not highly expressed in the brain, it was shown to increase in the presence of central nervous system stressors such as ischemia, seizures and hyperthermia [211–216]. Moreover, there are many ongoing

studies suggesting a protective role for HSP27 in neurodegenerative diseases, such as Huntington's disease, Alzheimer's disease, Parkinson's disease, due of its inhibitory effect on protein aggregation formation [217].

Opposite to these protective roles, HSP27 was reported to have an effect on the initiation of cardiovascular diseases. The reason for this effect is because it has a potential to induce proinflammatory autoimmune response against endothelial cells. However, it was also demonstrated to inhibit aggregation of platelets and promote cardio protection in ischemia [218].

1.5.1.2 HSP27 and Cancer

Previous studies demonstrated an increase in HSP27 level in various cancer types, such as endometrial cancer, ovarian cancer, breast cancer, endometrial cancer and osteosarcoma [219]. Additionally, phosphorylation profile of HSP27 was shown to differ in tumor cells compared to their non-transformed partners and has a direct association with metastasis [220, 221]. An increasing number of studies suggest that HSP27 is a potential candidate to target in cancer therapy, but its complex structure complicates finding a molecule to neutralize [189].

1.5.1.3 Quercetin as HSP Inhibitor

In the previous studies, flavonoids were shown to inhibit HSP synthesis [222]. Quercetin, one of the well-known flavonoids, has various biological roles in mammalian cells. In addition to its HSP inhibitory effect, quercetin is known to inhibit many different protein kinases and *in vitro* cell growth [223–227]. One study reported that quercetin activity depends on the cell type and in breast cancer cells it inhibits HSP induction without affecting DNA-binding activity of HSF [228]. Additionally, quercetin was suggested to have negative effects on major biological pathways, such as enzyme synthesis, glycolysis, and cell cycle, in order to abolish the growth of malignant cells [229–231]. Moreover, it is well known that quercetin has

antioxidative and anti-inflammatory effects [232, 233]. A recent study demonstrated that quercetin is protective against LPS-induced lung injury [234].

1.6 Aim of the Study

This study was organized in two main sections to investigate the molecular mechanisms underlying liver cancer and liver fibrosis with a common purpose of presenting novel therapeutic approaches to prevent their progression.

In our previous studies, we suggested that FLT3 contributes liver regeneration and hepatocellular carcinogenesis. This was achieved by demonstrating that subcellular localization of FLT3 changes upon PH and contributes cellular proliferation that takes place during liver regeneration [107]. Additionally, we were able to show that FLT3 inhibition results in a diminution in the aggressiveness of HCC cells suggesting a potential role for FLT3 as a link between liver regeneration and hepatocellular carcinogenesis [110]. In addition to these, our ongoing studies suggest us a possible activity for FLT3 in liver fibrogenesis, acting like a potential link between liver regeneration, fibrosis, cirrhosis, and HCC. Therefore, in this study we aimed to support our previous findings on the potential role of FLT3 in liver cancer and its potential candidacy as a biomarker for the diagnosis of this widespread disease. Besides, we aimed to support the effect of K-252a tyrosine kinase inhibitor on hepatocellular carcinogenesis with further experiments on the role of FLT3 in this process to present K-252a as a therapeutic candidate for liver cancer patients.

The second part of this study was devoted to exploration of molecular mechanisms lying beneath liver fibrogenesis, which is an initiation point for cirrhosis and HCC in most cases. Our initial purpose was to generate an *in vivo* liver fibrosis model and confirm its validity by analyzing the changes in the cardinal markers that are being commonly used in liver fibrosis studies. Then we aimed to investigate the expression pattern of TLRs in our model and confirm previous findings, which had reported that TLR expression increases following fibrosis induction [138, 235]. After showing that TLRs were upregulated in our liver fibrosis model, we intended to examine if the

suppressive ODN A151 demonstrates an immunosuppressive effect on liver fibrosis progression similar to previous findings due to the high immune response bearing nature of liver fibrosis [175, 236, 237]. Overall, we aimed to present a novel liver fibrosis suppressive agent to prevent its progression and reverse it, thus block its progression into cirrhosis and even HCC.

Previous studies demonstrated that HSP family members HSP60 and HSP70 are capable of activating TLR signaling pathway under certain circumstances [238, 239]. Moreover, another HSP family member, HSP27 is also a potential candidate to have a role in liver fibrogenesis due to its actin filament stabilization and maintenance roles inside the cell, which are important events that take place during this process under the framework of cytoskeletal rearrangements and cellular motility [189, 207, 208]. Therefore, our final purpose was to investigate the expression patterns of HSPs, especially HSP27 in liver fibrosis for the first time due to a lack of findings about HSP27 in liver fibrogenesis and target them to present a novel therapeutic approach if they are potential candidates for being liver fibrosis markers.

Chapter 2

2 Materials and Methods

2.1 Materials

2.1.1 General Laboratory Reagents

General laboratory reagents such as ethanol, methanol, 2-propanol, acetic acid, hydrochloric acid, Tris-base, SDS, acrylamide, glycine, β-mercaptoethanol were mostly supplied from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA) and Merck (Darmstadt, Germany). DMSO was purchased from Applichem (Darmstadt, Germany).

2.1.2 Cell Culture Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified eagle medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin/streptomycin, non-essential amino acids (NEAA), HEPES, L-Glutamine, sodium pyruvate, trypsin were purchased from Lonza (Basel, Switzerland). Cell culture plastic ware such as flasks, multiple well plates, serological pipettes, cryotubes were supplied from Sarstedt (Nümbrecht, Germany). Transfection reagent X-tremeGENE HP DNA was purchased from Roche (Roche Applied Science,

Mannheim, Germany). FLT3 shRNA plasmids were purchased from Openbiosystems (Alabama, USA) and GFP plasmid was supplied from Clontech (Clontech Laboratories, California, USA). Puromycin used in transfection experiments was purchased from Sigma (Sigma-Aldrich, Missouri, USA).

Different chemicals used in treatment experiments were purchased from different companies, such as K-252a from Calbiochem (EMD Millipore, Billerica, Massachusetts, USA), Quercetin from Sigma (Sigma-Aldrich, Missouri, USA).

2.1.3 Reagents for Total RNA Isolation

Nucleospin RNA isolation kit used for RNA isolation from cells was purchased from Macherey-Nagel (Duren, Germany). On the other hand, Trizol Reagent used for RNA isolation from tissues was supplied from Invitrogen (Carlsbad, CA, USA). DEPC was purchased from Sigma (Sigma-Aldrich, Missouri, USA).

2.1.4 RT-PCR Reagents and Primers

cDNA Synthesis kit and *Taq* DNA Polymerase were purchased from New England Biolabs (New England BioLabs Inc., Massachusetts, USA). Primers were designed using the online Primer3 tool v.0.4.0 (http://frodo.wi.mit.edu/primer3/) and NCBI primer BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from Alpha DNA (Montreal, Canada). The list of the primers is shown in Table 2.1.

Table 2.1. Primers used in mouse and human RT-PCR experiments

Primer		Sequence	Product Size
GAPDH (human)	Forward Reverse	5'-GGCTGAGAACGGGAAGCTTGTCAT-3' 5'-CAGCCTTCTCCATGGTGGTGAAGA-3'	119 bp
FLT3	Forward Reverse	5'-ATGGATTCGGGCTCACCT-3' 5'-GCTGATTGACTGGGATGC-3'	184 bp
GAPDH (mouse)	Forward Reverse	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'	452 bp
αSMA	Forward Reverse	5'-CTGACAGAGGCACCACTGAA-3' 5'-AGAGGCATAGAGGGACAGCA-3'	127 bp
TLR1	Forward Reverse	5'-TTTGGGGGAAGCTGAAGACATC-3' 5'-CTTCGGCACGTTAGCACTGAGAC-3'	400 bp
TLR2	Forward Reverse	5'-TCTCTGGGCAGTCTTGAACATTTG-3' 5'-CGCGCATCGACTTTAGACTTTG-3'	320 bp
TLR3	Forward Reverse	5'-GGGGCTGTCTCACCTCCAC-3' 5'-GCGGGCCCGAAAACATCCTT-3'	222 bp
TLR4	Forward Reverse	5'-TGCCGTTTCTTGTTCTTCCTCT-3' 5'-CTGGCATCATCTTCATTGTCCTT-3'	240 bp
TLR5	Forward Reverse	5'-TGGGGCAGCAGGAAGACG-3' 5'-AGCGGCTGTGCGGATAAA-3'	380 bp
TLR6	Forward Reverse	5'-GCCCGCAGCTTGTGGTATC-3' 5'-GGGCTGGCCTGACTCTTA-3'	650 bp
TLR7	Forward Reverse	5'-TTAACCCACCAGACAAACCACAC-3' 5'-TAACAGCCACTATTTTCAAGCAGA-3'	700 bp
TLR8	Forward Reverse	5'-CTTGGGCATTAACTTTATTGAGAA-3' 5'-TATTGGCATTGAAGGACAGATTTA-3'	341 bp
TLR9	Forward Reverse	5'-GATGCCCACCGCTCCCGCTATGT-3' 5'-TGGGGTGGAGGGGCAGAGAATGAA-3'	430 bp
HSP70	Forward Reverse	5'- AATCCAAGCTGCCAACCTT -3' 5'- CAAGAGTTCCTCCACCCAAG -3'	137 bp
HSP27	Forward Reverse	5'-TCCCTGGATGTCAACCACTT-3' 5'-GATGTAGCCATGCTCGTCCT-3'	111 bp

2.1.5 Agarose Gel Electrophoresis Reagents

Agarose was purchased from Prona (Condalab, Madrid, Spain) and Gene Ruler DNA ladder was purchased from Thermo (Thermo Fisher Scientific, Massachusetts, USA).

2.1.6 Antibodies

The list of antibodies used in Western blotting, immunostaining, ELISA, and flow cytometry experiments with their working dilutions/concentrations and catalog numbers are given in Table 2.2, Table 2.3, Table 2.4, and Table 2.5, respectively.

Table 2.2. Primary and secondary antibodies used in Western blotting experiments

Antibody	Catalog #	Dilution	Blocking Solution
FLT3 (130 kDa & 160 kDa) *(Cell Signaling Technology)*	3462	1:1000	5% BSA
CALNEXIN (90 kDa) *(Santa Cruz Biotechnology, Inc.)*	sc-6465	1:1000	5% milk powder
αSMA (42 kDa) *(Abcam)*	ab5694	1:400	5% milk powder
HSP27 (27 kDa) *(Santa Cruz Biotechnology, Inc.)*	sc-1048	1:400	5% milk powder
GAPDH (37 kDa) *(Santa Cruz Biotechnology, Inc.)*	sc-20357	1:1000	5% milk powder
anti-rabbit-HRP *(Cell Signaling Technology)*	7074	1:2000	5% milk powder
anti-goat-HRP *(Santa Cruz Biotechnology, Inc.)*	sc-2033	1:5000	5% milk powder

Table 2.3. Primary and secondary antibodies used in immunostaining experiments

Antibody	Catalog #	Dilution
FLT3 *(Santa Cruz Biotechnology, Inc.)*	sc-340	1:200
αSMA *(Abcam)*	ab5694	1:200
HSP27 *(Santa Cruz Biotechnology, Inc.)*	sc-1048	1:200
anti-rabbit-FITC *(Sigma-Aldrich)*	F-9887	1:200
anti-rabbit-Alexa Fluor 568 *(Invitrogen)*	A11036	1:1000

Table 2.4. Antibodies used in ELISA experiments

Antibody	Catalog #	Working Concentration
IL-6 *(Biolegend)*	504502	5 μg/ml
IL-12 *(Biolegend)*	505202	2 μg/ml
Biotinylated IL-6 *(Biolegend)*	504602	1 μg/ml
Biotinylated IL-12 *(Biolegend)*	505302	1 μg/ml

Table 2.5. Antibodies used in flow cytometry experiments

Antibody	Catalog #	Working Concentration
MHC-II-FITC *(Biolegend)*	107606	1 µg/ml
CD54-PE *(Biolegend)*	116108	1 µg/ml
CD69-FITC *(Biolegend)*	104506	1 µg/ml
CD80-FITC *(Biolegend)*	104706	1 µg/ml

2.1.7 Reagents for Protein Isolation

Igepal CA-630 and protease inhibitor cocktail used in the preparation of lysis buffer for protein isolation were purchased from Sigma (Sigma-Aldrich, Missouri, USA) and Roche (Roche Applied Science, Mannheim, Germany), respectively.

2.1.8 Western Blotting Reagents

PVDF membrane was purchased from Roche (Roche Applied Science, Mannheim, Germany). Bovine Serum Albumin (BSA) was supplied from Sigma (Sigma-Aldrich, Missouri, USA). PageRuler prestained protein ladder and SuperSignal West Femto Chemiluminescent Substrate were purchased from Thermo (Thermo Fisher Scientific, Massachusetts, USA).

2.1.9 Immunostaining Reagents

BSA was purchased from Sigma (Sigma-Aldrich, Missouri, USA). DakoCytomation LSAB 2 System-HRP kit used in immunohistochemistry experiments was supplied from Dako (DakoCytomation, Glostrup, Denmark). UltraCruz Mounting Medium used in immunofluorescence experiments was purchased from (Santa Cruz Biotechnology Inc., California, USA). Picrosirius Red Stain kit was purchased from Polysciences (Polysciences Inc., Pennsylvania, USA) and Sirius Red/Fast Green Collagen Staining kit was purchased from Chondrex (Chondrex Inc., Washington, USA).

2.1.10 Reagents for Proliferation, Migration and Invasion Assays

MTT cell proliferation kit was purchased from Roche (Roche Applied Science, Mannheim, Germany). Matrigel Basement Matrix and Diff Quick Staining Solution kit used in invasion experiments were purchased from BD (BD Biosciences, New Jersey, USA) and IMEB (IMEB Inc. California, USA), respectively.

2.1.11 TLR Ligands and CpG ODNs

Different TLR ligands were purchased from different vendors; *E.coli* derived LPS from Sigma (Sigma-Aldrich, Missouri, USA), R848 from InvivoGen (California, USA) and phosphorothioated backbone K3 (5'-ATCGACTCTCGAGCGTTCTC-3') and mixed backbone D35 (5'-GGtgcatcgatgcaggggGG-3') CpG ODNs (capital letters are linked with phosphorothioate linkages and small letters are linked with phoshodiester linkages) were purchased from Alpha DNA (Montreal, Canada).

2.1.12 Reagents for ELISA

Immulon 2HB microtiter plates, streptavidin-AP and PNPP were purchased from Thermo (Thermo Fisher Scientific, Massachusetts, USA).

2.1.13 Reagents for Flow Cytometry

Fix & Perm Medium A used for the fixation of cells was purchased from Invitrogen (California, USA). BSA and sodium azide used in flow cytometry experiments were supplied from Roche (Basel, Switzerland) and Merck (New Jersey, USA), respectively.

2.1.14 Standard Solutions and Media

2.1.14.1 General Solutions

Ingredients and preparation instructions for general laboratory solutions are given in the Appendices section, Table 7.1.

2.1.14.2 RNA and Protein Isolation Solutions

Ingredients and preparation instructions for RNA and protein isolation solutions are given in the Appendices section, Table 7.2.

2.1.14.3 Western Blotting Solutions

Ingredients and preparation instructions for Western blotting solutions are given in the Appendices section, Table 7.3.

2.1.14.4 Immunostaining Solutions

Ingredients and preparation instructions for immunostaining solutions are given in the Appendices section, Table 7.4.

2.1.14.5 ELISA Solutions

Ingredients and preparation instructions of ELISA solutions are given in the Appendices section, Table 7.5.

2.1.14.6 Cell Culture Solutions

Ingredients and preparation instructions of cell culture solutions are given in the Appendices section, Table 7.6.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Trypsinization and Cell Counting

When the cells reach approximately 90% confluency, they were washed with 1X PBS and then incubated with 1X trypsin at 37•C for approximately 3 minutes. Trypsin inactivation was performed using cell culture medium supplemented with FBS. After spinning the cells, they were resuspended in 10 ml complete RPMI and 10 µl was used for cell counting using a hemocytometer. After counting the cells in the outer 4 largest squares, number of the cells was calculated using the formula given below:

Total # of cells in 1 ml = (# of cells counted/4) x 10 (ml; dilution factor) x 10^4

2.2.1.2 Hepatocellular Carcinoma Cell Lines

Human HCC cell lines Huh7, Hep40, and SK-Hep-1 were cultured in low-glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin. On the other hand, Snu398 cells were cultured in RPMI supplemented with 10% FBS, 100 U/ml penicillin/streptomycin and 0.1 mM non-essential amino acids by refreshing every 2-3 days. Cells were incubated at 37°C with 5% CO_2 and after reaching 90% confluency, they were sub-cultured using trypsin.

Vector transfected Snu398 cells were cultured in complete RPMI supplemented with 0.75 µl puromycin per 1 ml of medium to maintain stable transfection.

2.2.1.3 Preparation of Spleen Cell Suspension

Spleens were surgically removed after cervical dislocation of C57BL/6 mice. By smashing the spleen using the back part of a syringe in 5% FBS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 0.11 mg/ml sodium pyruvate and 0.5 mM β-mercaptoethanol containing RPMI, single cell suspension was obtained and centrifuged at 1500 rpm for 5 minutes for 3 times. Finally, splenocytes were resuspended in fresh complete RPMI at a density of approximately $5x10^6$/ml.

2.2.1.4 K-252a Treatment of Cells

Cells were treated with K-252a for 2 hours prior to the following experiment at a concentration of 200 nM. Same amount of DMSO was used as a control for K-252a since the stock solution for K-252a is prepared in DMSO according to the manufacturer's protocol.

2.2.1.5 Stable Transfection of Snu398 Cells

The day before transfection, Snu398 cells were seeded on 6-well plates. For each well together with appropriate amount of X-tremeGENE HP DNA Transfection Reagent, 1 μg plasmid either with or without FLT3 specific shRNA was used. X-tremeGENE HP DNA Transfection Reagent and plasmid DNA were equilibrated to +15°C to +25°C before the transfection. Using serum-free RPMI, FLT3 specific shRNA and GFP plasmids were diluted to 1 μg plasmid DNA /100 μl medium (0.01 μg/μl) at a 1:3 ratio. 2 μl X-tremeGENE HP DNA Transfection Reagent was mixed with 100 μl of plasmid DNA diluent and then this mixture was incubated for 15 minutes at room temperature. Next, mixture was added drop-wise on top of the cells and then cells were left 48 hours for incubation. RT-PCR was done using the half of the transfected cells in each well in order to detect the FLT3 mRNA expression. After confirming a decrease in FLT3 expression in shRNA transfected but not no change in empty vector transfected cells, each well of transfected cells were transferred into two 75 cm^2 flasks

separately and supplied with fresh RPMI that contains puromycin at a final concentration of 0.75 μg/ml. Two weeks after incubating in puromycin-positive RPMI, cells were harvested and diluted in the same media in order to be able to seed one cell per each well of a 96-well plate. Cells that had taken up plasmids were expected to form colonies with either reduced or unchanged expression of FLT3 after approximately three weeks.

2.2.2 Determination of Gene Expression

2.2.2.1 Total RNA Isolation from Cultured Cells

Cells were harvested using trypsin after reaching 80% confluency. Nucleospin RNA isolation kit was used according to the manufacturer's protocol in order to isolate total RNA from the spun cells. Concentration, OD260 and OD280 values of the RNA samples were measured using NanoDrop ND-1000 (NanoDrop Technologies Inc., Delaware, USA). For long-term storage, RNA samples were kept at -80•C.

2.2.2.2 Total RNA Isolation from Frozen Tissue

Total RNA was isolated from frozen tissue samples using TRIzol® Reagent according to the manufacturer's protocol. 100 mg of frozen tissue sample was homogenized in 1 ml TRIzol and incubated at room temperature for 5 minutes in order to allow dissociation of nucleoprotein complex. After adding 0.2 ml chloroform and shaking well, mixture was left at room temperature for 15 minutes. 15 minutes of centrifugation at 12000 g was performed at +4°C and three phases were obtained. Upper aqueous RNA containing phase was transferred into a fresh tube, 0.5 mL of 100% isopropanol was added. Tube was inverted for a few times and left at room temperature for 10 minutes. Following 10 minutes of centrifugation at 12000 g at +4°C, supernatant was removed and the pellet was resuspended in 1 mL of 75% DEPC-ethanol. A final centrifugation was performed at 7500 g for 5 minutes at +4°C,

supernatant was removed and the RNA pellet was air-dried for max. 10 minutes. Finally, pellet was resuspended in appropriate amount (generally 100 µl depending on the size of the RNA pellet) of DEPC-treated water. Concentration, OD260 and OD280 values of the RNA samples were measured with NanoDrop. For long-term storage, RNA samples were kept at -80•C.

2.2.2.3 cDNA Synthesis

cDNA was generated from the isolated RNA sample using ProtoScript® First Strand cDNA Synthesis kit according to the manufacturer's protocol. Briefly, 2 µg RNA was used for each reaction to obtain 40 µl of solution in total. RNA was diluted with DEPC-treated water up to 14 ul. 2 µl of oligodT primer was added, and incubated at 70•C for 5 minutes. After the incubation step, 20 µl of M-MuLV reaction mix and 4 ul of M-MuLV enzyme mix were added. 40 µl cDNA synthesis mixture was incubated at 25•C for 10 minutes, 40•C for 45 minutes and 85•C for 5 minutes. cDNA samples were kept at -20•C.

2.2.2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reaction mixture used in RT-PCR experiments is given in Table 2.6.

Table 2.6. Reaction mixture used in RT-PCR experiments

Ingredient	Volume
10X Taq Reaction Buffer	2.5 µl
50 mM $MgCl_2$	1.5 µl
10 mM dNTPs	0.5 µl
10 uM Forward Primer	1.0 µl
10 uM Forward Primer	1.0 µl
5 U/ul Taq Polymerase	0.5 µl
ddH_2O	17.0 µl
cDNA	1.0 µl
Total	**25.0 µl**

RT-PCR reaction conditions were variable depending on the primer set given in Table 2.1. Initial denaturation step was performed at 95°C for 30 seconds, which was followed by denaturation-annealing-extension steps with varying cycle numbers between 23 and 35 depending on the primer set used. Denaturation step was done for 30 seconds at 95°C for all primer sets, annealing step was performed for 30 seconds at different temperatures for different primer sets, which was followed by extension step for 1 minute at 68°C for all primer sets. Final extension was done at 68°C for 5 minutes for all primer sets. Annealing temperatures and cycle numbers for different primer sets are given in Table 2.7.

Table 2.7. Annealing temperatures and cycle numbers for each primer set used in RT-PCR experiments

Primer	Annealing Temperature	Cycle Number
GAPDH (human)	60°C	30
FLT3	60°C	30
GAPDH (mouse)	63°C	23
αSMA	62°C	35
TLR1	60°C	35
TLR2	60°C	35
TLR3	60°C	35
TLR4	60°C	35
TLR5	60°C	35
TLR6	60°C	35
TLR7	60°C	35
TLR8	60°C	35
TLR9	60°C	35
HSP70	60°C	35
HSP27	60°C	30

2.2.2.5 Agarose Gel Electrophoresis

Using 1X TAE, agarose and 1 mg/ml ethidium bromide solution, 1.5% agarose gel was prepared. RT-PCR products were mixed with 6X loading dye and 10 µl of sample mixture was loaded into each well of the agarose gel. Gene Ruler DNA Ladder Mix was used as a marker. Gel was run at 80V-120V for approximately 30-45 minutes depending on the size of the gel and expected products. Visualization of the gels was done using Transilluminator (Vilber Lourmat, Paris, France) and images of the gels

were taken using the ChemiCap software. Semi-quantitative analysis of mRNA expression was performed using ImageJ software on the gel photos obtained. Housekeeping genes were used to normalize the expression of genes of interest and cDNA-negative samples were used to remove the background on the gel image.

2.2.3 Determination of Protein Expression

2.2.3.1 Total Protein Isolation from Cultured Cells

Cells were harvested using a scraper in 1X PBS. Cell pellet was resuspended in 100 ul (may change depending on the size of the pellet) lysis buffer. This mixture was left on ice for 30 minutes and occasional vortexing was done to achieve complete lysis of the cells. Finally, the cell lysate was centrifuged at 13000 rpm for 20 minutes at 4•C. Supernatants were collected and protein concentrations were measured with Bradford Assay.

2.2.3.2 Total Protein Isolation from Frozen Tissue

Total protein was isolated from frozen tissue samples using Camiolo Buffer. 100 mg frozen tissue sample was homogenized in 1 ml Camiolo Buffer. Homogenates were centrifuged for 20 minutes at 12000 g and 4•C. Protein concentrations of supernatants were determined with Bradford assay.

2.2.3.3 Protein Quantification with Bradford Assay

Different concentrations of BSA were prepared according to the Table 2.8 in order to generate a standard curve. Depending on the protocol used for the lysis, different reagents such as lysis buffer and Camiolo Buffer were used as blank. OD values of the

samples were measured using Beckman DU 640 Spectrophotometer (Beckman Coulter Inc., California, USA). Protein concentrations were calculated after generating a standard curve using OD values of different BSA concentrations. 2 µl of protein was mixed with 98 µl ddH_2O and 900 µl Bradford Reagent for the proteins with unknown concentrations.

Table 2.8. BSA standard curve solutions prepared for Bradford Assay

BSA Standard	Bradford Reagent	ddH_2O	BSA (1ug/ µl)	Total
1	900 µl	100 µl	0 µl	1000 µl
2	900 µl	97.5 µl	2.5 µl	1000 µl
3	900 µl	95 µl	5 µl	1000 µl
4	900 µl	92.5 µl	7.5 µl	1000 µl
5	900 µl	90 µl	10 µl	1000 µl
6	900 µl	87.5 µl	12.5 µl	1000 µl
7	900 µl	85 µl	15 µl	1000 µl
8	900 µl	80 µl	20 µl	1000 µl
9	900 µl	75 µl	25 µl	1000 µl
10	900 µl	65 µl	35 µl	1000 µl

2.2.3.4 Western Blotting

2.2.3.4.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

After determining the concentration of proteins with Bradford Assay, 25 µL of 30 µg of protein was prepared in cracking buffer and denatured at 90°C for 5-10 minutes. After denaturation, proteins samples were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prepared before the experiment according to the Table 2.9 and Table 2.10.

Different resolving gel percentages were chosen depending on the size of the protein of interest; generally 7.5% for proteins larger than 100 kDa, 10-12% for proteins around 30-90 kDa, 15% for proteins around or smaller than 20 kDa. 5% resolving gel was prepared for all proteins.

Table 2.9. Resolving gel preparation for SDS-PAGE

Ingredients	**7.5% Resolving Gel**	**10% Resolving Gel**	**12% Resolving Gel**	**15% Resolving Gel**
30% Acrylamide Mix	2.5 ml	3.33 ml	4 ml	5 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.1 ml
10% APS	0.1 ml	0.1 ml	0.1 ml	0.1 ml
TEMED	0.008 ml	0.008 ml	0.008 ml	0.008 ml
ddH_2O	4.79 ml	3.96 ml	3.29 ml	2.29 ml
Total	**10 ml**	**10 ml**	**10 ml**	**10 ml**

Table 2.10. Stacking gel preparation for SDS-PAGE

Ingredients	**5% Stacking Gel**
30% Acrylamide Mix	0.83 ml
1 M Tris pH 6.8	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml
ddH_2O	3.43 ml
Total	**5 ml**

Gel was placed into Bio-Rad Mini PROTEAN Tetra Cell system (Bio-Rad Laboratories, Inc., California, USA) after loading proteins and PageRuler protein

ladder, and run for 2-3 hours at 80-120V in cold 1X Running Buffer. Stacking gel was removed and washed gently with ddH_2O before the transfer of proteins to the PVDF membrane. Generally wet transfer was done for large proteins and semi-dry for small proteins.

2.2.3.4.2 Wet Transfer of Large Proteins

Bio-Rad Mini PROTEAN Tetra Cell system was used for the wet transfer of proteins. Whatman papers and PVDF membrane were cut depending on the size of the gel. PVDF membrane was put into methanol for 30 seconds, then ddH_2O for 2 minutes and finally into the Wet Transfer Buffer. Sponges and Whatman papers were soaked into the transfer buffer. One sponge, two Whatman papers, PVDF membrane, gel, two Whatman papers and another sponge were aligned carefully and transfer of proteins was performed overnight (might be more but no more than 22 hours) at 16V at 4^oC in 1X cold Wet Transfer Buffer. After the transfer of proteins, PVDF membrane was washed with 1X TBS-T (0.1%) and the gel was put into Commasie Solution for 30 minutes and de-stained with the De-staining Solution in order to check the efficiency of transfer.

2.2.3.4.3 Semi-Dry Transfer of Small Proteins

For the semi-dry transfer of small proteins, Whatman papers, PVDF membrane and gel were prepared and aligned similarly without sponges. Since the size of the membrane determined according to the size of the gel, different amount of current was applied depending on the size of the membrane. Amount of current was calculated by multiplying the area of the membrane in cm^2 with 3.5 mA. Transfer was done for approximately 25-40 minutes depending on the size of the protein. After the transfer of proteins, PVDF membrane was washed with 1X TBS-T (0.1%) and the gel was put into Commasie Solution for 30 minutes and de-stained with the De-staining Solution in order to check the efficiency of transfer.

2.2.3.4.4 Blocking and Antibody Incubation

Blocking of membrane was performed either overnight at 4°C or 2-4 hours at room temperature. Blocking solution was prepared in 1X TBS-T (0.1%) either with non-fat milk powder or BSA depending on the type of the antibody used. Primary and secondary antibodies and their working conditions are given in the Table 2.2. After blocking the membrane, primary antibody incubation was done overnight at 4°C. The membrane was washed three times with 1X TBS-T (0.1%) for 10 minutes. Secondary antibody incubation was done for 1 hour at room temperature and then again the membrane was washed three times with 1X TBS-T (0.1%) for 10 minutes.

2.2.3.4.5 PVDF Membrane Development

Finally, SuperSignal West Femto Maximum Sensitivity Substrate was applied on top of the membrane for 1 minute and washed slightly with 1X TBS-T (0.1%). Membrane was placed on a glass, covered with stretch film, put into a film cassette and the film was developed for different time periods between 5 seconds to 5 minutes with Amersham Hyperprocessor Automatic Film Processor (GE Healthcare Life Sciences, Pennsylvania, USA).

2.2.4 Immunostaining Procedures

2.2.4.1 Immunofluorescence Staining for Cultured Cells

Cells were grown until 80% confluency on coverslips before the staining. After removing the medium and washing with 1X PBS, cells were fixed with ice-cold methanol for 15 minutes at room temperature. Then, the cells were washed with 1X PBS-T (0.1%) and blocked with Blocking Solution for 1 hour at room temperature. After the blocking step, cells were treated with the primary antibody solution prepared in the Blocking Solution for either 1 hour at room temperature or overnight at 4°C

depending on the antibody. Then the cells were washed with 1X PBS-T (0.1%) and incubated in secondary antibody solution for 1 hour at room temperature in dark. Working concentration and conditions of the antibodies for immunostaining procedures are given in Table 2.3. After washing with 1X PBS-T (0.1%), cells on the coverslips were mounted on slides using UltraCruz mounting medium with DAPI. Photos of the slides were taken using Leica TCS/SP5 microscope (Tokyo, Japan). Excitation wavelength for DAPI, FITC, and Alexa Fluor 568 are 359 nm, 490 nm, and 561 nm respectively.

2.2.4.2 Immunofluorescence Staining for Frozen Tissue Sections

5 μm frozen tissue sections were prepared prior to the experiment and placed into a humidified chamber. Tissues were fixed with 4% paraformaldehyde solution for 30 minutes at room temperature. After washing with 1X PBS-T (0.1%) for 5 minutes, tissues were blocked with Blocking Solution for 1 hour at room temperature and then primary antibody solution was applied for either 1 hour at room temperature or overnight at 4^{o}C ensuring that tissues do not dry. After washing with 1X PBS-T (0.1%) for 5 minutes, secondary antibody solution was applied for 1 hour at room temperature in dark. Working concentration and conditions of the antibodies for immunostaining procedures are given in Table 2.3. Finally, tissues were washed with 1X PBS-T (0.1%) and mounted using UltraCruz mounting medium with DAPI. Photos of the tissues were taken using Leica TCS/SP5 microscope. Excitation wavelength for DAPI and FITC are 359 nm and 490 nm, respectively.

2.2.4.3 Immunohistochemistry Staining for Paraffin Embedded Tissue Sections

De-paraffinization of paraffin embedded tissues was done by incubating tissues in xylene for 15 minutes at room temperature. In order to hydrate the tissues, samples were incubated in decreasing concentrations of ethanol prepared in ddH_2O. After washing the samples with ddH_2O, antigen retrieval was done by treating tissues with Sodium Citrate Buffer for 10 minutes at 90^{o}C. Then the tissues were treated with 3%

H_2O_2 prepared in methanol for 30 minutes at room temperature and washed with 1X PBS-T (0.1%) for 5 minutes. Tissues were placed in a humidified chamber and blocked for 1 hour at room temperature with Blocking Solution. Then, primary antibody solution was applied for either 1 hour at room temperature or overnight at 4^{o}C. Working concentration and conditions of the antibodies for immunostaining procedures are given in Table 2.3. After washing the tissues with 1X PBS-T (0.1%) for 5 minutes, Biotin-link anti-mouse & anti-rabbit IgG was applied for 30 minutes at room temperature. Then, Streptavidin-HRP solution was applied for another 30 minutes at room temperature. After washing the tissues with 1X PBS-T (0.1%) for 5 minutes, DAB+Chromogen Substrate was applied on top of the tissues for approximately 1 minute (until observing a color change) and then the tissues were washed with tap water to stop the color change. Hematoxylene was applied for 1 minute as a counterstain and then the samples were washed with tap water. Tissues were mounted with glycerol and covered. Photos of the tissues were taken using Leica TCS/SP5 microscope.

2.2.4.4 Picrosirius Red Staining

Frozen tissue samples were prepared as 5 μm sections to stain collagen types I and III using Picrosirius Red Stain Kit according to the manufacturer's protocol. Tissues were placed in a humidified chamber and without fixation, Solution A was applied for 2 minutes at room temperature. After washing with ddH_2O, Solution B was applied for 1 hour at room temperature. Finally, Solution C was applied for 2 minutes at room temperature and washed with 70% ethanol in ddH_2O. Samples were mounted with glycerol and their photos were taken with Leica TCS/SP5 microscope.

2.2.5 Sirius Red/Fast Green Collagen Staining

Frozen liver tissue samples were prepared as 5 μm sections (approximately 40-50 mm^2) in order to quantitate the collagen and non-collagenous protein content in each section using Sirius Red/Fast Green Collagen Staining Kit according to the

manufacturer's protocol. Without fixation, tissues were placed in a humidified chamber and treated with Dye Solution for 30 minutes at room temperature. After removing the Dye Solution, samples were washed with ddH_2O. Finally, Dye Extraction Buffer was applied on top of the tissues by pipetting and collected in a plate for measurement. OD values at 540 nm and 605 nm were read with Synergy H1 microplate reader (BioTek Instruments Inc., Vermont, USA). Amount of collagen and non-collagenous protein was calculated using the equation below:

Collagen (µg/section) = [OD 540 value - (OD 605 value x 0.291)] / 0.0378

Non-collagenous proteins (µg/section) = OD 605 value / 0.00204

2.2.6 MTT Cell Proliferation Assay

$5x10^3$-10^4 cells/well were seeded into a 96-well plated depending on the experiment and cell type. Cells were incubated at 37°C for 24-72 hours depending on the course of the experiment. Cell Proliferation Kit I (MTT) was used according to the manufacturer's protocol. After the incubation step, MTT Labeling Reagent was added on top of the cells and 4 hours incubation was performed at 37°C. Finally, cells were treated with Solubilization Solution and incubated overnight at 37°C. OD values at 551 nm were read using Synergy H1 microplate reader.

2.2.7 Wound Healing Assay

Cells were seeded into 6-well plates prior to the experiment and when they reached 100% confluency, three vertical scratches were made using a micropipette tip. Cells were incubated either in 10% FBS or 2% FBS containing medium to compare normal and serum starvation conditions. Photos of the wounds were taken at three time points, 0 hour, 24 hours, and 48 hours. Distance between the initial and final wound was measured at ten different points and the average was calculated to obtain the overall healed distance.

2.2.8 Matrigel Invasion Assay

Matrigel Basement Membrane Matrix was diluted in the cell culture medium, put carefully into 24-well transwell plate and incubated at 37°C for 4-5 hours. After coating the transwells, 10^5 cells were put into each well and the lower chamber was filled with RPMI containing 5 μg/ml. 20-24 hours incubation was done at 37°C. Non-invaded cells were removed with a cotton swab and invaded cells were stained with Diff-Quick Staining Solution kit. Invaded cells were counted under a light microscope.

2.2.9 Model Animals

Animals were housed under controlled environmental conditions (22ºC) with a 12-hour light and 12-hour dark cycle and received unlimited access to food and water in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University. Protocols in this study were performed according to the guidelines of Bilkent University Local Animal Ethic Committee (BILHADYEK) on humane care with the ethic committee permission number 2012/7 and European Convention (ETS 123) criteria about the use of laboratory animals.

2.2.9.1 Liver Fibrosis Mouse Model

In order to generate an *in vivo* liver fibrosis model, 1 year old male C57BL/6 mice were administered with 10% CCl_4 intraperitoneally (ip) twice a week with the dose of 8 μl /g body weight for 4 weeks. Mice were sacrificed two days after the last injection, blood samples, livers, and spleens were collected.

2.2.9.2 Nude Mouse Tumor Xenograft Model

$6x10^6$ cells were prepared in PBS for each side of a male CD1 nude mouse and kept on ice until the injection. After stabilizing the animal, cells were injected subcutaneously (sc) on one side. Control cells were injected on the other side of the same mouse. Tumor sizes were measured every 3 days. Mice were sacrificed after termination of the experiment.

2.2.9.3 Injection of Animals with Suppressive ODN A151

Each of the male C57BL/6 mouse was ip injected with 150 μg A151 solution three times a week for one week. Mice were sacrificed two days after the last injection.

2.2.9.4 Quercetin Treatment

Male C57BL/6 mice were ip injected with quercetin at 5 mg/ml concentration three times a week for one week. Mice were sacrificed two days after the last injection.

2.2.9.5 Blood Collection and Serum Isolation for ALT and AST Level Measurement

On the day of termination of the experiment, blood samples were collected by cardiac puncture from the animals. Collected blood samples were incubated at 37°C for 1 hour and centrifuged for 10 minutes at 5000 rpm at 4°C to isolate serum. ALT (alanine transaminase), AST (aspartate aminotransaminase) levels were measured using isolated sera in Hacettepe University Faculty of Medicine.

2.2.10 Proinflammatory Cytokine Enzyme Linked-Immunosorbent Assay

5 µg/ml IL-6 and 2 µg/ml anti-mouse IL-12 coating of Immulon 2HB microtiter plates were done for 4 hours at room temperature. After the incubation, plates were blocked with blocking buffer containing 5% BSA in 1X PBS for 2 hours at room temperature and washed 5 times with 1X PBS-T (0.025%). Cell supernatants from stimulated cells were added along with serially diluted standards and incubated for 2 hours at room temperature. First, 1 µg/ml biotinylated IL-6 or 1 µg/ml IL-12 was added after washing as described above and then 50 µl streptavidin-AP was added. After adding 50 µl PNPP substrate, plates were developed and read at 405 nm until complete development of the standards yielding an S-shaped curve. In order to follow cytokine concentrations of the samples, a 4-parameter curve setting was adjusted using an ELISA reader (Molecular Devices, California, USA). Details of the antibodies used in ELISA experiments are given in Table 2.4.

2.2.11 Flow Cytometry Analysis of Cell Surface Markers

After isolating splenocytes just after the removal of spleens, $5x10^6$ splenocytes were fixed using Fix & Perm Medium A. Then the cells were stained with different antibodies (MHC-II-FITC, CD54-PE, CD69-FITC, and CD80-FITC) at 1 µg/ml concentration in 1X PBS supplemented with 1% BSA and 0.5 µg/ml Na-Azide. After incubating cells with antibodies for 30 minutes at room temperature in dark, cells were washed and analyzed using BD Accuri C6 flow cytometer (BD Biosciences, California, USA). Details of the antibodies used in flow cytometry experiments are given in Table 2.5.

2.2.12 Statistical Analyses

Statistical significance analyses were performed using GraphPad software. In order to determine the significance between treatments, Student's t-test was applied with a significance level of p-values < 0.05 and p-values < 0.01.

Chapter 3

3 Results

3.1 Role of FLT3 in Hepatocellular Carcinoma

Previous studies claimed a potential role for FLT3 in proliferation and liver regeneration. In addition to this, our previous findings in HCC cell lines directed us to a different potential role of FLT3 in hepatocellular carcinogenesis. In our previous studies, both *in vitro* and *in vivo* experiments were performed to investigate the effect of K-252a, a tyrosine kinase inhibitor, on the migratory and tumorigenic capacity of HCC cells [110]. In this sense, we performed additional *in vitro* and *in vivo* experiments to further investigate the effect of FLT3 in hepatocellular carcinogenesis. This was achieved by knocking down FLT3 in Snu398 cells, a poorly differentiated (PD) HCC cell line after analyzing the expression of *FLT3* in both well differentiated (WD) and PD HCC cell lines.

3.1.1 Expression of FLT3 in Well Differentiated and Poorly Differentiated HCC Cell Lines

We have chosen two WD (Huh7 and Hep40) and two PD (Snu398 and SK-Hep-1) HCC cell lines. First of all, we analyzed *FLT3* mRNA expression in these cell lines by performing RT-PCR. We did not see any *FLT3* expression in Huh7 and SK-Hep-1 cells at mRNA level, but our results showed that Hep40 cells slightly express *FLT3*

whereas Snu398 cells express at higher levels (Figure 3.1). Previously, FLT3 expression analysis in HCC cells had also been done at protein level and we found that Hep40 cells are not capable of expressing FLT3 at protein level, but Snu398 cells are [110]. For this reason, we decided to continue with knockdown experiments using Snu398 cells.

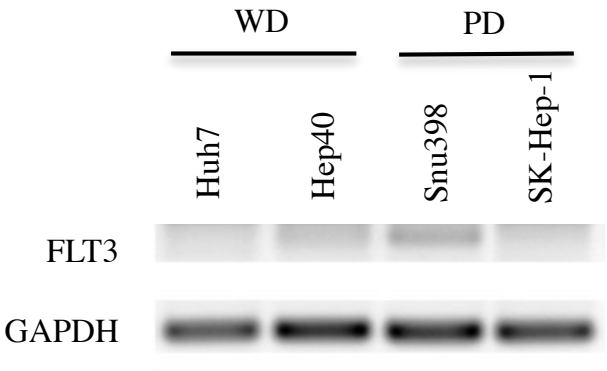


Figure 3.1. Expression of FLT3 in HCC cell lines

FLT3 expression in WD and PD HCC cell lines at mRNA level detected by RT-PCR. *GAPDH* was used as loading control.

3.1.2 FLT3 Knockdown in Snu398 Cells

Considering our previous findings about the inhibitory effect of K-252a on the migratory and tumorigenic capabilities of Snu398 cells, we also wanted to investigate the effect of FLT3 downregulation on these features in addition to proliferative and invasive abilities of these cells [110]. To do so, we stably transfected Snu398 cells either with FLT3 specific shRNA or pGIPz empty vector control. Then, we measured FLT3 expression after transfection both at mRNA and protein levels by performing RT-PCR and immunofluorescence staining, respectively (Figure 3.2 and Figure 3.3). As a result of these experiments, we observed approximately 45% lower *FLT3* mRNA expression in FLT3 shRNA transfected Snu398 cells (Figure 3.2). Moreover, less number of FLT3 positive cells was detected following FLT3 shRNA transfection compared to pGIPz control (Figure 3.3).

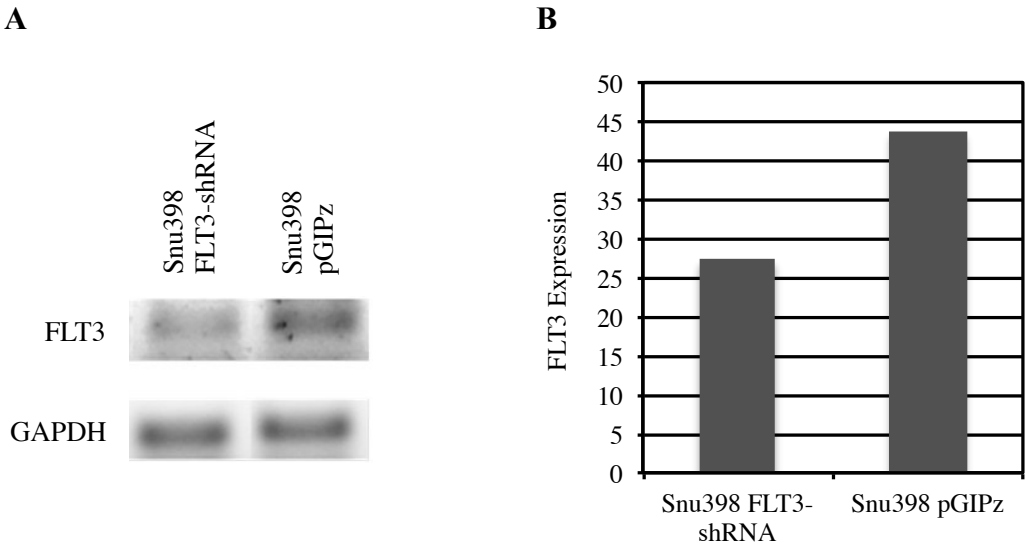


Figure 3.2. Expression of *FLT3* mRNA in Snu398 cells after transfection

(A) *FLT3* mRNA expression in Snu398 cells following FLT3 specific shRNA (Snu398 FLT3-shRNA) and empty vector (Snu398 pGIPz) transfection detected by RT-PCR. *GAPDH* was used as loading control. (B) Graphical representation of *FLT3* mRNA expression quantification by ImageJ on a single gel image.

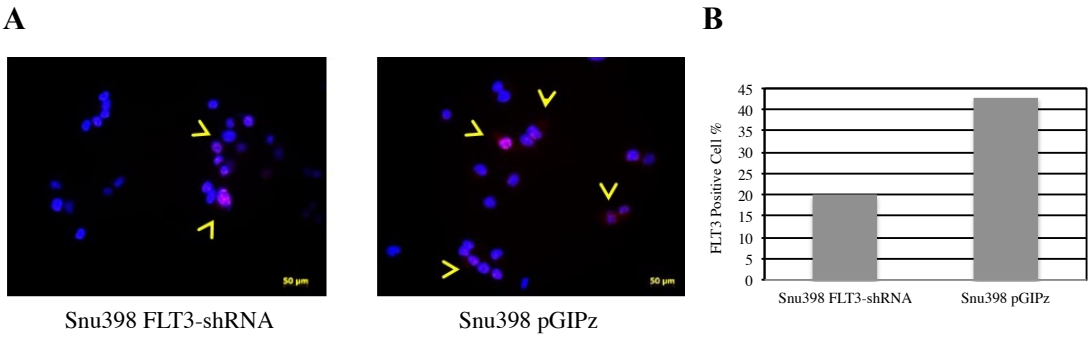


Figure 3.3. Expression of FLT3 protein in Snu398 cells after transfection

(A) FLT3 protein (Alexa Flour 568-labeled) expression in Snu398 cells following FLT3 specific shRNA (Snu398 FLT3-shRNA) and empty vector (Snu398 pGIPz) transfection shown by immunofluorescence staining. DAPI was used to stain nuclei, and arrowheads indicate FLT3 positivity. Images were taken using 40X objective. (B) Graphical representation of FLT3 protein expression percentage calculated by dividing FLT3 positive cells with total number of cells based on DAPI staining on a single immunofluorescence image.

3.1.3 Effect of FLT3 Knockdown in Snu398 Cells *in vitro*

Next, we decided to investigate the changes in the proliferative, migratory and invasive capacities of Snu398 cells after shRNA transfection. Initially, we performed MTT assay in order to analyze the changes in the proliferative ability of Snu398 cells after both K-252a treatment and transfection. We found out that proliferation rate of K-252a treated Snu398 cells significantly decreases compared to DMSO treated control cells (Figure 3.4A; **: p-value=0.001). We also observed a change in the rate of proliferation following FLT3 shRNA transfection compared to empty vector transfection (Figure 3.4B).

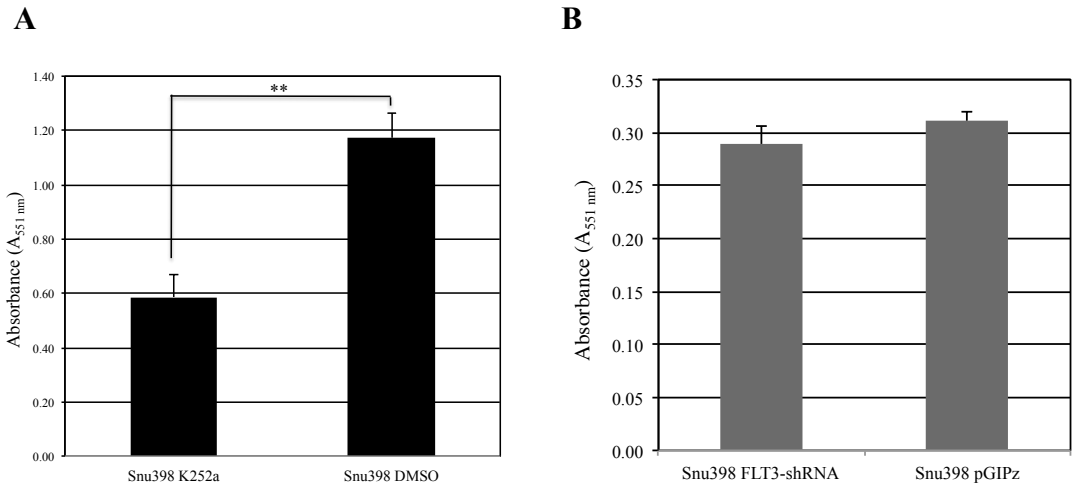


Figure 3.4. Effect of inhibitor treatment and FLT3 knockdown on *in vitro* proliferation of Snu398 cells

Proliferation rate of Snu398 cells measured by MTT assay following (A) either 2 hours of K-252a or DMSO treatment (**: p-value=0.0011) and (B) either FLT3 specific shRNA (Snu398 FLT3-shRNA) or empty vector (Snu398 pGIPz) transfection (p-value=0.1285). Measurements were done in triplicates.

Previously, we were able to demonstrate that migration capacity of Snu398 cells decreases following K-252a treatment under both normal and serum starvation conditions with wound healing assay [110]. Thus, we performed wound healing assay again under both normal (10% FBS) and serum starvation (2% FBS) conditions in order to see the effect of transfection on the migratory capacity of Snu398 cells. There was a significant reduction in the migration rate of Snu398 cells transfected with FLT3 specific shRNA compared to the control cells under both normal and serum starvation

conditions at the end of 48 hours (Figure 3.5; for normal medium *: p-value=0.0344 and for serum starvation *: p-value=0.0231). Representative images of the healed wounds are shown in Figure 7.1.

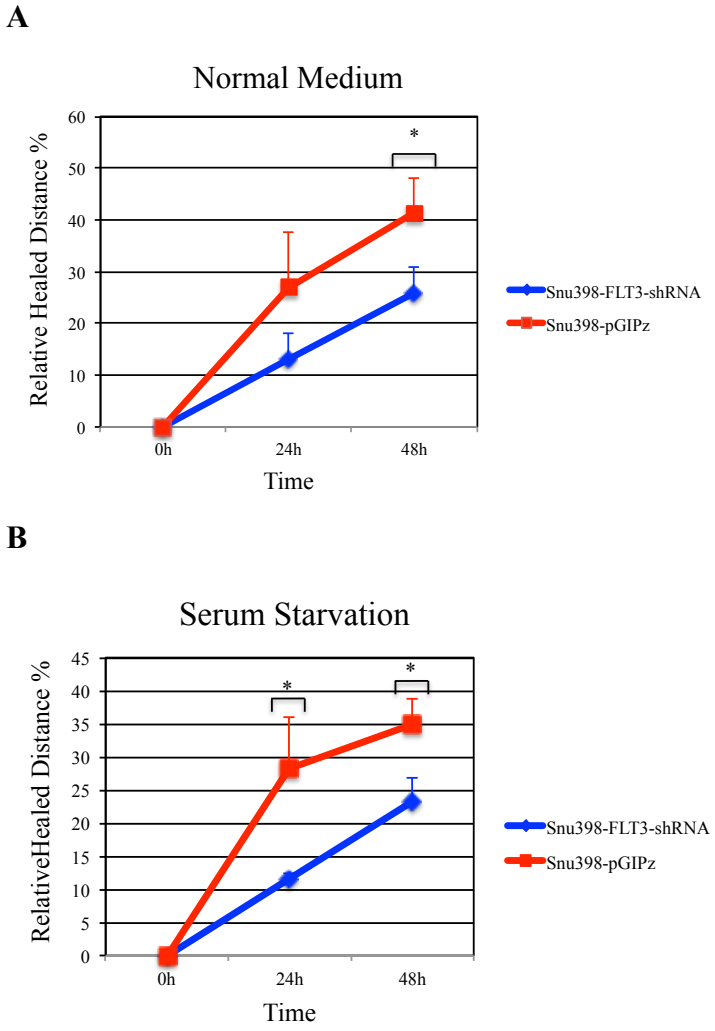


Figure 3.5. Effect of FLT3 knockdown on *in vitro* migration of Snu398 cells

Wound healing capacity of Snu398 cells analyzed by wound healing assay under (A) normal (10% FBS) and (B) serum starvation (2% FBS) conditions following FLT3 specific shRNA (Snu398 FLT3-shRNA; blue lines) and empty vector (Snu398 pGIPz; red lines) transfection at 0 hour, 24 hour and 48 hour time points (for normal medium 24 hour p-value=0.1161, 48 hour *: p-value=0.0344; for serum starvation 24 hour *: p-value=0.0231, 48 hour *: p-value=0.0192). Measurements were done in triplicates.

Finally, we also wanted to investigate the effect of K-252a treatment and FLT3 knockdown on the invasiveness of Snu398 cells by performing matrigel invasion

assay. Number of invaded cells through matrigel was significantly lower in the case of K-252a treatment compared to DMSO control (Figure 3.6; *: p-value=0.0411). Also, we observed a change in the number of colonies invaded following FLT3 shRNA transfection compared to empty vector control (Figure 3.6). Representative images of invaded colonies through matrigel are shown in Figure 7.2.

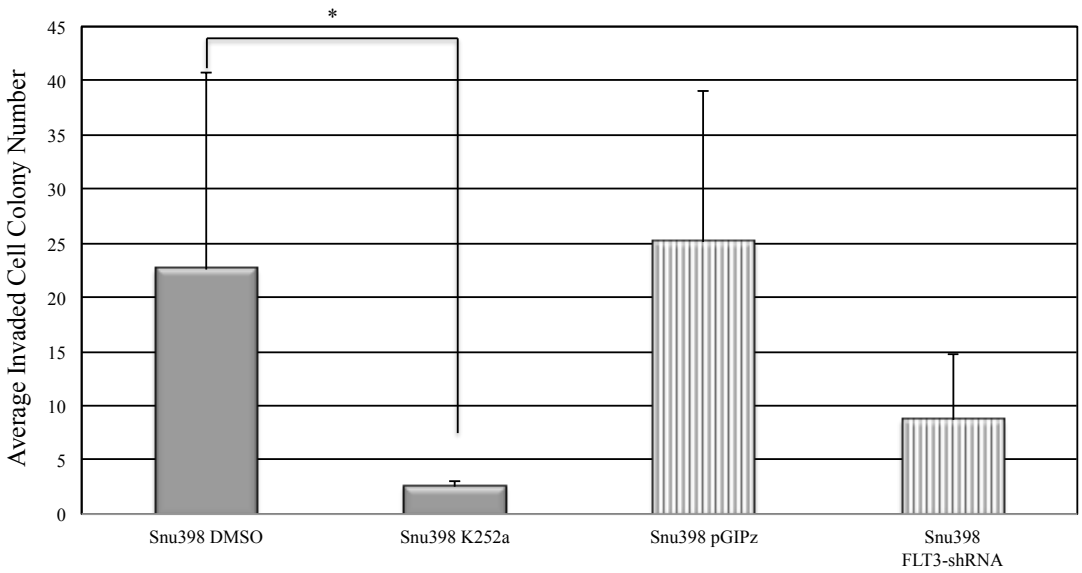


Figure 3.6. Effect of inhibitor treatment and FLT3 knockdown on *in vitro* invasion capacity of Snu398 cells

Invaded colonies following matrigel invasion assay in control medium (Snu398 DMSO), inhibitor-containing medium (Snu398 K-252a) (*: p-value=0.0411), following empty vector (Snu398 pGIPz) and FLT3 specific shRNA (Snu398 FLT3-shRNA) transfection (p-value=0.2680). Calculations were done in triplicates for K-252a and DMSO, and in duplicates for FLT3-shRNA and pGIPz.

Overall, these and our previous results show that inhibition of FLT3 activity with inhibitor leads to a significant diminution in the proliferative, migratory and invasive capacities of Snu398 cells *in vitro*. Additionally, FLT3 shRNA transfection of Snu398 cells results in a significant decrease in the *in vitro* wound healing capacity of these cells and changes in their proliferation rate and invasiveness.

3.1.4 Effect of FLT3 Knockdown in Snu398 Cells *in vivo*

In order to investigate if these *in vitro* effects of FLT3 knockdown are parallel to its *in vivo* effects, we decided to perform further experiments on nude mice. Previously, we injected K-252a and DMSO treated Snu398 cells to the different sides of the nude mice to generate tumor xenografts and showed that K-252a treatment results in the significant inhibition of tumor forming ability of Snu398 cells compared to DMSO control cells ($p<0.05$) [110]. Therefore, as a next step we generated tumor xenografts on nude mice by injecting FLT3 shRNA and empty vector transfected Snu398 cells to the opposite sides of the mouse (Figure 3.7A). After tumor xenograft generation, average volume of each tumor was calculated. Average tumor xenografts volume was found to be 48% lower in the case of FLT3 shRNA transfection compared to empty vector transfection (Figure 3.7C).

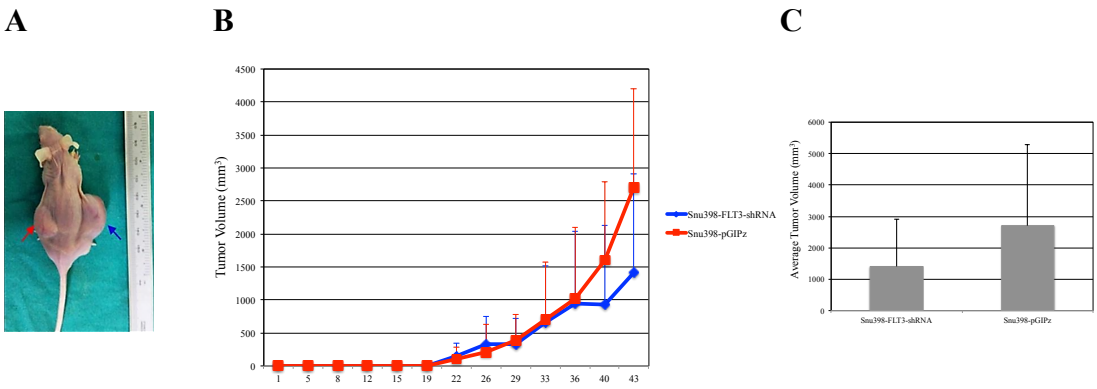


Figure 3.7. Effect of FLT3 knockdown on the *in vivo* tumorigenicity of Snu398 cells

(A) Representative image of a mouse injected with FLT3 specific shRNA transfected Snu398 cells (Snu398 FLT3-shRNA) or empty vector transfected Snu398 cells (Snu398 pGIPz). Blue arrow indicates empty vector and red arrow indicates FLT3-shRNA transfected cells' injection sites taken at the end of 43 days after injection. (B) Tumor growth curve of the tumors generated by injection of FLT3-shRNA and empty vector transfected Snu398 cells at different time points until 43 days. (C) Average tumor volumes of xenografts at the end of day 43 in the presence of either FLT3-shRNA or empty vector transfection (p-value=0.4953). Three mice were used per group.

Tumor xenografts were removed after the termination of experiment at day 43 and analyzed at molecular level by performing immunofluorescence staining to detect the expression of FLT3 and alpha smooth muscle actin (αSMA), which was used to show the invasiveness of cells. We observed fewer cells expressing FLT3 protein in tumor sections generated by injection of Snu398 cells transfected with FLT3 shRNA compared to the control tumor sections (Figure 3.8).

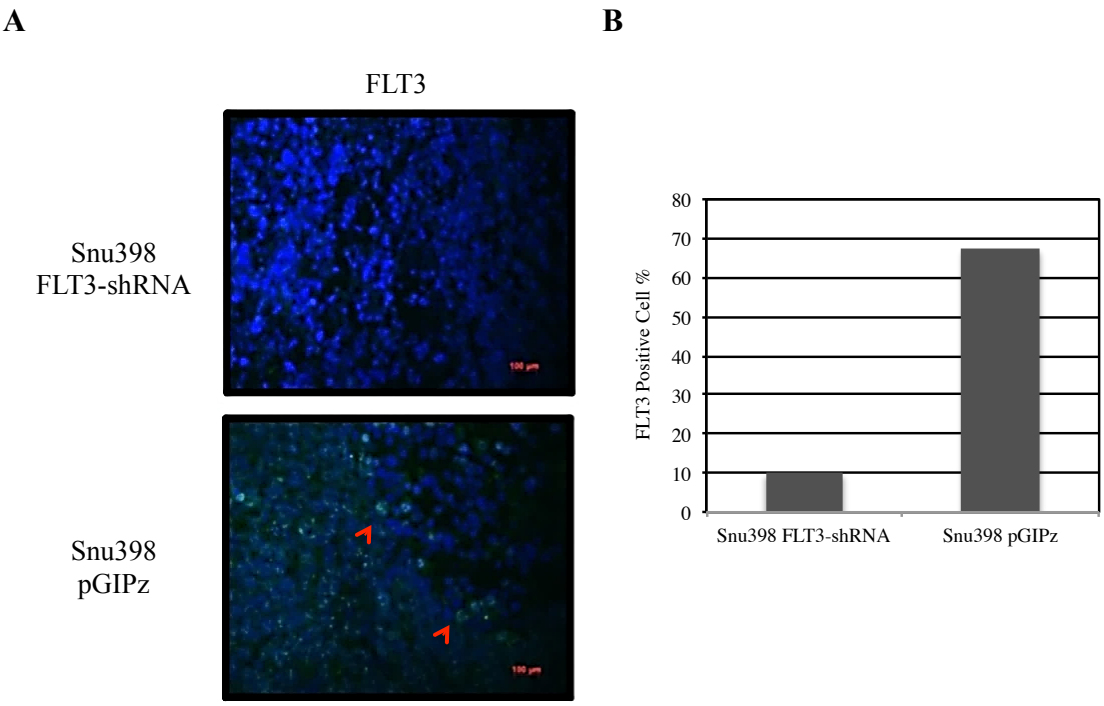


Figure 3.8. Effect of FLT3 knockdown on FLT3 expression in tumor sections generated by transfected Snu398 cells

(A) FITC labeled FLT3 protein expression in the nude mice tumor section generated by Snu398 cells transfected with either FLT3 shRNA (Snu398 FLT3-shRNA) or empty vector (Snu398 pGIPz); merged pictures of the sections with DAPI stained nuclei are shown and arrowheads denote FITC labeled FLT3 positive cells. Images were taken using 40X objective. (B) Quantification of FLT3 positive cells with ImageJ on a single immunofluorescence image.

Moreover, less number of cells was found to express αSMA in the tumor sections of Snu398 cells transfected with FLT3 shRNA compared to the controls suggesting less invasive potential of these cells (Figure 3.9).

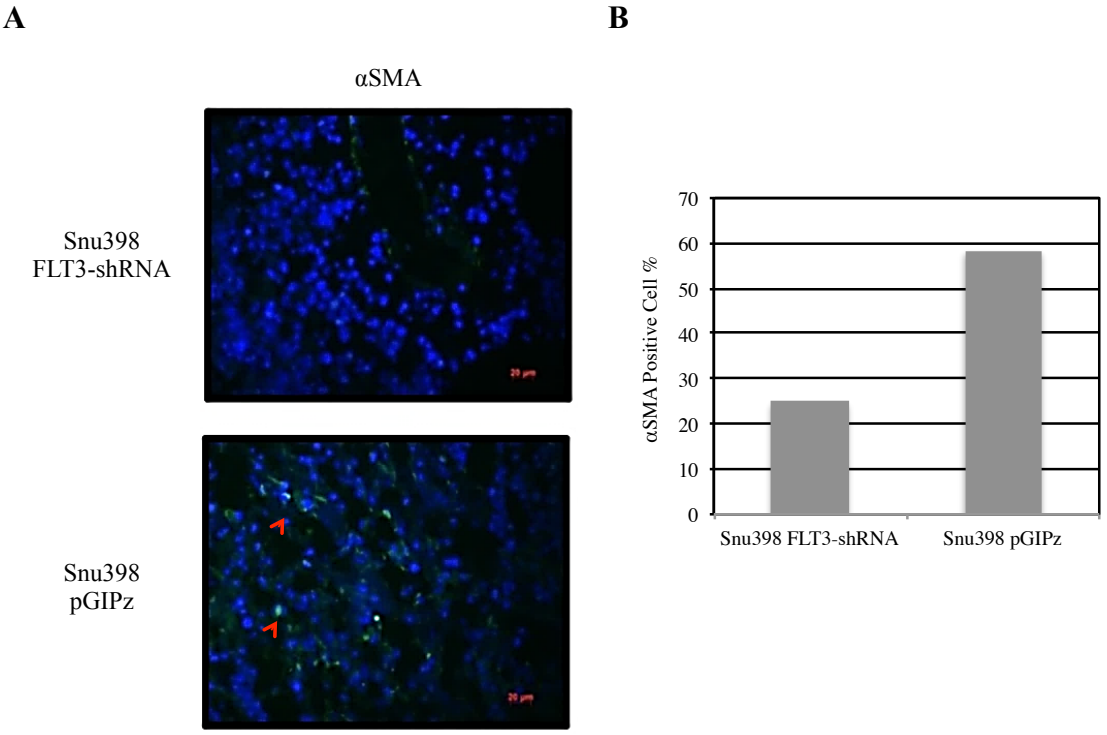


Figure 3.9. Effect of FLT3 knockdown on αSMA expression in tumor sections generated by transfected Snu398 cells

(A) FITC labeled αSMA protein expression in the nude mice tumor section generated by Snu398 cells transfected with either FLT3 shRNA (Snu398 FLT3-shRNA) or empty vector (Snu398 pGIPz); merged pictures of the sections with DAPI stained nuclei are shown and arrowheads denote FITC labeled αSMA positive cells. Images were taken using 40X objective. (B) Quantification of αSMA positive cells with ImageJ on a single immunofluorescence image.

To sum up, in addition to the inhibitory effect of FLT3 knockdown on the invasive capacity of Snu398 cells *in vitro*, we were able to show that this effect was also parallel *in vivo* following both shRNA transfection and inhibitor treatment.

3.2 Liver Fibrosis

3.2.1 Generation of *in vivo* Liver Fibrosis Model

In vivo mouse liver fibrosis model was generated by CCl_4 administration for 4 weeks. CCl_4 treatment resulted in the formation of visible fibrotic lesions on the liver, which are not present on the normal liver (Figure 3.10A vs. Figure 3.10B).

A **B**

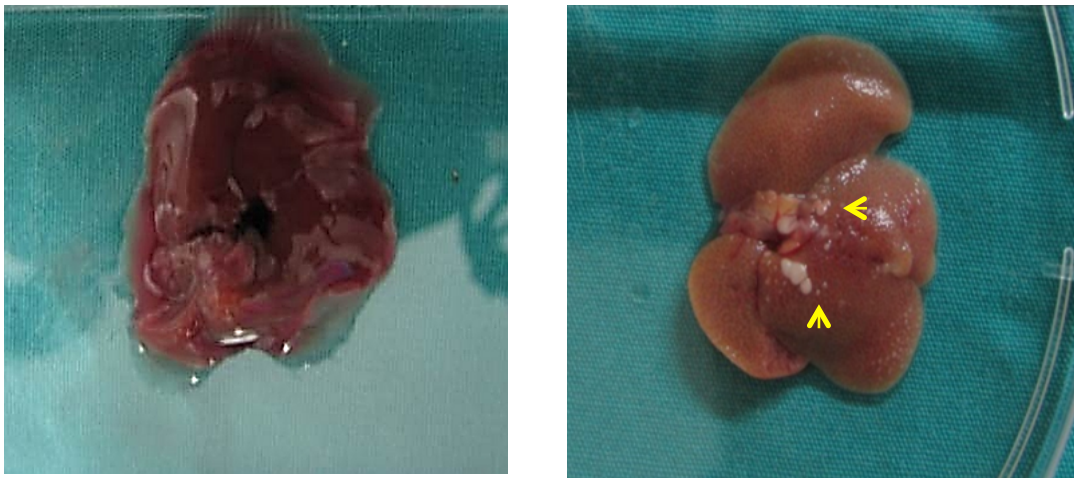


Figure 3.10. Generation of *in vivo* liver fibrosis model

Representative images of (A) normal liver (B) CCl_4 induced fibrotic liver. Arrowheads indicate fibrotic lesions.

Generation of liver fibrosis was confirmed by measuring serum levels of liver enzymes ALT and AST that are crucial for the functioning of liver. There was a significant increase in ALT level after the induction of liver fibrosis (Figure 3.11; *: p-value=0.0303).

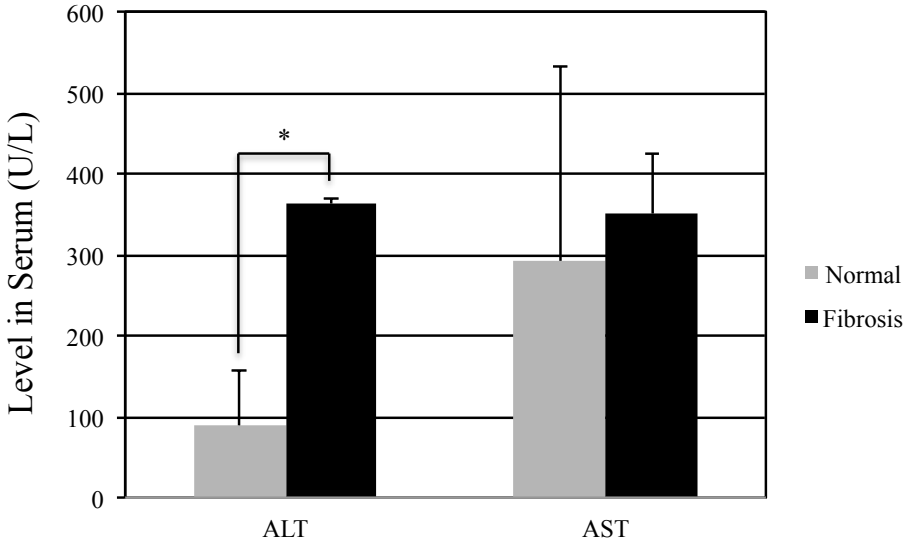


Figure 3.11. Serum ALT and AST levels of normal and fibrotic mice

Level of liver enzymes ALT and AST in the serum. Three mice were used per group. (ALT *: p-value=0.0303 and AST p-value=0.7719).

Moreover, we examined the changes in αSMA expression in the liver since it is a well-known and commonly used marker for the detection of liver fibrosis (Figure 3.12).

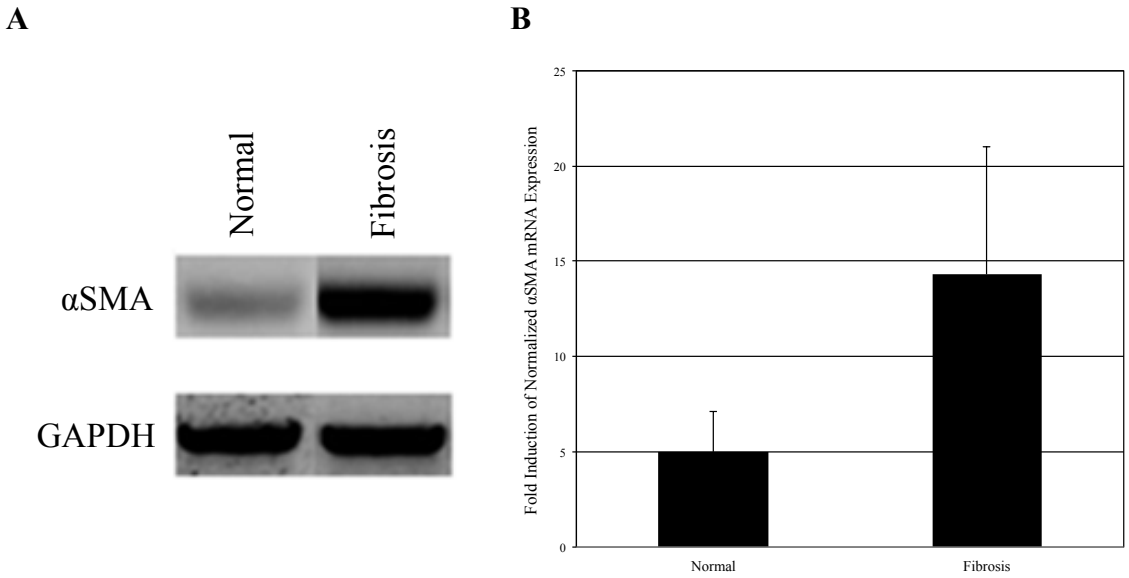


Figure 3.12. Expression of *αSMA* mRNA in the liver before and after fibrosis induction

(A) *αSMA* mRNA levels in the normal and fibrotic liver detected by RT-PCR. *GAPDH* was used as loading control. (B) Quantification of *αSMA* mRNA expression by ImageJ. Three mice were used per group (p-value=0.0835).

Additionally, we also examined the αSMA protein expression in both normal and fibrotic livers by performing immunofluorescence staining and Western blotting (Figure 3.13).

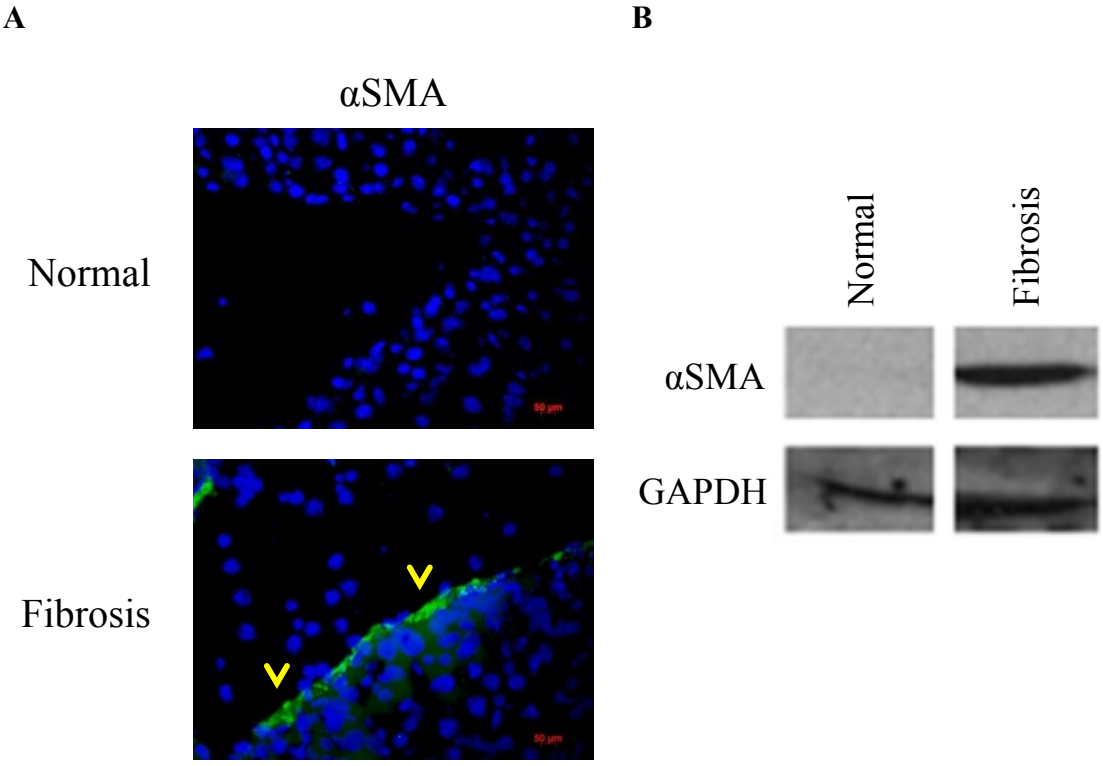


Figure 3.13. Expression of αSMA protein in the liver before and after fibrosis induction

(A) FITC labeled αSMA protein expression in the liver section of normal and fibrotic mice; merged pictures of the sections with DAPI stained nuclei are shown. Arrowheads indicate αSMA positivity. Images were taken using 40X objective. (B) αSMA protein levels in the normal and fibrotic liver detected by Western blot. GAPDH was used as loading control.

Overall, we observed changes in αSMA expression both at mRNA and protein levels in the fibrotic liver confirming the validity of this CCl_4 induced liver fibrosis model.

3.2.2 TLR Expression in the CCl_4-induced Fibrotic Liver

We performed RT-PCR in order to see the changes in the expression of *TLRs (TLR1-9)* following fibrosis induction with CCl_4 compared to the normal liver. Our findings showed that expression of *TLR1*, *TLR3*, *TLR6*, *TLR8* and *TLR9* at the mRNA level significantly increases in the liver after the initiation of fibrotic response (Figure 3.14; for TLR1 *: p-value=0.0119; for TLR3 *: p-value=0.0487; for TLR6 *: p-value=0.0232; for TLR8 *: p-value=0.0477; for TLR9 *: p-value=0.0293).

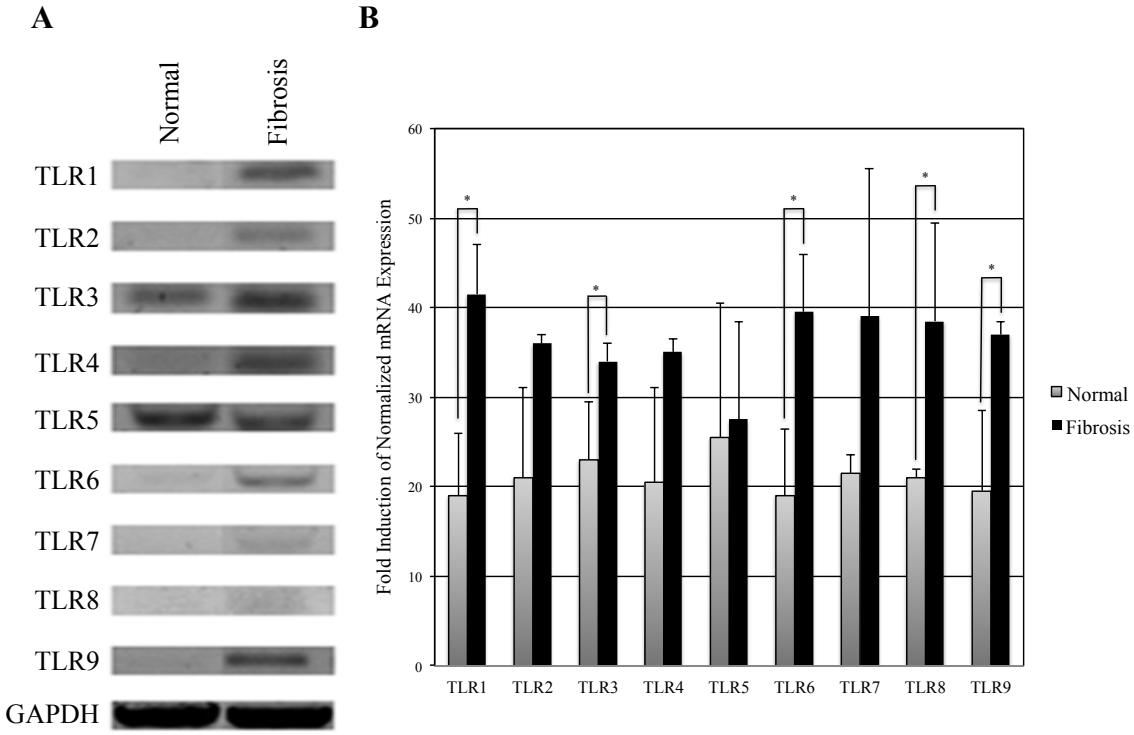


Figure 3.14. mRNA expression of *TLRs* in the liver before and after fibrosis induction

(A) RT-PCR results showing the mRNA expression of *TLRs (TLR1-9)* in the normal and fibrotic livers. *GAPDH* was used as loading control. (B) Quantification of mRNA expression of *TLRs* by ImageJ. Three mice were used per group (TLR1 *: p-value=0.0119; TLR2 p-value=0.0610; TLR3 *: p-value=0.0487; TLR4 p-value=0.0770; TLR5 p-value=0.8613; TLR6 *: p-value=0.0232; TLR7 p-value=0.1423; TLR8 *: p-value=0.0477; TLR9 *: p-value=0.0293).

3.2.3 Effect of Suppressive ODN A151 on Liver Fibrosis

3.2.3.1 Inhibitory Effect of Suppressive ODN A151 on Liver Fibrosis Progression

In order to investigate the role of A151, a suppressive ODN, on the progression of liver fibrosis we generated four treatment groups (G1-G4; Table 3.1). All mice were age matched. Group G1 mice were normal. Group G2 mice were injected with CCl_4 for 4 weeks to induce fibrosis. Mice in groups G3 and G4 also received CCl_4 for 4 weeks; additionally, group G3 mice were injected with suppressive ODN A151 one week after fibrosis induction for one week, whereas group G4 mice received A151 directly after fibrosis induction for one week in order to see the effect of suppressive ODN A151 administration at different time points following fibrosis initiation. Three mice were used per group (Figure 3.15).

Table 3.1: Treatment groups used to investigate the effect of suppressive ODN A151 on liver fibrosis

Group 1 (G1)	Normal; no treatment
Group 2 (G2)	Fibrosis; 4 weeks CCl_4 injection
Group 3 (G3)	Fibrosis; 4 weeks CCl_4 injection + 1 week no treatment + 1 week A151 treatment
Group 4 (G4)	Fibrosis; 4 weeks CCl_4 injection + 1 week A151 treatment

Severity of fibrosis in all groups was analyzed after the termination of treatments. Fibrotic lesions were visible on the livers of all fibrosis groups (G2-G4) (Figure 3.16B-D), but suppressive ODN A151 treatment (G3 and G4) resulted in less intense lesion formation compared to only CCl_4 administration (G2) (Figure 3.16C and Figure 3.16D vs. Figure 3.16B).

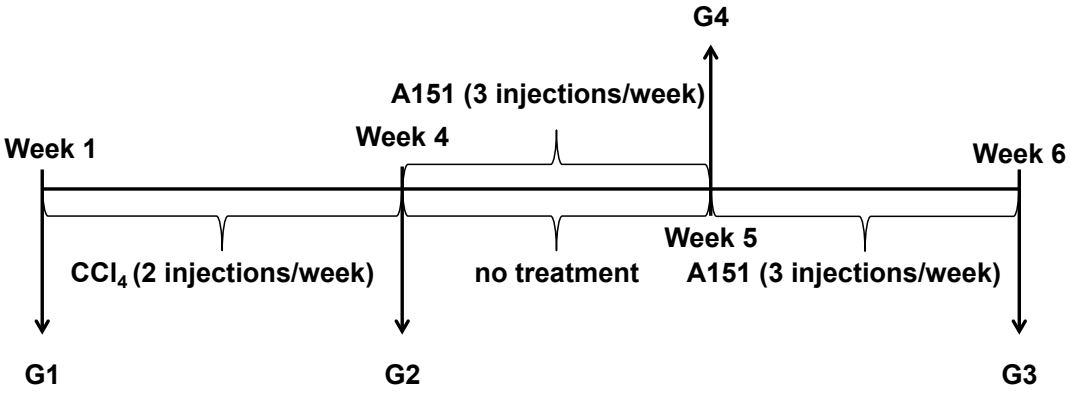


Figure 3.15. Timeline of treatments used to identify the effect of suppressive ODN A151 on liver fibrosis

(G1: normal, G2: 4 weeks CCl_4 injection, G3: 4 weeks CCl_4 injection + 1 week no treatment + 1 week A151 treatment, and G4: 4 weeks CCl_4 injection + 1 week A151 treatment). Three mice were used per group.

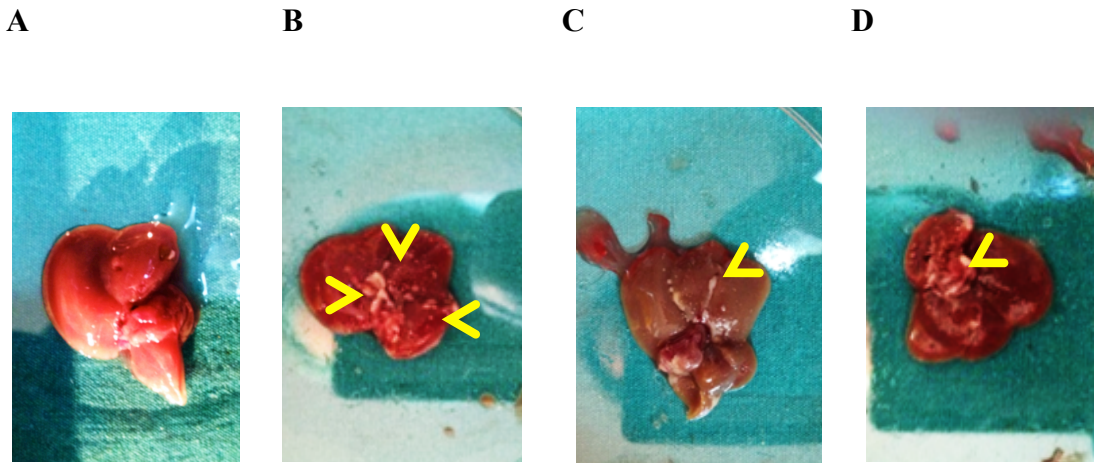
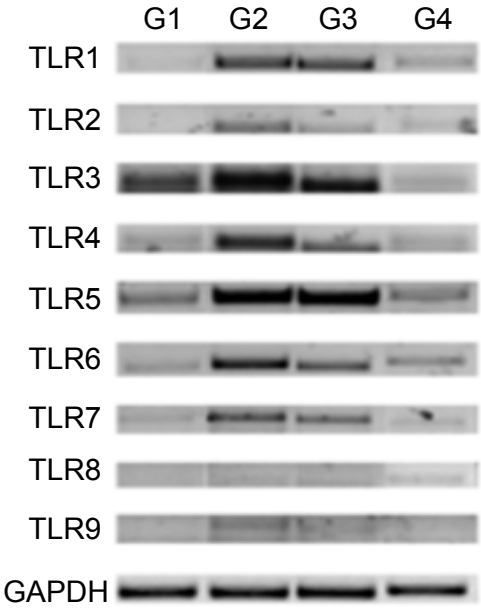


Figure 3.16. Representative liver images of each group generated to investigate the effect of suppressive A151 ODN on liver fibrosis

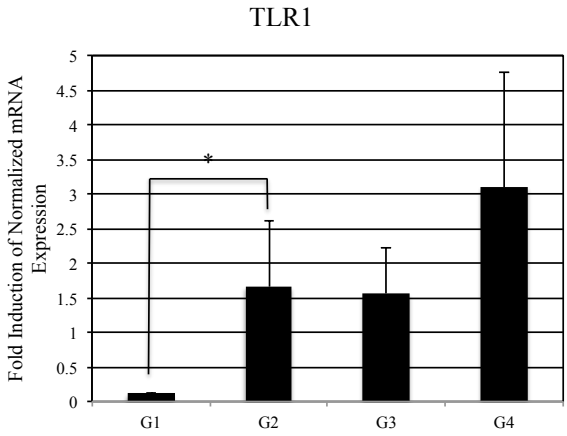
Livers taken from (A) G1 (normal), (B) G2 (4 weeks CCl_4 injection), (C) G3 (4 weeks CCl_4 injection + 1 week no treatment + 1 week A151 treatment), and (D) G4 (4 weeks CCl_4 injection + 1 week A151 treatment) groups mice. Arrowheads indicate fibrotic lesions.

Initially, we wanted to analyze the potential suppressive effect of A151 on the expression of TLRs following A151 administration and observed a significant diminution in *TLR4* (**: p-value=0.0013), and *TLR9* (*: p-value=0.0375) mRNA expression in G4 livers (Figure 3.17E and Figure 3.17J; G2 vs. G4).

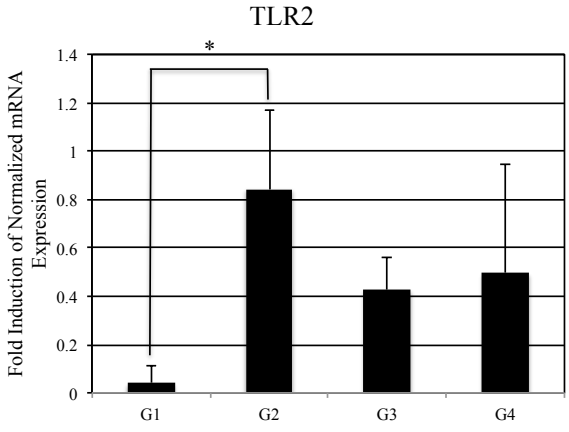
A


G1
G2
G3
G4
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
GAPDH

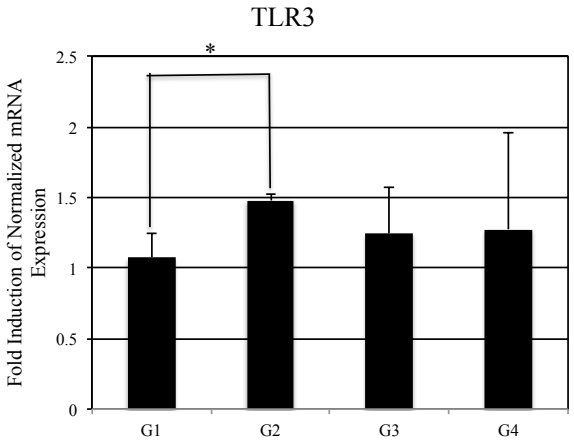
B


TLR1
Fold Induction of Normalized mRNA Expression
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
G1
G2
G3
G4

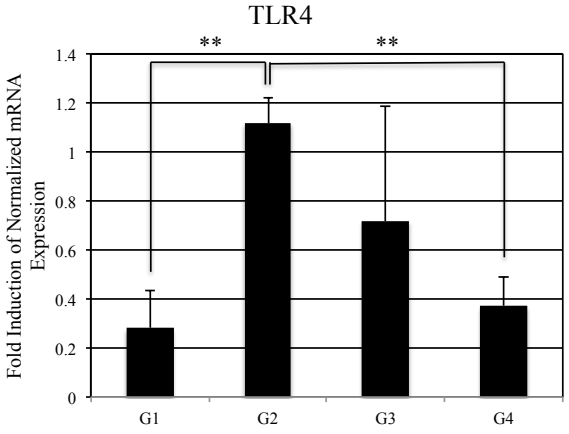
C


TLR2
Fold Induction of Normalized mRNA Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
G1
G2
G3
G4

D


TLR3
*
Fold Induction of Normalized mRNA Expression
2.5
2
1.5
1
0.5
0
G1
G2
G3
G4

E


TLR4
**
**
Fold Induction of Normalized mRNA Expression
1.4
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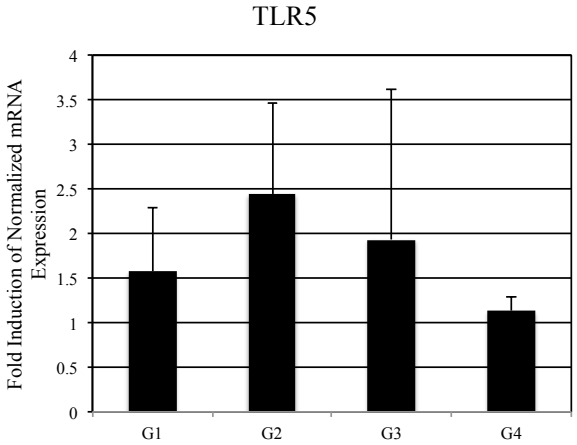
F


TLR5
Fold Induction of Normalized mRNA Expression
4
3.5
3
2.5
2
1.5
1
0.5
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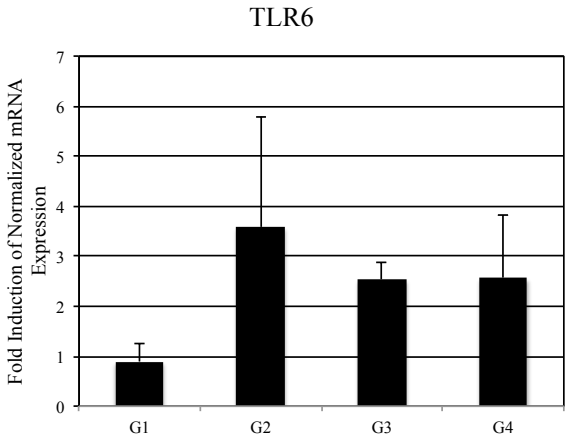
G1
G2
G3
G4

G


TLR6
Fold Induction of Normalized mRNA Expression
0
1
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3
4
5
6
7
G1
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G4

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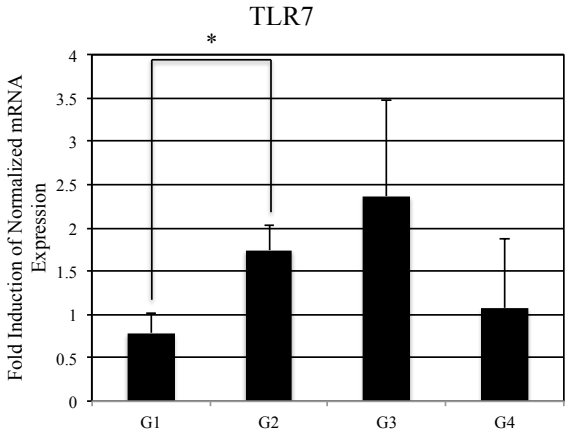
TLR7
*
Fold Induction of Normalized mRNA Expression
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0.5
1
1.5
2
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4
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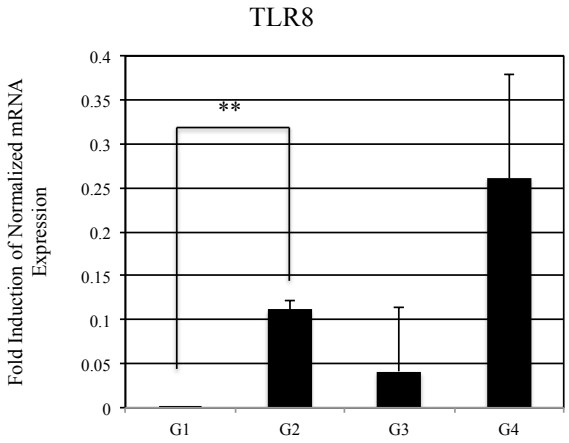
G3
G4

I


TLR8
**
Fold Induction of Normalized mRNA Expression
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0.05
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0.15
0.2
0.25
0.3
0.35
0.4
G1
G2
G3
G4

J

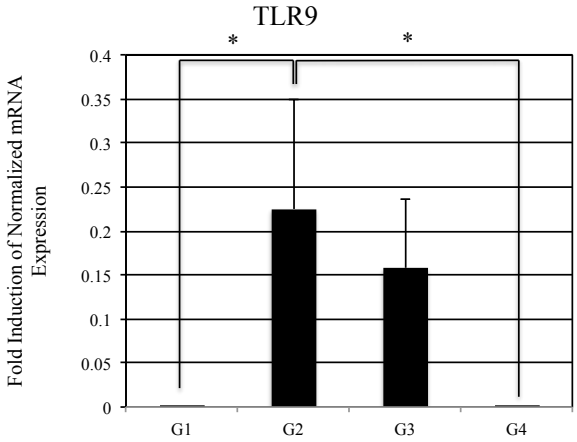


Figure 3.17. Effect of A151 treatment on *TLR* mRNA expression

(A) *TLR* mRNA expression in livers of mice in all treatment groups detected by RT-PCR; *GAPDH* was used as loading control (G1: normal, G2: 4 weeks CCl_4 injection, G3: 4 weeks CCl_4 injection + 1 week no treatment + 1 week A151 treatment, and G4: 4 weeks CCl_4 injection + 1 week A151 treatment). Three mice were used per group for all TLRs. (B) Quantification of *TLR1* mRNA expression by ImageJ (G1 vs. G2 *: p-value=0.0499; G2 vs. G3 p-value=0.8780; G2 vs. G4 p-value=0.2661). (C) Quantification of *TLR2* mRNA expression by ImageJ (G1 vs. G2 *: p-value=0.0148; G2 vs. G3 p-value=0.1158; G2 vs. G4 p-value=0.3384). (D) Quantification of *TLR3* mRNA expression by ImageJ (G1 vs. G2 *: p-value=0.0183; G2 vs. G3 p-value=0.2793; G2 vs. G4 p-value=0.6378). (E) Quantification of *TLR4* mRNA expression by ImageJ (G1 vs. G2 **: p-value=0.0013; G2 vs. G3 p-value=0.2325; G2 vs. G4 **: p-value=0.0012). (F) Quantification of *TLR5* mRNA expression by ImageJ (G1 vs. G2 p-value=0.2939; G2 vs. G3 p-value=0.6777; G2 vs. G4 p-value=0.0926). (G) Quantification of *TLR6* mRNA expression by ImageJ (G1 vs. G2 p-value=0.1059; G2 vs. G3 p-value=0.4617; G2 vs. G4 p-value=0.5264). (H) Quantification of *TLR7* mRNA expression by ImageJ (G1 vs. G2 *: p-value=0.0109; G2 vs. G3 p-value=0.3986; G2 vs. G4 p-value=0.2443). (I) Quantification of *TLR8* mRNA expression by ImageJ (G1 vs. G2 **: p-value=0.0001; G2 vs. G3 p-value=0.1616; G2 vs. G4 p-value=0.0972). (J) Quantification of *TLR9* mRNA expression by ImageJ (G1 vs. G2 *: p-value=0.0375; G2 vs. G3 p-value=0.4715; G2 vs. G4 p-value=0.0375).

Next, we also checked the αSMA protein expression in the liver of all group animals by performing Western blotting since it is an established marker for liver fibrosis (Figure 3.18).

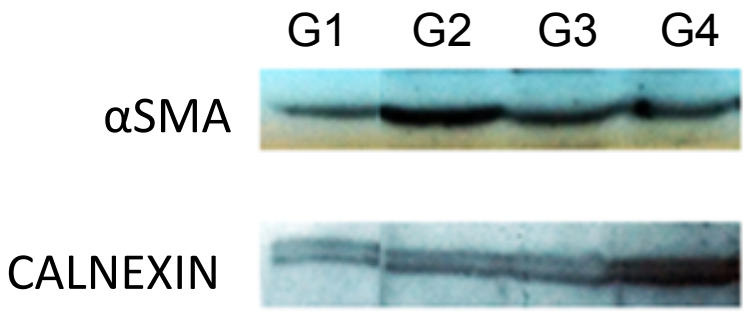


Figure 3.18. Effect of A151 treatment on αSMA protein expression

αSMA protein expression in livers of all treatment groups detected by Western blotting; CALNEXIN was used as loading control (G1: normal, G2: 4 weeks CCl_4 injection, G3: 4 weeks CCl_4 injection + 1 week no treatment + 1 week A151 treatment, and G4: 4 weeks CCl_4 injection + 1 week A151 treatment).

In addition to these, we performed αSMA immunofluorescence staining and observed less positivity in G3 and G4 liver sections compared to G2 (Figure 3.19A). Moreover, we performed Picrosirius red collagen staining in order to see the collagen accumulation in the liver, which is another common way of showing the severity of liver fibrosis. Supporting our previous results about the liver fibrosis model, there was more intense collagen staining in the livers of fibrotic group G2 mice, which was less following A151 treatment at different time points (Figure 3.19B). We also measured the collagen levels with Sirius red/Fast green assay in these liver tissue sections and found a significant decrease in both G3 and G4 livers compared to G2 (Figure 3.19C; G1 vs. G2 *: p-value=0.0138; G2 vs. G3 *: p-value=0.0211; G2 vs. G4 *: p-value=0.0184).

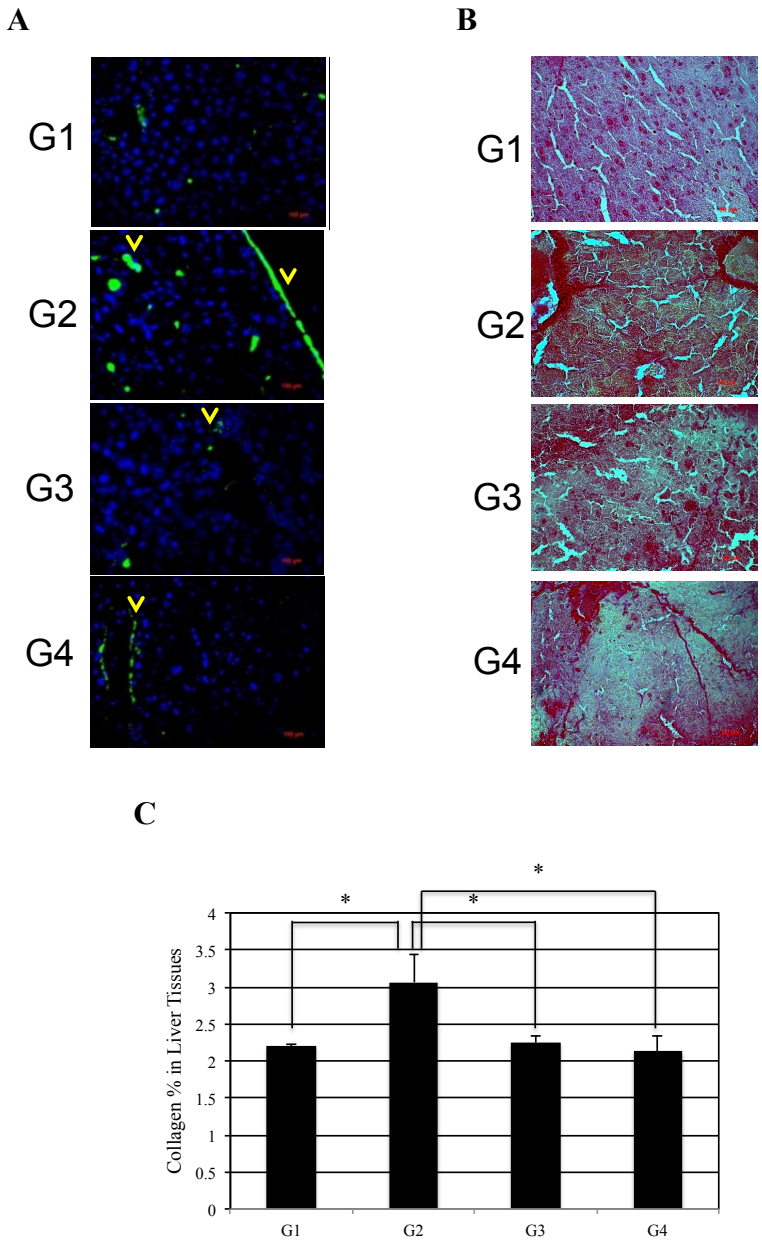


Figure 3.19. Effect of A151 treatment on αSMA protein expression and collagen accumulation in the liver

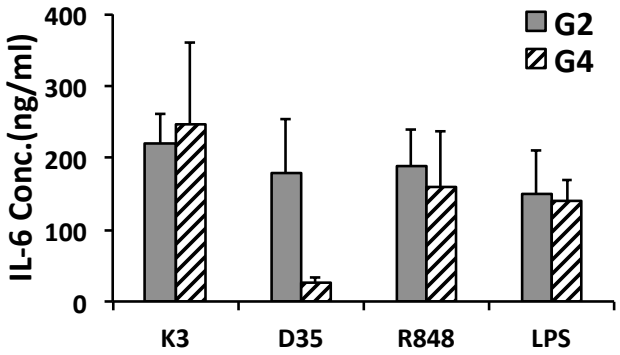
(A) αSMA protein accumulation in livers of all treatment groups shown by immunofluorescence staining; FITC labeled αSMA protein expression in the liver sections of mice in each treatment group; merged pictures of the sections with DAPI stained nuclei are shown and yellow arrowheads indicate αSMA staining. All images are taken using 40X objective. (B) Collagen accumulation detected by Picrosirius staining. All images are taken using 40X objective. (C) Quantification of collagen accumulation in the liver with Sirius red/Fast green staining. Three mice were used per group (G1: normal, G2: 4 weeks CCl_4 injection, G3: 4 weeks CCl_4 injection + 1 week no treatment + 1 week A151 treatment, and G4: 4 weeks CCl_4 injection + 1 week A151 treatment), (G1 vs. G2 *: p-value=0.0138; G2 vs. G3 *: p-value=0.0211; G2 vs. G4 *: p-value=*0.0184).

3.2.3.2 Systemic Effect of Suppressive ODN A151

Previous studies showed that suppressive A151 ODN results in the *in vivo* downregulation of both TLR dependent and independent proinflammatory immune responses [175, 185, 236, 237]. Even though our findings suggest that there is a local immune suppression in the fibrotic liver following A151 administration, we also wanted to examine the systemic effect that could help reversing the fibrosis progression in the liver. Thus, we analyzed the changes in IL-6 and IL-12 secretion profiles of splenocytes. After the isolation of splenocytes from the spleens of animals in all four groups, we tested the effect of four different TLR ligands; K3 (TLR9L, B or K-type CpG ODN), D35 (TLR9L, A or D-type CpG ODN), R848 (TLR7L), and LPS (TLR4L), on IL-6 and IL-12 secretion from these cells.

Since the suppressive activity of A151 was more effective in group G4, we decided to continue analyzing its systemic effect by comparing groups G2 and G4. G2 and G4 splenocytes stimulated with TLR4L, TLR7L, and TLR9L showed similar patterns in IL-6 secretion (Figure 3.20A). On the other hand, IL-12 secretion by G2 splenocytes after K3, R848, and LPS stimulation increased dramatically, which then significantly decreased following A151 administration (Figure 3.20B; G2 vs. G4; for K3 *: p-value=0.0117, for R848 *: p-value=0.0187, for LPS *: p-value=0.0186). Additionally, we observed a change in IL-12 secretion following stimulation of splenocytes with D35 between G2 and G4. Overall, our results suggest that A151 has a systemic effect on the suppression of PAMP-associated immune response evidenced by changes in cytokine secretion profiles upon A151 administration following fibrosis induction.

A



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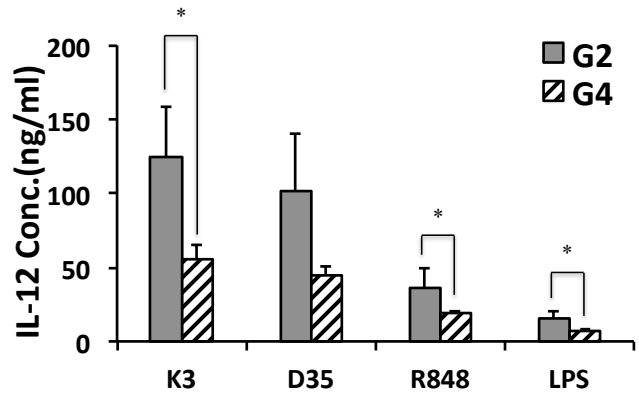

Figure 3.20. Effect of A151 treatment on IL-6 and IL-12 secretion in response to TLR ligands

Levels of (A) IL-6 and (B) IL-12 secreted by splenocytes in response to stimulation with K3 and D35: TLR9L, R848: TLR7L and LPS: TLR4L shown by ELISA analysis of 36 hours culture supernatants. Three mice were used per group (G2: 4 weeks CCl_4 injection and G4: 4 weeks CCl_4 injection + 1 week A151 treatment), (IL-6; K3 p-value=0.4160, D35 p-value=0.3042, R848 p-value=0.7273, LPS p-value=0.9232), (IL-12; K3 *: p-value=0.0117, D35 p-value=0.1007, R848 *: p-value=0.0187, LPS *: p-value=0.0186).

3.2.3.3 Effect of Suppressive ODN A151 on APC Function

In addition to unraveling the systemic effect of suppressive A151 ODN in liver fibrosis, we also aimed to investigate the changes in the expression of critical surface markers that are capable of contributing tissue destruction process, such as CD54, CD69, CD80, and MHC-II. According to our flow cytometry data, A151

administration resulted in a significant reduction in CD54, and MHC-II positive cell percent in G4 splenocytes compared to G4 (Figure 3.21). Changes were approximately 8.3% for CD54 (20.7±1.6 vs. 12.4±1.1; **: p-value=0.0018), 4.3% for CD80 (14.7±3.1 vs. 10.6±1.8), and 11.2% for MHC-II (17.9±2.9 vs. 6.7±1.9; **: p-value=0.005) (Figure 3.21A and Figure 3.21C). Moreover, we were able to show a change from 14.5% to 8.4% in CD54-CD69 double positivity after A151 treatment (Figure 3.21B). These results suggest that A151 administration causes a downregulation in T-cell priming by APCs resulting in the prevention of liver fibrosis progression and an increase in liver regeneration induction.

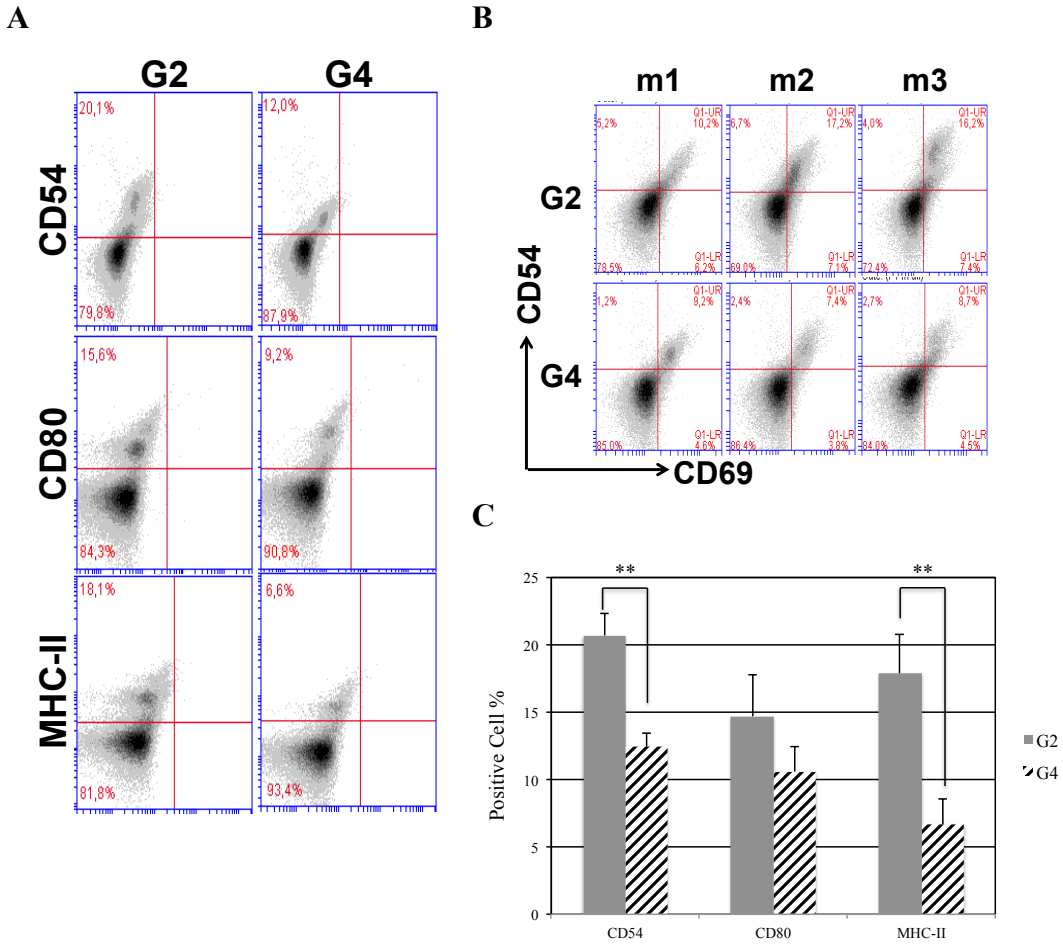

Figure 3.21. Effect of A151 treatment on APC function

Surface marker expression following A151 treatment seen on plots showing (A) MHC-II, CD54, and CD80 positive cells and (B) CD54-CD69 double positivity. (C) Comparative percent positivity of CD54, CD80, and MHC-II positive cells between fibrotic (G2: 4 weeks CCl_4 injection) and A151 treated (G4: 4 weeks CCl_4 injection + 1 week A151 treatment) animals. Three mice were used per group (CD54 **: p-value=0.0018; CD80 p-value=0.1187; MHC-II **: p-value=0.005).

3.2.4 Heat Shock Proteins in Liver Fibrosis

Our previous results were able to support the findings of different studies on the contribution of TLRs to liver fibrosis progression and present novel approaches for this field. In addition to TLRs and their potential effects on liver fibrosis, we also focused on HSPs, another family of proteins with vital functions in maintaining cellular metabolism. According to the previous studies, both TLR2 and TLR4 are known to respond certain HSPs, such as HSP60 and HSP70 [238, 239]. Thus, we checked whether there is a change in the expression profile of HSP70 in the fibrotic liver. In our experimental CCl_4 induced liver fibrosis model, there was no difference in the expression of HSP70 between normal and fibrotic livers (Figure 3.22).

Next, we aimed to investigate other members of this family, to see if they have any contribution to liver fibrosis. The reason for this was because HSPs are known to have a wide range of roles in different cellular events under stress conditions, especially in regulating intracellular environment. Previous studies showed that HSP27 is an essential member of this family having different roles in various cancer types, especially in breast cancer [228]. Moreover, it was reported to contribute to the organization and rearrangement of cytoskeletal elements, such as intermediate filaments inside the cell [207, 208]. Since liver fibrosis is an event that involves various cytoskeletal rearrangement events ongoing inside the cell at different stages and under different environmental conditions, we hypothesized that HSP27 is a potential candidate for the regulation and progression of liver fibrosis.

3.2.4.1 Expression of Heat Shock Proteins in the Fibrotic Liver

After inducing liver fibrosis with CCl_4 in mice and confirming its validity with changes in αSMA expression both at mRNA and protein levels, we checked if there is a change in the expression of HSP27. According to our results, we observed a change in HSP27 mRNA and protein expression (Figure 3.22 and Figure 3.23). Thus, we aimed to continue investigating the potential contribution of HSP27 to liver fibrosis progression.

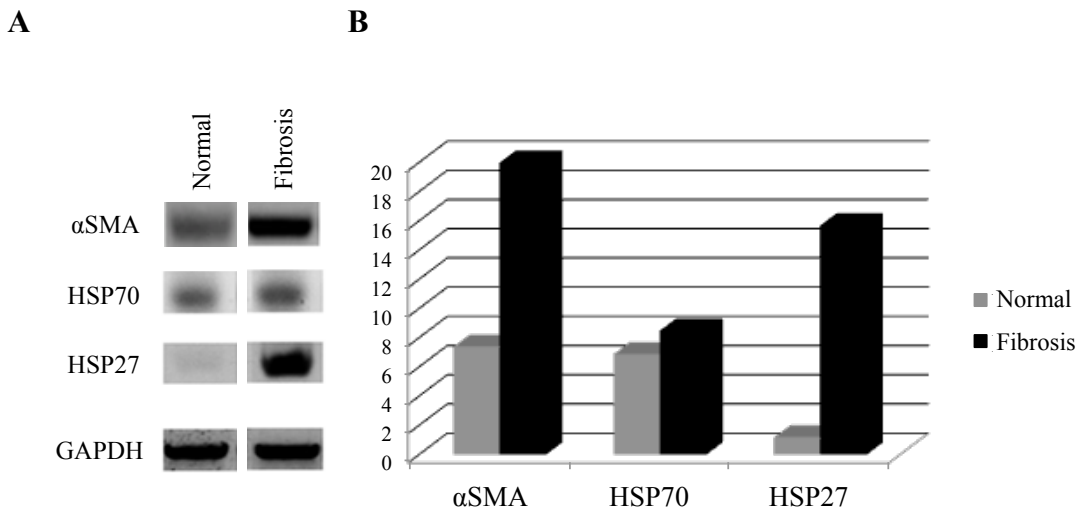

Figure 3.22. mRNA expression of *HSPs* in the fibrotic liver

(A) Expression of *αSMA*, *HSP70*, and *HSP27* mRNA in the normal and fibrotic liver detected by RT-PCR. *GAPDH* was used as loading control. (B) Quantification of *αSMA*, *HSP70*, and *HSP27* mRNA expression by ImageJ on a single gel image.

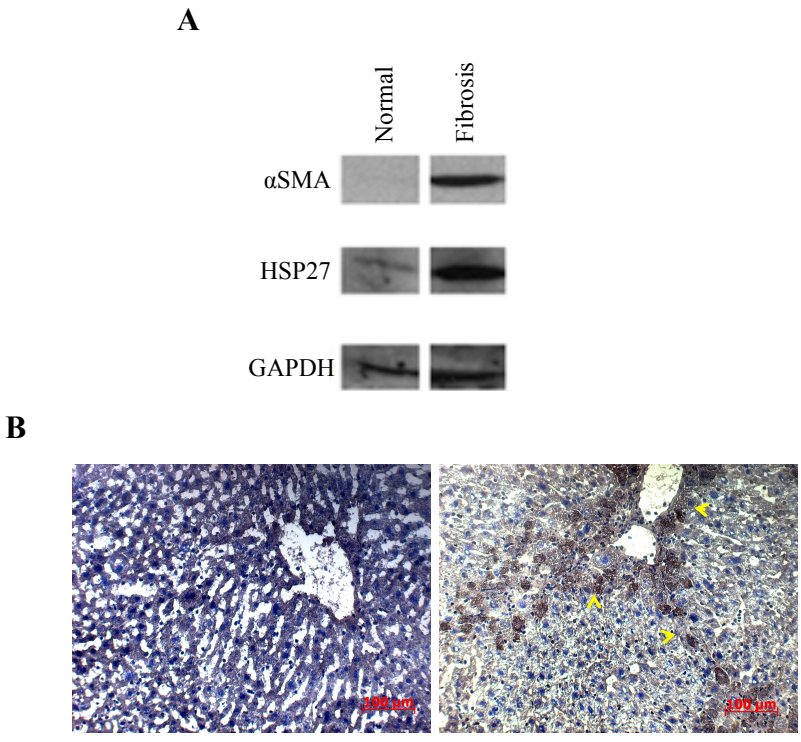

Figure 3.23. HSP27 protein expression in the fibrotic liver

(A) Protein expression of αSMA and HSP27 in the normal and fibrotic livers detected by Western blot. GAPDH was used as loading control. (B) HSP27 expression in the normal (on the left) and fibrotic liver (on the right) sections showed by immunohistochemistry staining. Arrowheads indicate HSP27 positive regions in the fibrotic liver section. Images were taken using 20X objective.

3.2.4.2 Effect of HSP27 Inhibition on the Progression of Liver Fibrosis

Quercetin is a well-known flavonoid that is found in plants and used in the clinics for many different purposes due to its antihistaminic, antioxidant, and anti-inflammatory properties. In addition to these, quercetin was reported to inhibit the expression of HSP27, and its clinical use was suggested to be an important therapeutic approach for breast cancer patients [228].

In our study, mice received quercetin for one week after the completion of CCl_4 administration, thus fibrosis induction. In addition to a change in HSP27 expression, we also observed a difference in αSMA expression after quercetin treatment following fibrosis induction (Figure 3.24).

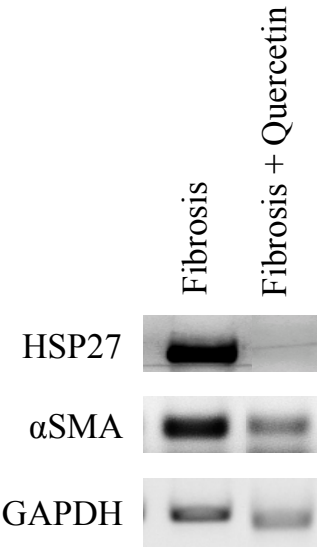

Figure 3.24. Effect of quercetin administration on *αSMA* and *HSP27* expression in the fibrotic liver

RT-PCR results showing *αSMA* and *HSP27* mRNA expression in the fibrotic liver before and after one week quercetin treatment. *GAPDH* was used as loading control.

Overall, our preliminary findings about the role of HSP27 in liver fibrosis suggest that use of quercetin might be a potential and a novel therapeutic approach to the prevention of liver fibrosis progression, and even its regression. Therefore, further research should be conducted on the potential role of HSP27 in liver fibrosis to clarify this hypothesis.

Chapter 4

4 Discussion

For many years, both medical and biological studies have been conducted on understanding the complex nature of the liver using divergent approaches. What makes this area of research very fertile is the organized cooperation of complicated mechanisms lying beneath the heterogeneous structure of the liver. Indeed, the curiosity of researchers about understanding the liver has started in ancient times with a Greek legend of Prometheus. According to this legend, Prometheus was punished by Zeus and chained to a rock to make his liver eaten by an eagle everyday, but his liver was regenerating at night time [240].

Liver is vital for its wide range of metabolic functions, such as drug detoxification, production of hormones and plasma proteins, glycogen storage and many more. Thus, because of its excessive burden, changing environmental conditions have been impairing the natural processing of the liver more compared to most of the other organs in the body. In spite of the medical and scientific developments and its regenerative capacity, liver failure has been more widespread in the recent years. With increasing number of liver failure cases, number of the patients waiting for liver transplantation increases everyday.

HCC is known to be responsible for most of the deaths caused by cancer worldwide [52]. Even though the science and technology advances, since environmental conditions become worse, the tolerability of the liver against external factors decreases. Liver fibrosis is an initial response generated against these risk

factors in order to help liver to fight and regenerate. However, due to shortage of studies on liver fibrosis, it is not highly possible to prevent its progression into cirrhosis and ending up with liver cancer yet. On the other hand, HCC has been more focus of interest in the last decades. However, since there are various potential causes of HCC and it progresses through different stages, most of the findings have been restricted to certain individual cases [53].

This study mainly focused on presenting potential therapeutic approaches to serve two vital liver related problems, liver cancer and fibrosis. According to our previous findings, FLT3, a RTK, was suggested to contribute liver regeneration and has a potential role in hepatocellular carcinogenesis [107, 110]. Moreover, since FLT3 is expressed in hematopoietic stem cells, which are well known for their contribution to liver development, our hypothesis about the potential role of FLT3 in hepatocellular carcinogenesis becomes more substantial. In addition to these, findings suggest that FLT3 and other tyrosine kinases are involved in the development of malignancies, especially in leukemias such as AML, ALL, CLL, and CML [94, 95]. Therefore, in the first part of our study we aimed to continue examining the potential role of FLT3 in the invasiveness and aggressiveness of HCC and present a therapeutic approach to find a candidate for FLT3 inhibition, thus HCC progression.

In our previous study about the potential role of FLT3 in hepatocellular carcinogenesis, we were able to show that K-252a, a RTK inhibitor, impairs the invasive ability of HCC cells acting through FLT3 signaling [110]. In order to confirm the effect of K-252a on FLT3 and contribution of FLT3 in hepatocellular carcinogenesis, we decided to investigate both *in vitro* and *in vivo* changes in HCC cells after silencing FLT3.

Initially, we investigated the expression pattern of FLT3 in two WD (Huh7 and Hep40) and two PD (Snu398 and SK-Hep-1) HCC cell lines to find an FLT3-positive HCC cell line to continue our silencing experiments. Hep40 and Snu398 cells were positive for *FLT3* expression mRNA level, which was absent in Hep40 cells at protein level. In addition to FLT3, Snu398 cells are known to be positive for FLT3L, which is required for the activation of FLT3 signaling [241]. Thus, we moved on to FLT3

silencing experiments using Snu398 cells and obtained approximately 45% less *FLT3* expression following stable transfection.

As a first step, we analyzed if the proliferative capacity of either K-252a treated or FLT3 specific shRNA transfected Snu398 cells changes since abnormal proliferation rate is a common characteristic for cancer cells. Our previous findings with K-252a treatment that had revealed an inhibitory effect on the migratory ability of Snu398 cells were parallel to our observations after FLT3 knockdown. This was achieved by performing wound healing assay under both normal (medium supplied with 10% FBS) and serum starvation (medium supplied with 2% FBS) conditions. The reason for examining the migratory capacity of cells grown in serum starved media in addition to regular culture media is because serum starvation prevents the cellular proliferation, thus helps only the migration ability of cells to be traced. Another characteristic of cancer cells that reflects their aggressiveness is their invasive capacity. Data obtained from matrigel invasion assay following both K-252a treatment and FLT3 knockdown revealed changes in the invasive potential of Snu398 cells, supporting our findings on the potential contribution of FLT3 in HCC progression.

In AML cells FLT3 was reported to act through apoptosis *in vitro,* because its inhibition resulted in a decrease in Mcl-1 levels and an increase in Bax activation [242]. Additionally, in another study human meningioma cells showed diminished migration and survival capacity upon PDGFR and FLT3 targeting [243]. Therefore, further studies might be conducted to see if this *in vitro* effect of FLT3 on hepatocellular carcinogenesis involves apoptosis.

Previous tumor xenograft studies on the contribution of FLT3 in tumor growth revealed that FLT3 has a potential tumor suppressive role *in vivo*. Among these studies, one study used a different inhibitor MLN0518 to inhibit FLT3 activity and demonstrated a diminution in tumor growth in mouse glioma xenograft models [244]. Additionally, another study claimed that inhibiting activity of FLT3 with a multikinase inhibitor LY2801653 yields antitumor activities in various xenograft models, namely lung, colon, and bile duct [245].

In addition to these studies and our *in vitro* findings, our *in vivo* results were also promising in terms of supporting the tumorigenic role of FLT3 in liver cancer. Indeed, our tumor xenograft experiments with K-252a and FLT3 specific shRNA might be an evidence for the contribution of FLT3 not only to the invasive and migratory potential of Snu398 cells, but also to their tumor formation abilities. In fact, these findings direct us to think that FLT3 might be a potential cancer stem cell marker, but still more studies are needed to support this hypothesis.

Hypoxia is another known contributors of increased tumorgienesis in HCC and FLT3 was previously suggested to have a role in tumor microenvironment regulation [54]. FLT3 activity inhibition with C-1311 showed a reduction in both HIF-1 dependent reporter gene and endogeneous HIF-1 transcription, as well as anticancer activity [246]. Thus, we might speculate on potential hypoxia reversing effects of FLT3 inhibition or silencing in HCC, yet more studies and findings are required to confirm this.

In this study, we were also able to show for the first time that FLT3 inhibition resulted in less αSMA expression in the tumor xenografts. These finding supports the potential effect of FLT3 on *in vivo* invasiveness, since αSMA is a known EMT marker having roles in development and tumor invasiveness in addition to the contribution of EMT to cancer metastasis [9, 247]. Additionally, this finding might also be informative for the further studies that need to be conducted to understand the role of FLT3 in liver fibrosis since αSMA is also a marker for liver fibrosis.

Overall, our findings support the potential presence of a link between FLT3 and hepatocellular carcinogenesis. Thus, targeting FLT3 to inhibit its activity in HCC might be a promising therapeutic approach for lowering its aggressiveness and progression rate. Drugs that are already being used in the clinics, such as a multikinase inhibitor Sorafenib (Nexavar), for the treatment of advanced stage HCC have adverse side effects like hand-foot skin reaction and diarrhea [248], [249, 250]. Therefore, blocking the activity of one kinase at a time, such as inhibiting the activity of FLT3, might be a much more advantageous and reasonable therapeutic approach for intermediate/advanced stage HCC patients.

In the second part of our study, we focused on the molecular mechanisms underlying liver fibrosis and potential candidates to favor its regression. Liver fibrosis, especially in its advanced form, is an important clinical condition since it has a potential to turn into cirrhosis and even HCC if not prevented. Fortunately, recent studies on liver fibrosis have been able to find potential antifibrotic candidate therapies mainly targeting the accumulation of ECM proteins and fibrogenic cells responsible for the origination of this process [23], [251]. Both innate and adaptive immune systems are effective on the regulation of liver fibrosis based on the activities of HSCs, KCs and endothelial cells after being stimulated by the remnants of damaged hepatocytes [252].

Being one of the most important contributors of innate immune response generation under wide range of conditions, TLRs have also been considered to be responsible for the activation and generation of fibrogenic response in the liver as a result of continuous antigen exposure. Several TLRs were reported to have both inhibitory and triggering effects on the initiation and progression of liver fibrosis. Among these TLR4, TLR3 and TLR9 have been the focus of interest of the studies conducted to attribute roles to TLRs in liver fibrosis [30], [34, 253–255]. Additionally, these studies already demonstrated that TLR3, TLR4, TLR7/8, and TLR9 expression increases in the fibrotic liver, but they have not revealed any data about TLR1 and TLR2 in liver fibrosis. This study was first to present that TLR1 and TLR2 expression increases in the liver when there is a fibrotic response generation. Since TLR1/2 was shown to recognize bacterial PGNs and lipopeptides and bacterial components derived from intestines are capable of inducing liver fibrosis, our findings seem to be supportive [256–258].

After generating an experimental CCl_4 induced liver fibrosis model in mice, we examined the validity of the model by analyzing the changes in the established liver fibrosis markers. αSMA and collagen accumulation and fibrotic lesion formation are important signs of induction of liver fibrosis. In addition to these, liver enzyme levels in the serum are expected to increase in the presence of a fibrotic response in the liver. Our results about the validation of CCl_4 induced liver fibrosis model were parallel to these specified parameters. Previous findings reported that TLR mRNA expression is

very low in the healthy liver, but upregulated following fibrosis induction contributing its progression [235],[138].

Here we report changes in TLR1-9 mRNA expression in the fibrotic liver parallel to previous findings. After analyzing TLR expression in our liver fibrosis model, we examined the effect of suppressive ODN A151, previously demonstrated to downregulate TLR dependent and independent inflammatory immune responses *in vivo*, administration on liver fibrosis progression [175, 236, 237]. Notably, our findings present a potential candidate for the suppression of liver fibrosis that is capable of lowering αSMA and collagen accumulation in addition to TLR expression in the liver. Besides, A151 treatment introduced a systemic effect on liver fibrosis progression and APC function as supported by changes in IL-12 secretion profiles and expression of cell surface markers known to trigger tissue destruction, CD54, CD69, CD80, and MHC-II, by splenocytes.

With these findings we are presenting a novel therapeutic approach to suppress the progression of fibrogenic response in the liver that might even drive its regression. Moreover, in contrast to other immune suppressive drugs that are being used in the clinics, this suppressive ODN A151 based approach overcomes potential systemic side effects. Since liver fibrosis might be an initiation point for cirrhosis and HCC, further studies should be conducted to understand if this effect of suppressive ODN A151 is similar in cirrhosis and HCC.

In the final part of our study, we presented preliminary data about the effect of another potential therapeutic candidate for the prevention of liver fibrosis progression for the first time, but still more research should be conducted to support our hypothesis. HSP family members are chaperones expressed in the presence of stress-causing factors for the cell and trigger a wide range of metabolic events to maintain the cellular homeostasis [188]. In addition to these metabolic functions, HSP60 and HSP70 were reported to activate TLR signaling pathway in a previous study [239][238].

Our results, which are parallel to the findings of other studies, showing changes in TLR expression in the fibrotic liver directed us to examine if HSPs play a role in this

vital stress based process. Unfortunately, we were not able to see a significant change in *HSP70* expression, which was our potential candidate due to its TLR activating role. Even though there has been no finding on the potential association of HSP27 and TLRs, we focused on HSP27 because of its roles in actin cytoskeleton organization, wound healing and cell motility, which are among the cardinal cellular events that take place during fibrogenesis [207, 208]. We observed a change in HSP27 expression after the induction of fibrosis with CCl_4 both at mRNA and protein levels. Thus, we suggested HSP27 as a potential candidate that contributes liver fibrosis and searched for a specific inhibitor to see the changes in the severity of fibrosis.

Quercetin is a clinically used flavonoid due to its anti-inflammatory, antihistaminic and antioxidant effects. Initially, inhibitory effect of quercetin on HSP27 expression was reported in a breast cancer study, after which it was also demonstrated under different conditions and for different cell types [228]. Quercetin treatment changed the expression of HSP27 in the fibrotic liver using quercetin. More strikingly, this change was also true for αSMA accumulation, assigning a potential and novel antifibrotic effect to quercetin. Therefore, further studies should be conducted in order to investigate this promising therapeutic approach for the prevention and even regression of liver fibrosis.

Chapter 5

5 Future Perspectives

Data presented in this study about the role of FLT3 in hepatocellular carcinogenesis support its candidacy as a biomarker for HCC. Moreover, these findings support our previous studies suggesting the inhibitory effect of K-252a on hepatocellular carcinogenesis acting through FLT3.

Previous studies reported that FLT3 acts through apoptotic pathways in different cells. Thus, further studies could be done to investigate if this activity is through apoptosis also in HCC cells. In addition to the role of FLT3 in HCC, further investigation might be done using liver fibrosis and cirrhosis models to understand if this role is gradual and initiated with the first insult to the liver. Moreover, our findings about changes in αSMA protein expression, a known marker for liver fibrosis in addition to EMT, following FLT3 knockdown and our ongoing studies on the role of FLT3 in liver fibrosis might be supportive for the potential contribution of FLT3 in liver fibrosis that need to be investigated further.

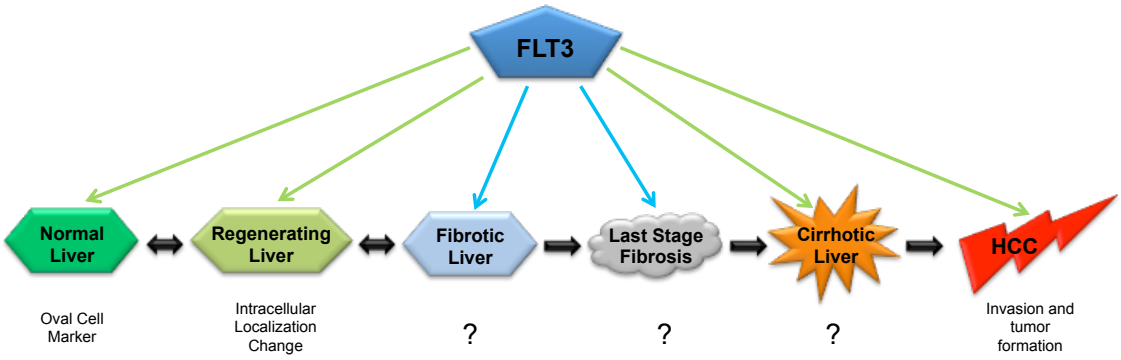

Figure 5.1. Contribution and roles of FLT3 in different states of the liver

Question marks denote the potential but not yet established activities of FLT3 in fibrosis and cirrhosis. Further studies need to be conducted to reveal these potential roles.

Our findings on the potential role of FLT3 in hepatocellular carcinogenesis would be helpful for future studies need to be conducted to see the effect of drugs used in the clinics for the treatment of HCC. Sorafenib is a multikinase inhibitor commonly used for this purpose by inhibiting different pathways such as KIT, PDGFR, VEGFR, RET, and FLT3 in different tumor types [67, 68, 109]. However, this effect has not been investigated by focusing on FLT3 signaling in HCC. Therefore, future investigations might be done to understand if the therapeutic effect of sorafenib is through FLT3 signaling in HCC. Moreover, another multikinase inhibitor sunitinib was demonstrated to be used in HCC treatment by targeting same pathways as sorafenib and its effect on FLT3 signaling in HCC might also be investigated [243, 259]. Erlotinib is on the other hand, targets EGFR pathways and being used for HCC [260]. Thus, examining the effect of erlotinib on FLT3 signaling might be helpful to further understand the role of FLT3 in hepatocellular carcinogenesis.

Findings of this study about liver fibrosis indicate that even thought there have been increasing amount of data accumulated in this field of research, there are still a lot more questions need to be answered than thought. Although too many studies have been conducted in the last decades in order to understand the underlying mechanisms of cirrhosis and HCC so that present both diagnostic and therapeutic approaches for

these fatal diseases, targeting them at much earlier stages especially at the fibrotic level is definitely more reasonable.

According to our data, suppressing liver fibrosis with suppressive ODN A151 is a rational way of decreasing the severity of fibrogenic response. In addition to our findings, more analysis should be done to understand the effect of suppressive ODN A151 at different stages of this gradually progressing disease. Moreover, this suppressive effect might also be examined using varied doses of suppressive ODN A151. Since liver fibrosis has a potential to progress into cirrhosis and end up with HCC, investigating the effect of suppressive ODN A151 on cirrhosis and hepatocellular carcinogenesis might be supportive for our findings about liver fibrosis. In this way, suppressive ODN A151 treatment might be a potential and novel therapeutic approach for HCC.

Furthermore, here we are presenting our data using CCl_4 induced liver fibrosis model. In the human body, there are various reasons for the induction and progression of liver fibrosis. Therefore, testing the suppressive action of suppressive ODN A151 on different liver fibrosis models may enlighten and support our findings.

On the other hand, our preliminary but novel data about HSP27 and liver fibrosis should be extended by focusing on the effect of quercetin regarding the changes in the inflammatory responses because of its anti-inflammatory features. Similarly, changes in the course of fibrotic events might also be observed following quercetin treatment at different stages and using varied doses on different liver fibrosis models. Moreover, further research may be conducted to investigate if there is an association between TLRs and HSP27, especially in liver fibrosis, cirrhosis, and HCC.

Considering both suppressive ODN A151 and quercetin as potential therapeutic candidates for liver fibrosis, additional studies need to be conducted to examine their effect on cirrhosis and HCC. Overall, both suppressive ODN A151 and quercetin might be potential candidates for the treatment of liver diseases at various stages and arising as a result of different causes. In this way, both might be therapeutic alternatives to liver transplantation.

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Appendices

Appendix A: Supplementary Figures

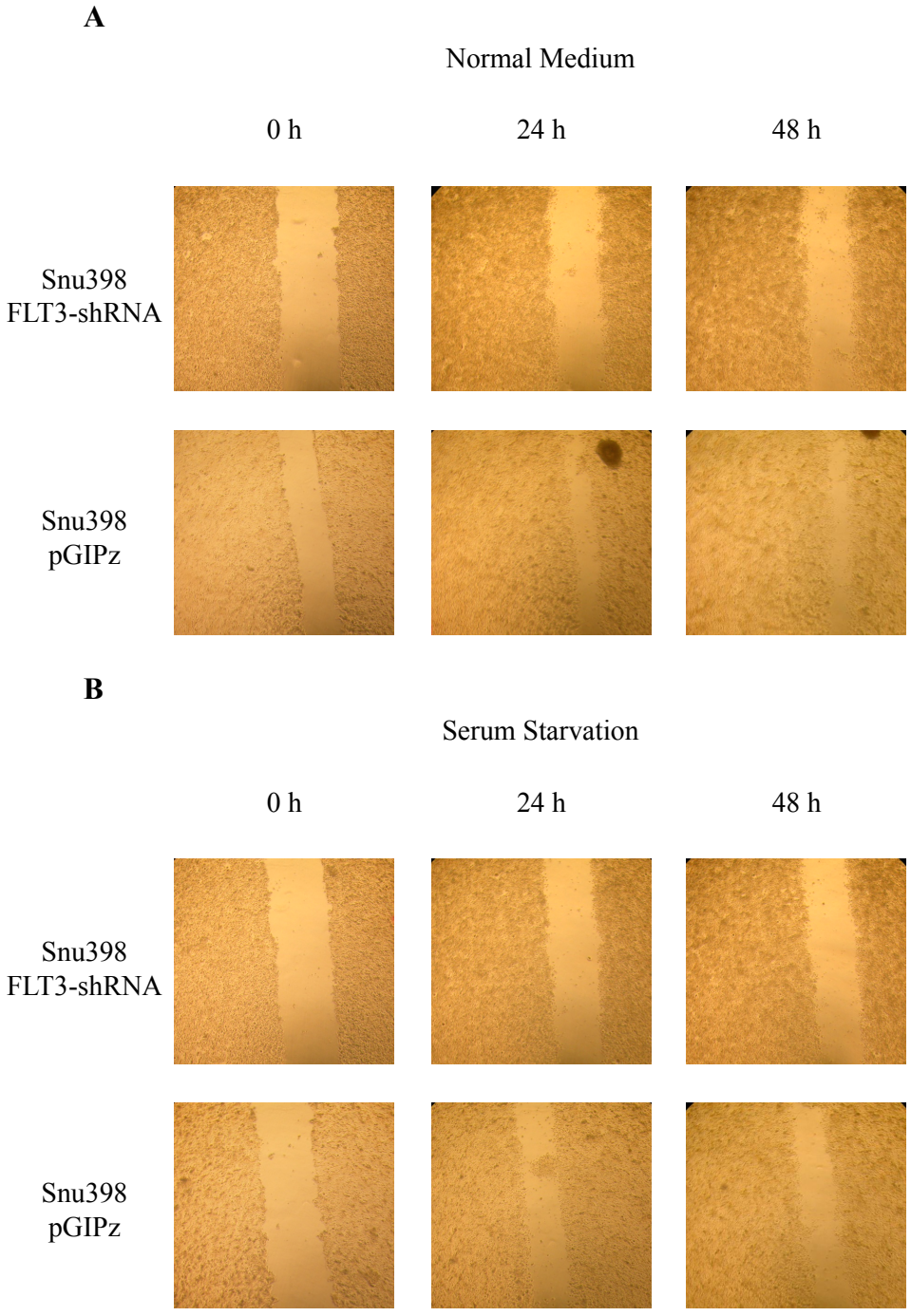

Figure 7.1. Effect of FLT3 knockdown on *in vitro* wound healing capacity of Snu398 cells

Representative wound healing images of Snu398 cells under (A) normal (10% FBS) and (B) serum starvation (2% FBS) conditions following FLT3 specific shRNA (Snu398 FLT3-shRNA) and empty vector (Snu398 pGIPz) transfection.

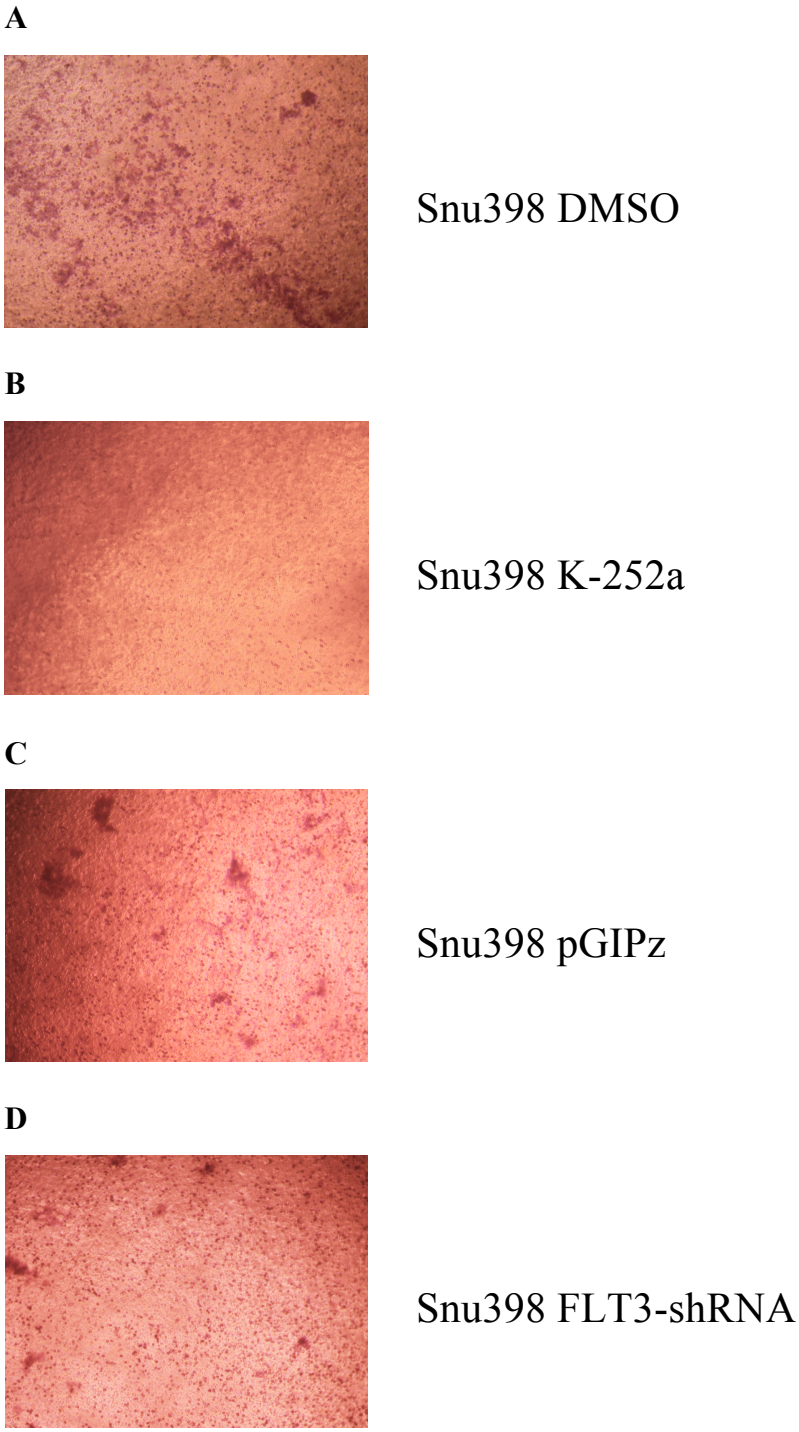

Figure 7.2. Effect of FLT3 knockdown on *in vitro* matrigel invasion of Snu398 cells

Representative images of invaded colonies of Snu398 cells through matrigel in control medium (Snu398 DMSO), inhibitor-containing medium (Snu398 K-252a), following empty vector (Snu398 pGIPz) and FLT3 specific shRNA (Snu398 FLT3-shRNA) transfection. Images were taken using 4X objective.

Appendix B: Standard Solutions and Media

Table 7.1. Ingredients and preparation instructions for general laboratory solutions

10X PBS	80 g NaCl, 2 g KCl, 8.01 g $Na_2HPO_4.2H_2O$ in 1 L ddH_2O; Working dilution: 1X
10X TBS	12.19 g Tris-base, 87.76 g NaCl in 1 L ddH_2O; pH: 8.0; Working dilution: 1X
50X TAE	242 g 2 M Tris-base, 57.1 ml Glacial Acetic Acid, 50 mM EDTA in 1 L ddH_2O; Working dilution: 1X
10X Agarose Gel Loading Dye	0.009 g BFB, 0.009 g XC, 1.2 ml 0.5 M EDTA, 2.8 ml ddH_2O up to 15 ml with glycerol; 1:10 diluted with sample before loading
0.5M EDTA	93.05 g EDTA, 300 ml ddH_2O; Adjusted to pH 8.0 with NaOH and completed to 500 ml with ddH_2O
1M Tris	60.55 g Tris-base, 300 ml ddH_2O, 21 ml 37% HCl; Adjusted to pH 8.0 with HCl and completed to 500 ml with ddH_2O

Table 7.2. Ingredients and preparation instructions for RNA and protein isolation solutions

DEPC-Treated ddH_2O	1 ml DEPC in 1 L ddH_2O; Stirred 1 hour under the hood and autoclaved
Lysis Buffer	75 µl 2 M NaCl, 50 µl 1 M Tris pH: 8.2, 10 µl 0.9% Igepal CA-630, 100 µl 10X Protease Inhibitor Cocktail, 715 µl ddH_2O
Camiolo Buffer	0.368 g KAc, 0.8765 g NaCl, 1 ml 0.5 M EDTA, 125 µl Triton X-100, 0.871 g L-arginine in 50 ml ddH_2O; Filter sterilized

Table 7.3. Ingredients and preparation instructions for Western blotting solutions

Bradford Reagent	100 mg Coomassie Brilliant Blue, 100 ml 85% Phosphoric acid, 50 ml 95% Ethanol, 850 ml ddH_2O; Filtered through Whatman paper
10% SDS	1 g SDS in 10 ml ddH_2O
10% APS	1 g APS in 10 ml ddH_2O
30% Acrylamide Mix	145 g Acrylamide, 5 g Bis-acrylamide in 500 ml ddH_2O; Filtered and stored in dark at 4^oC
1M Tris-HCl	12.1 g Tris-base in 100 ml ddH_2O; pH: 6.8

1.5M Tris-HCl	18.1 g Tris-base in 100 ml ddH_2O; pH: 8.8
1X TBS-T (0.1%)	50 ml 10X TBS, 950 ml ddH_2O, 1 ml Tween-20
5% Milk Powder Blocking Solution	0.5 g milk powder in 10 ml 1X TBS-T (0.1%)
5% BSA Blocking Solution	0.5 g BSA in 10 ml 1X TBS-T (0.1%)
Cracking Buffer (2X Protein Loading Buffer)	50 mM Tris-HCl (pH:6.8), 2 mM EDTA (pH:6.8), 1% SDS, 20% Glycerol, 0.02% BFB; 1% β-mercaptoethanol prior to use
5X Running Buffer	15 g Tris-base, 72 g Glycine, 5 g SDS in 1 L ddH_2O; Working dilution: 1X; Stored at 4^oC
Semi-dry Transfer Buffer	2.5 g Glycine, 5.8 g Tris-base, 0.37 g SDS, 200 ml Methanol, 800 ml ddH_2O
Wet Transfer Buffer (for large proteins)	43.2 g Glycine, 9 g Tris-base, 300 ml Methanol, 1.5 ml 10%SDS complete up to 1.5 L with ddH_2O
Coomassie Blue Satining Solution	0.25 g Coomassie Brilliant Blue, 45 ml Methanol, 45 ml ddH_2O, 10 ml Glacial acetic acid

Destaining Solution	100 ml Methanol, 35 ml Acetic acid, 365 ml ddH_2O

Table 7.4. Ingredients and preparation instructions for immunostaining solutions

4% Paraformaldehyde	4 g Paraformaldehyde in 100 ml ddH_2O; Dissolved at 130^{o}C for 1 hour; Cooled prior to use
Blocking Solution	4 µl 2% BSA, 4 µl 1X PBS, 20 µl Tween-20
1X PBS-T (0.1%)	50 ml 10X PBS, 450 ml ddH_2O, 0.5 ml Tween-20
Sodium Citrate Buffer	10 mM Sodium citrate, 0.05% Tween-20; pH: 6.0

Table 7.5. Ingredients and preparation instructions for ELISA experiments

Blocking Buffer	500 ml 1X PBS, 25 g BSA; 250 µl Tween-20; Stirred 20-30 minutes; Stored at -20^{o}C
Wash Buffer	500 ml 10X PBS; 2.5 ml Tween-20; 4.5 L ddH_2O

Table 7.6. Ingredients and preparation instructions for cell culture solutions

DMEM	10% FBS, 1% Penicillin/streptomycin in 500 ml; Stored at 4^{o}C
RPMI	10% FBS, 1% Penicillin/streptomycin, 1% NEAA in 500 ml; Stored at 4^{o}C

Appendix C: Curriculum Vitae

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EDUCATION

2009 –……. **Ph.D.,** Molecular Biology and Genetics,
İhsan Doğramacı Bilkent University

Advirsors: Prof. Dr. İhsan Gürsel

Prof. Dr. Kamil Can Akçalı

Standing: High Honour

CGPA: 3.86/4.00

2005 – 2009 **B.Sc.,** Molecular Biology and Genetics,
İhsan Doğramacı Bilkent University

Standing: High Honour

CGPA: 3.75/4.00

2002 – 2005	**High School of Science,** Özel Arı Fen Lisesi
	CGPA: 5.00/5.00
1997 – 2002	**Primary School,** Özel Arı İlköğretim Okulu

HONOURS & AWARDS & SCHOLARSHIPS

2014	EASL Young Investigator Full Bursary Award (EASL HCC Summit (Basic & Clinical Science): Molecular Pathogenesis & Translational Research in Liver Cancer, and Liver Cancer Management, Geneva, Switzerland)
2014	EASL Young Investigator Course Participation Award (EASL Basic School of Hepatology Course 9: Epithelial Mesenchymal Interactions in Liver Development, Diseases and Cancer, Milan, Italy)
2013	Best Oral Presentation Award (9th Biennial Meeting of the Turkish Association For the Study of the Liver, Istanbul, Turkey)
2013	EASL Young Investigator Full Bursary Award (EASL Monothematic Conference: Systems Biology of the Liver: Systems Biology and Clinics Face-à-Face, Luxembourg, Luxembourg)
2012	EASL Young Investigator Registration Bursary Award (The International Liver Congress 2012, 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain)
2009	TUBITAK PhD Scholarship
2008	High Success Scholarship Bilkent University, Ankara, Turkey
2007	High Success Scholarship Bilkent University, Ankara, Turkey
2005	3rd on High School Graduation Özel Arı Fen Lisesi, Ankara, Turkey

TEACHING EXPERIENCE

MBG 102 - Biology II, Bilkent University, Department of Molecular Biology and Genetics

MBG 110 - Introduction to Modern Biology, Bilkent University, Department of Molecular Biology and Genetics

MBG 472 - Introduction to Stem Cells, Bilkent University, Department of Molecular Biology and Genetics

RESEARCH EXPERIENCE

2009 – ……	Ph.D. Study on the Molecular Biology of Liver Fibrosis and Cancer
	Supervised by Prof. Dr. İhsan Gürsel and Prof. Dr. Kamil Can Akçalı
	Bilkent University, Department of Molecular Biology and Genetics, Ankara, Turkey
2008 – 2009	Senior Project on the Genetics of Uner Tan Syndrome
	Supervised by Prof. Dr. Tayfun Özçelik
	Bilkent University, Department of Molecular Biology and Genetics, Ankara, Turkey
2008	Summer Practise on the Retinal Transduction Profiles of Adeno Associated Virus (AAV) in the Transgenic Rat Model of Retinitis Pigmentosa Compared with the Wild Type Retina
	Supervised by Prof. John G. Flannery and Dr. Deniz Dalkara Mourot
	University of California, Berkeley, Helen Wills Neuroscience Institute, Berkeley, USA
2007	Summer Practise on the Analysis of Predisposing Genes in Breast Cancer Supervised by Prof. Annika Lindblom and Dr. Simone Picelli

Karolinska Institute Stockholm, Center of Molecular Medicine and Surgery, Clinical Genetics, Stockholm, Sweden

2006 Summer Practise on Bioinformatics

Supervised by Assoc. Prof. Rengül Çetin Atalay

Bilkent University, Ankara, Turkey

2006 "Design of Vaccines Against Tuberculosis With CpG Motifs"

TUBITAK Project With Contributions of Bilkent University, Middle East Technical University, Hacettepe Medical School Undergraduate Students TUBITAK, Ankara, Turkey

2006 "Research on the Unknown Morphologic Features of Praying Mantis"

TUBITAK Project With the Contributions of Bilkent University, Middle East Technical University and Hacettepe Medical School Undergraduate Students TUBITAK, Ankara, Turkey

PUBLICATIONS

Published Papers:

- M.M. Aydin, N.S. Bayin, T. Acun, M.C. Yakicier, K.C. Akcali, "Role of FLT3 in the Proliferation and Aggressiveness of Hepatocellular Carcinoma", Turk. J. Med. Sci., 2015.

- M. Ceyhan, Y. Ozsurekci, M.M. Aydin, K.C. Akcali, B. Talim, M. Celik, E. Oncel Karadag, V. Gurbuz, A.E. Aycan, I. Onbasilar, T. Buzgan, "Determination of the presence of diphtheria toxin in the myocardial tissue of rabbits and a female subject by using an immunofluorescent antibody method", J. Clin. Med. Res., 2015 Jun; 7(6): 472-8.

Paper(s) In Revision:

- M.M. Aydin, T. Kahraman, F.C. Yagci, E. Akhan, K.C. Akcali, I. Gursel, "Regression of Liver Fibrosis by Suppressive Oligonucleotide", Mol. Ther. Nucleic Acids, 2015.

CONFERENCES

2014	EASL Basic School of Hepatology Course 9: Epithelial Mesenchymal Interactions in Liver Development, Diseases and Cancer, Milan, Italy
2013	9th Biennial Meeting of the Turkish Association for the Study of the Liver Istanbul, Turkey
2013	EASL Monothematic Conference: Systems Biology of the Liver: Systems Biology and Clinics Face-à-Face Luxembourg, Luxembourg
2012	The International Liver Congress 2012, 47th Annual Meeting of the European Association for the Study of the Liver Barcelona, Spain
2011	BIOMED 2011, 17th Biomedical Science Technology Symposium Ankara, Turkey
2011	EASL Monothematic Conference: Liver Fibrogenesis - Common and Organ Specific Mechanisms Petersberg, Germany
2009	MediMedGen, Mediterranean Medical Genetics Meeting Ankara, Turkey
2009	Conference on “Horizons in Molecular Biology and Genetics II” Bilkent University, Ankara, Turkey
2008	Conference on “Horizons in Molecular Biology and Genetics” Bilkent University, Ankara, Turkey
2006	Workshop on "Emerging Topics in Human Functional Genomics and Proteomics" Antalya, Turkey

ABSTRACTS & PRESENTATIONS & POSTERS

2014 **Abstract Submitted & Accepted:** "REVERSING THE PROGRESS OF LIVER FIBROSIS USING MEMBRANE VESICLES SECRETED FROM COMMENSAL BACTERIA"

The International Liver Congress 2014, 49th Annual Meeting of the European Association for the Study of the Liver, London, United Kingdom

2014 **Abstract Submitted & Accepted:** "ROLE OF FLT3 IN HEPATOCELLULAR CARCINOMA"

EASL HCC Summit (Basic & Clinical Science): Molecular Pathogenesis & Translational Research in Liver Cancer, and Liver Cancer Management, Geneva, Switzerland

2013 **Oral Presentation:** "TOLL BENZERİ RESEPTÖRLERİNİN BASKILANMASI VE HSP27 İNHİBİSYONU İLE KARACİĞER FİBROZİSİ OLUŞUMUNUN GERİ DÖNDÜRÜLMESİ"

9th Biennial Meeting of the Turkish Association For the Study of the Liver, Istanbul, Turkey

2013 **Poster Presentation:** "REVERSING THE PROGRESS OF LIVER FIBROSIS BY SUPPRESSION OF TLRS AND INHIBITION OF HSP27"

EASL Monothematic Conference: Systems Biology of the Liver: Systems Biology and Clinics Face-à-Face, Luxembourg, Luxembourg

2012 **Poster Presentation:** "ROLE OF FLT3 AND ITS POTENTIAL INTERACTION PARTNER HSP27 IN HEPATOCELLULAR CARCINOMA" (Journal of Hepatology 2012 vol. 56, S71-S224)

Poster Presentation: "NANOPARTICLE LABELING: A NEW ERA IN VIVO TRACING THE CELLS IN LIVER STUDIES" (Journal of Hepatology 2012 vol. 56, S71-S224)

The International Liver Congress 2012, 47th Annual Meeting of the European Association for the Study of the Liver, Barcelona, Spain

2011 **Poster Presentation:** "ROLE OF TLR2 AND TLR4 AND THEIR ENDOGENOUS LIGANDS IN THE PROGRESSION OF LIVER FIBROSIS"

EASL Monothematic Conference: Liver Fibrogenesis - Common and Organ Specific Mechanisms, Petersberg, Germany

SCIENTIFIC MEMBERSHIPS

European Association for the Study of the Liver (EASL)

LANGUAGES

- English (Advanced)
- German (Intermediate)
- Turkish (Native)

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