

**CONTRIBUTION OF NOTCH SIGNALING ON HCC
STEM CELL STATUS AND UTILIZING TLR
AGONISTS AND NOTCH INHIBITION TO IMPROVE
HCC THERAPY**

**A THESIS
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FOR THE DEGREE OF
MASTER OF SCIENCE**

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ABSTRACT

CONTRIBUTION OF NOTCH SIGNALING ON HCC STEM CELL STATUS AND UTILIZING TLR AGONISTS AND NOTCH INHIBITION TO IMPROVE HCC THERAPY

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Hepatocellular carcinoma (HCC) is the seventh most common cancer type worldwide, and ranked third place among cancer-related deaths within both sexes. As in many solid tumors, HCC shelters a cancer stem cell subpopulation, and is held responsible for the resistance developed during chemo-and-radio-therapy of HCC. The only option to cure HCC is liver transplantation, which is the bottleneck to provide a remedy to patients due to limited availability. Understanding the stem cell behavior of HCC would critically contribute to develop effective eradication strategies. In this study, a panel of 17 HCC cell lines was evaluated for their CSC status. Of these cell lines, six of them were determined to be positive for CD133 expression, a cardinal CSC marker. Next, HepG2, Huh7 and Hep3B-TR, (a desensitized TGF-beta-1 receptor clone) were selected and Notch activity vs. CSC fraction was investigated by analyzing CD133+/EpCAM+ levels. Our results revealed that DAPT (a notch inhibitor) led to a drop in CD133+/EpCAM+ levels in HepG2 and Huh7 by half, but not in Hep3B-TR cells, implicating a possible TGFβ1R involvement on CSC generation/maintenance. Treatment of cells with a notch ligand, Jagged-1, however, had little or no positive effect on CD133+/EpCAM+ expressions in all tested cells. Additionally, HCC cells' response to different TLR ligands and the resulting transcript expressions of TLRs were investigated by PCR. Of note, TLRs are widely used in immunotherapy of cancers. Here we aimed to combine Notch inhibitor along with selected TLR ligand, thereby improving tumor clearance in athymic mice xenografted with HCC. We found that in three selected cell lines upon TLR2 ligand stimulation, TLR5 and TLR7 were highly upregulated. Afterwards, treating these HCC cell lines with these ligands we observed that TLR3, TLR7/8 and TLR9 levels were activated. In the final part of this study, tumor-bearing mice with Huh7, were subjected to a combination therapy with TLR ligands +/- DAPT. We demonstrated that combination therapy comprising TLR3, 7/8 and 9 ligands and DAPT (only two injections, a week apart) induced significant tumor regression.

Keywords: Hepatocellular carcinoma, Cancer stem cells, Notch signaling pathway, Toll-like receptors, CD133, EpCAM, Therapy.

ÖZET

NOTCH YOLAĞININ HCC KÖK HÜCRELERİNE KATKISI VE HCC TEDAVİSİ İÇİN TLR ULAKLARIYLA NOTCH BASKILAMASININ KULLANILMASI

Yusuf İsmail ERTUNA

Moleküler Biyoloji ve Genetik Yüksek Lisansı

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Hepatosellüler karsinom (HSK) dünya genelinde en sık görülen kanserler sıralamasında yedinci, kanser kaynaklı ölümler göz önünde bulundurulduğunda ise üçüncü sırada yer almaktadır. Birçok solid tümörde görüldüğü gibi, HSK tümörlerinde de bir kanser kök hücresi altpopulasyonu barındırdığı bilinmektedir. Bu nedenle, HSK tümörlerinin radyoterapi ve kemoterapiye direnç geliştirmeleri bu kanser kök hücrelerinin düzeyleriyle sağlanmakta olduğu düşünülmektedir. Günümüzde HSK tedavisi için en etkin yol karaciğer naklidir. Ne yazık ki, uygun donör azlığı bu yaklaşımın etkin sağaltım sağlamasını sınırlı kılmaktadır. Bu bağlamda, karaciğer kanseri kök hücre altgruplarının düzeyini belirleyip miktarını azaltacak stratejiler geliştirmek, etkili tedavi yöntemleri geliştirilmesine önemli katkılarda bulunacaktır. Bu çalışmada, HSK kökenli 17 hücre hattı kullanılarak bir kanser kök hücresi paneli oluşturulmuştur. İmmünoperoksidaz ve Akım Sitometresi teknikleriyle gerçekleştirilen tarama sonunda altı KKH'nın kabul edilen bir kanser kök hücresi (KKH) markörü olan CD133 ifadesi bakımından oldukça pozitif olduğu saptanmıştır. Daha sonra üç hücre hattı HepG2, Huh7 ve Hep3B-TR (TGF-beta-1 almaçı duyarsızlaştırılmış) seçilerek Notch yolağının etkinleştirilmesi veya bastırılması, kök hücre altgrubu düzeyine olan etkisi belirlenmiştir.. Elimizdeki veriler, Huh7 ve HepG2 hücre hatlarının DAPT (bir tür gamma-sekretaz inhibitörü) ile muamelesi sonucunda CD133+/EpCAM+ KKH altpopulasyonun yarı yarıya azaldığını, buna karşın Hep3B-TR hücrelerinde herhangi bir değişim saptanmadığı gözlenmiştir. Bu veriler, TGFβ1 reseptörünün KKH oluşumunda ve devamlılığında rolü olabileceği ihtimalini önermektedir. Seçilen hücre hatlarının Jagged-1 (Notch ligandı) ile muamelesi sonucunda ise KKH altpopulasyonu üzerine bir etki oluşturmamıştır. Bu hücre hatlarının bazal toll-benzeri reseptör (TLR) ifade düzeyleri PZR metoduyla incelendiğinde, TLR5 ve TLR7 transkriptlerinin yüksek olduğu bulunmuştur. Bu ulaklarla hücreleri muamele edilince de, TLR3, 7/8 ve 9 genlerinin daha fazla arttırdığı gözlenmiştir. Bu sonuçların ışığında Notch inhibitörüyle, TLR ulaklarının kullanıldığı bir kombine tedavinin HSK tümörü geliştirilmiş atimik farede denenmesi planlanmıştır. Çalışmanın son bölümünde, Huh7 hücreleri farelere zenograft edilerek, ya sadece TLR ligand kokteyli, veya DAPT eklenmiş şekilde, iki kez peritümöral enjeksiyonu sonunda (bir hafta arayla)

tümör düzeyleri takip edilmiştir. Sonuç olarak, TLR3, TLR7/8 ve TLR9 ligandlarının DAPT ile kombine terapisinin uygulandığı hayvanlardaki tümör boyutlarının önemli derecede gerilediği bulunmuştur.

Anahtar sözcükler: Hepatosellüler karsinom, Kanser kök hücresi, Notch sinyal yolağı, Toll benzeri reseptör, CD133, EpCAM, Tedavi .

*To my parents Emine and Mehmet,
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Abbreviations

HBV	Hepatitis-B virus
HCV	Hepatitis-C virus
HCC	Hepatocellular carcinoma
TLR	Toll-like receptor
CSC	Cancer-stem cell
ROS	Reactive oxygen species
NAFLD	Non-alcoholic fatty liver disorder
NK	Natural killer
FCS	Fetal calf serum
LPS	Lipopolysaccharide
IFN	Interferon
PGN	Peptidoglycan
LTA	Lipoteichoic acid
Poly(I:C)	Polyinosinic-polycytidylic acid

Chapter 1

Introduction

1.1 Hepatocellular carcinoma

1.1.1 Epidemiology of Hepatocellular Carcinoma

Liver cancer has a 4.6% survival rate, which renders it to be the second most lethal cancer type worldwide in both sexes after pancreas cancer ¹. The severity of liver cancer stems from several aspects including the shortage of the markers used for pinpointing cancerous cells and highly resistant nature against radio and chemotherapy.

There are various subtypes of liver cancer encompassing intrahepatic bile duct carcinoma, hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma and last but with being the most common type of primary liver cancer type by consisting around the 85% of all cases, hepatocellular carcinoma(HCC) ².

HCC, *per se* enters the list of most common cancer types as seventh, and is the third upon evaluation of primary cancer related deaths worldwide (in both sexes) ³.

Although primary liver cancers including HCC show a differential variability worldwide, China makes up for the 55% of all primary liver cancer types due to its enormous population and very high age-standardized incidence rates. China is followed by the sub-Saharan countries Cameroon and Mozambique ⁴. Mediterranean countries including Italy and Greece show intermediate incidence ratios with 20 per 100.000 people (2%) ⁵. In Turkey, HBV incidence rate is 5-10% and HCV carrier rate is 1.5%. HCC incidence rate on the other hand is 1.9% in men, but 1.1% in women ⁶.

1.1.2 Etiologies and Risk Factors of Hepatocellular Carcinoma

HCC establishment comprises a lengthy, complicated, multi-step condition and exposure to numerous factors has a significant contribution in progression of HCC. These risk factors include but not limited to viral causes, in case of Hepatitis B or C infections (HBV and HCV hereafter), excessive alcohol consumption, frequent exposure to toxic substances like Aflatoxin B1 diabetes and chronic liver inflammation. The effects of risk factors are expectedly variable, while HBV is the leading cause of HCC in Africa, both HBV and HCV accounts for the prime risk factors in Asia, although HCV is the most risky factor in the US ⁷. Through triggering a set of mechanisms generally starting with inflammation due to damage to resident hepatocytes, combined effects of these risk factors finally result in chronic liver disease and fibrosis⁸. Additional factors including alcohol consumption, diabetes, exposure to toxic compounds such as aflatoxins or pesticides, reactive oxygen species (ROS) production, all has contribution in the progression of HCC and counted as significant risk factors ^{9,10}.

1.1.2.1 Virus Induced Hepatocellular Carcinoma

Viral hepato-carcinogenesis is mostly attributable to two distinct viral infections, one of which is HBV infection and the second one is HCV infection. Each year around 320.000 people die because of HBV related diseases, and HCC constitutes the 40-50% of HBV related deaths ⁸. Some of these infections might be acquired from child-birth but among the ones that get the disease during adulthood, 30% develops

chronic liver disease, which is followed by fibrosis, cirrhosis leading to hepatocellular carcinoma at a later stage ⁷. More than 60% of HCC cases are caused by HCV and around 20% by HBV in the US, Egypt and Europe. In Asia and Africa, the reciprocal scenario applies, however, 60% of all HCC cases is related to HBV and 20% is related with HCV infections. There are multiple factors, though, determining the rate for fibrotic progression, and there seems to be a variability among patients to enter fibrosis, yet the mean progression time is within one or two decades ¹¹.

HCV is a positive-sense single stranded RNA virus, the virus genome itself possess an open reading frame that encodes a polyprotein. The genes that form the structural parts are encoded from the regions close by to 5', the rest of the genes are encoded nearby 3' region ¹². HBV, on the contrary, possess a partially double stranded and circular DNA genome, which will eventually give rise to 4 totally different proteins that are Surface, Precore, X protein and DNA polymerase ¹³.

With the further contribution of excessive alcohol consumption, or additional infections such as HIV, the disease progression in HCV and HBV related cases becomes prevalent to acceleration.

1.1.2.3 Alcohol and Hepatocellular Carcinoma

Alcohol abuse is pointed as being one of the primary or else cofactor causing HCC. This variability is dependent on the circumstances that a patient bears with, in the first case scenario intensive consumption of alcohol may directly trigger HCC or through alcohol-induced cirrhosis. In the latter case, this being the cofactor, alcohol may act this time as an interplay for a patient, who was diagnosed with HBV or HCV ¹⁴. The significant matter at this stage is to determine the amount of alcohol that falls between the danger limit and this range is 60-80g of alcohol on a daily basis ¹⁵. With this definition, statistical data verified that around 7% of the people in US are within the alcohol abuse limits ¹⁶. In a study with 464 Italian patients, it was shown that people consuming 40-80g of alcohol on a daily basis had a possibility of progressing into HCC with an odds ratio of 1.5 compared to the ones, whose alcohol intake is 0-

40g. More catastrophically, this ratio climbs up to 7.3 fold when the alcohol intake passes over 80g per day ¹⁷. There are various mechanisms relating alcohol to HCC, some of which can be listed as the genototoxic effects ¹⁸, non-genototoxic effects, such as the activity of metabolized intermediate forms of alcohol on liver ¹⁹, alcohol leading to methylation abnormalities ²⁰, and finally immune surveillance prevention effect of alcohol in tumors ²¹. Some of the mechanisms will be covered in the following sections.

1.1.2.4 Aflatoxin Exposure Induced Hepatocarcinogenesis

Aflatoxins (*Aspergillus flavus* toxins) are in essence the metabolites of *Aspergillus parasiticus* and *A. flavus*. Upon infection of plants, which could be crops, these mycotoxins are also produced within the crops upon preservation in moisture areas ²². Since coming with an edible food, these toxins potentially exert cumulative effects on people, who are frequently consuming them, these effects include hepatotoxicity and immunotoxicity. Aflatoxin B1 is the most abundant and extremely toxic type of aflatoxins ²³. Aflatoxin B1-rich areas in Africa seem to be the ones with high rates of HCC. Moreover, research suggests that with an additional HBV infection Aflatoxin exposure increases the possibility of progression into HCC ^{24–26}. Therefore the research has suggested that, in majority of the regions that has a mutation in p53 gene coincides with the region that possess the highest Aflatoxin B1 exposure and further data shows Aflatoxin B1 is the responsible factor ^{27,28}.

1.1.2.5 Additional Factors Inducing Hepatocarcinogenesis

Along with all the risk factors listed and discussed in previous sections, there are additional factors with a lower frequency triggering a step forward hepatocellular carcinoma.

To begin with a high-incidence factor, diabetes is an important factor for HCC development, it may work as an independent factor, and data suggest that HCC development was seen in HCC patients with no other liver disease related factors ¹⁶. Such a tendency may stem from the accumulation of free-fatty acids in the liver following insulin resistance in diabetic patients. Build-up of tremendous amount of

lipid in the liver starts up a cascade of events leading to hepatocellular injury, oxidative stress through radical production by excess lipid metabolism, all of which may eventually end up with fibrosis ^{2,29}.

Non-alcoholic liver disorders such as Non-alcoholic fatty liver disorder (NAFLD) or non-alcoholic steatohepatitis are also risk factors that by inducing liver damage they lead to fibrosis and cirrhosis, which will progressively fire HCC. ³⁰.

HCC can be connected with metabolic disorders, as well, and one of these disorders is hereditary haemochromatosis. This disorder is originated from excess iron absorption of liver cells that will cause hepatocellular damage in return ³¹. Moreover, porphyria cutanea tarda, which is again associated with extreme iron absorption leads to fibrosis and cirrhosis ³².

1.1.3 Molecular Pathogenesis of Hepatocellular carcinoma

Hepatocellular carcinogenesis can be triggered through different risk factors as mentioned in previous sections, and these primary factors are known to be HCV and HBV infections, alcohol abuse, exposure to toxic factors like Aflatoxin B1 and metabolic abnormalities such as diabetes.

Either cumulative or individual effects of these risk factors on liver cause damage to hepatocytes which will induce necrosis, following excessive cell death, an increase in proliferation of hepatocytes is observed.

This cycle of necrosis-proliferation is known to be progressing in to Chronic liver disease ². Continuum of such intensive scarring foster a cirrhosis condition which is characterized by nodule formation covered by collagen fibers, during that process hepatocytes go under proliferative arrest due to telomere shortening ³³ (Figure 1.1)

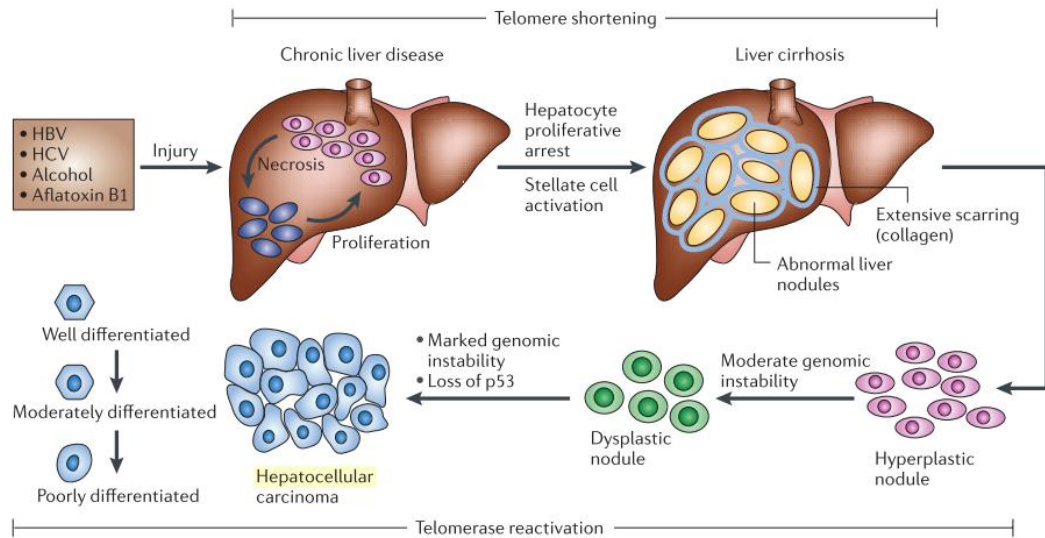


Figure 1.1: Multistep pathogenesis of hepatocellular carcinoma (adapted from Farazi & DePinho, 2006²).

With the presence of HBV or HCV, liver enters into a state of inflammation, although HCV is cytopathic to the hepatocytes, HBV seems to be non-cytopathic to the cells in cell culture^{34,35}. This viral-persistence creates an inflammatory environment that renders chronic liver disease continuous with no viral clearance, and even presence of virus-specific lymphocytes and natural-killer cells (NK cells) are detected at the inflammation sites, activity of those still is not effective enough to clear the virus³⁶. HBV and HCV coded proteins affect and change the host gene expression, even HBV genome integrates itself into the host genome through which it can cause the activation of oncogenes or telomerase activation³⁷. Moreover, the oxidative stress and environment created by immune activity is made use of by these viruses to activate different signaling pathway to escape apoptosis, cellular senescence, and sustain viral-persistence³⁸.

Following cirrhosis, continuously regenerating hepatocytes turn into hyperplastic nodules. The nodules are formed by rather normal cells, yet piled-up genetic mutations further develops these hyperplastic nodules into pre-malignant dysplastic nodules. With the increased mutations in TP53, MET or E-cadherin genes, these dysplastic nodules may progress into hepatocellular carcinoma².

1.1.4 Genetics of Hepatocellular Carcinoma

Genetic aberrations, although not being completely universal, are very critical components that regulate the progression into HCC. Having been discussed, the blueprint of HCC formation with rounds of necrosis, inflammation and regeneration render the cells to make mistakes during cell division and cell metabolism, as results of these defects, tumor suppressor genes may become inactivated and oncogenes may get activated ³⁹.

With developing sequencing techniques like exome-sequencing, next-generation sequencing and parallel sequencing, previously unknown genetic mutations, gene fusions or intra-chromosomal rearrangements are much easier to follow up with. Since HCC tumors show a heterogeneity in their genomic sequences, a rather targeted therapy should be a possible way to interfere with this mechanism ⁴⁰. Accumulating data collected from genomics and transcriptomics studies present intermingled subgroups of HCC in terms of genetic mutation tendency. One of the highly-mutated pathway seems to be Wnt-signaling pathway elements including CTNN1, AXIN1 and AXIN2 with around 50% mutation rate in collected samples, whereas TP53 turns out to be a frequently mutating tumor suppressor gene with mutations in 20% of tumors ⁴¹. Just as loss of functions mutations, there are also gain-of-function mutations, two of which take place at Chromosome 1p and 8q, which leads to the activation of oncogenic genes MYC, SHC1 and MLC1, respectively.

A different study suggests the alteration of genes involved in SWI/SNF complex and the genes that are members of interferon and oxidative stress pathways ⁴². Furthermore, integrated expression profiling analyses revealed the effects of HBV integration into the genome in HCC. First of these is that HBV integrates itself in the promoter region of TERT gene, FAR2, IRAK2, MAPK1, MLL2 and MLL4 ⁴³. Moreover, HBV integration breakpoints are found to be showing a tendency towards copy-number-variations, which implies a role for HBV integration in genomic instability of HCC ⁴⁴.

1.2 Cancer stem cells (CSCs)

Tumors are embodied from elaborately combined but differentially functioning subpopulations of cells. Some of the members of the subpopulations are rapidly dividing cells, differentiated cells or the ones that are post-mitotic. Within such diversity, a group of cells has been drawing the utmost attention, these are known as cancer stem cells (CSCs hereafter). According to the concept of cancer stem cells, these cells shelter some of the characteristics of tissue resident stem cells, which possess the self-renewal ability and potential to differentiate into more determined cell types, which will be investigated in detail in the following sections ⁴⁵.

1.2.1 Biology of Cancer Stem Cells

Presence of heterogeneity in tumors was long known, yet with the revisiting of this heterogeneity in leukemia fired back the attention on these cells. Engraftment studies on acute myeloid leukemia (AML) have shown that grafts could be established only in the presence of $CD34^+CD38^-$ cells, even more fascinating result was the determination of the responsible fraction which was calculated to be one in a million of tumor cells ⁴⁶. Application and translation into the solid tumor has come a bit later with the study in solid breast cancer tumors. This work has enlightened the case for bulk tumors, and a few $CD44^+CD24^-/low$ cells were sufficient to form tumors in transplanted xenografts, although thousands of other types were not able to drive the same effect ⁴⁷. There are two commonly proposed theories on CSCs, one is 'stochastic model' and the other one is 'hierarchy model'. In stochastic model, all the cells in a tumors are assumed to be "biologically equivalent", meaning they all hold the same potential to form a tumor and only differentiate in the on-time transcriptional factor levels, epigenetic regulations active within the present time and this state is not temporary, giving rise to an unpredictable state of cells in a tumor. Whereas the hierarchy model suggest that a tumor mass has certain stem cells of its own which will both give rise to its own clones, along with it a fraction will differentiate into less-potent cell types ⁴⁸.

The most prominent and suggested aspect of CSCs is their resistance against multiple therapeutic approaches, including radiotherapy and chemotherapy and studies show

their resistance is owed to having ABC drug pump expression, anti-apoptotic protein expression and resistance to DNA damage ⁴⁹. As expected it should be noted that this state of resistance to chemo and/or radiotherapy is not universal for all cancer types ⁵⁰.

Another suspect against CSCs is on their responsibility for tumor relapse upon surgical removal, or assumed clearance after chemo-or-radiotherapy. Even recurrences at different locations were detected, which opens the next characteristic; their role in metastasis. In pancreatic tumors, a subpopulation of CSCs, which were identified by the markers $CD133^{+}CXCR4^{+}$, were shown to be responsible for the type of tumors, it was the same study, which offered that upon ablation of $CD133^{+}CXCR4^{+}$ subpopulation metastasis ability in the cell lines were halted, although tumorigenesis was maintained ⁵¹.

1.2.2 Selected Markers of Cancer Stem Cells

Marker selection in determination of CSCs is one of the essential steps in pursuit of their presence and metabolism. Along with having various markers, marker definition is still a controversy between scientific communities. The markers for CSCs, in its essence, are also the markers used in the detection of tissue resident adult stem cells. CD34 and CD38, as examples, are very well-established hematopoietic stem cell markers, and are the most trusted markers of leukemia ⁴⁶. CD133 (Prominin-1) is a widely accepted CSC marker found in the stem cells of different tissues and organs, such as colon and brain ⁵². Figure 1.3. summarizes the percentage of potential CSCs and Table 1.1 describe function and phenotypes of commonly used cancer stem cell markers for hepatocellular carcinoma.

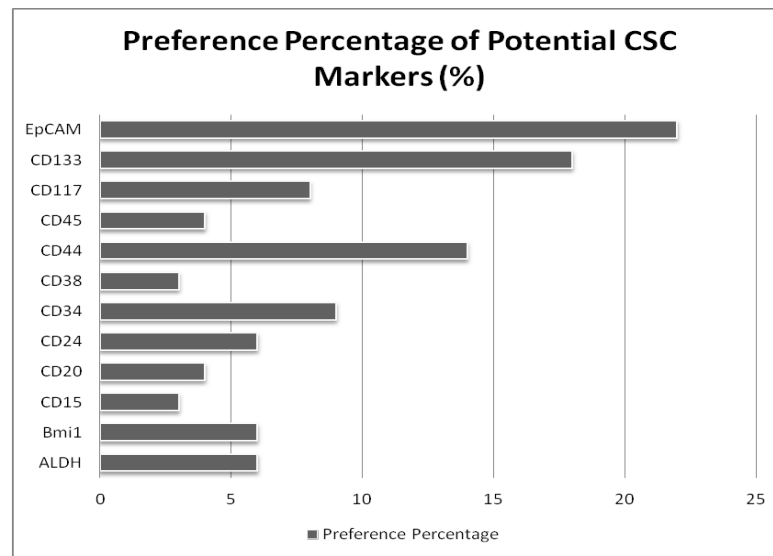


Figure 1.2: CSC marker preference percentage by the scientific communities (adapted from Razvi, 2014 ⁵³).

Among these markers, CD133 and EpCAM are the most frequently preferred and the most trusted markers for HCC ^{54–58}. CD133, within its origin, is a pentaspan membrane protein and is the first member to be identified in the prominin family of proteins, which specifically gives the name for it, Prominin-1 ⁵⁹. Although its exact association with a certain pathway is not established yet, expression in specific type of cells was observed, as an instance, CD133 expression was considered to be the co-expressed marker for CD34+ hematopoietic stem cells ⁶⁰.

Table 1.1: Commonly used cancer stem cell markers for hepatocellular carcinoma ⁵⁴.

Cell Surface Marker	Suggested Function in CSCs	Phenotypes of CSCs
CD13	ROS-induced DNA damage reduction	Tumorigenic, cell cycle arrest, chemoresistant
CD24	STAT3-mediated Nanog regulation	Tumorigenic, chemoresistant
CD44	Regulation of redox status	Tumorigenic, invasive
CD90	Unknown	Tumorigenic, metastatic

CD133	Neurotensin/IL-8/CXCL1 signaling	Tumorigenic, chemoresistant
DLK1	Unknown	Tumorigenic, chemoresistant
EpCAM	Activation of Wnt signaling	Tumorigenic, invasive, chemoresistant
OV6	Unknown	Tumorigenic, invasive, chemoresistant,

There has been several studies indicating involvement of CD133 expression in CSCs, in one of which CD133+/CD34+ cells has shown to be responsible for a rather improved clonogenic capacity when engrafted ⁶¹.

An alternative study suggested that CD133+/CD44+ double positive cells were more resistant to chemotherapy due to increased ABC super family transporters in HCC ⁵⁵.

EpCAM (CD326), an epithelial cell adhesion molecule, is initially identified as a liver stem/progenitor cell marker, found in oval cells ⁶². EpCAM association with CSCs is described with numerous studies, one of which states the concentrated presence of EpCAM+/AFP+ cell population at the tumor invasion border in HCC, and HCCs with such phenotype exhibit poorer survival. Moreover, EpCAM is shown to be a target for Wnt signaling pathway, and the study has shown that activation of beta-catenin has increased the spheroids formed in soft agar in HCC cell lines ⁶³.

1.2.3 Cancer Stem Cells and Hepatocellular Carcinoma

As seen in other cancer types, CSC markers were also detected in HCC samples and cell lines (Table 1.1). Cancer-stem-cells of the liver and/or HCC may expresses markers in different combinations depending of the cell lines or the sample. Yet, the most common feature of this population is to have the ability to form engrafted tumors upon injection in mice ⁶⁴. One of the clearest ways to point out the existence of CSC in HCC is through markers

Normally, hepatoblasts, the progenitor cells of hepatocytes and cholangiocytes, express cytokeratin-19 (CK19), EpCAM and AFP ⁶⁵. CSCs and liver stem cells also share a set of markers, for instance although EpCAM and CD133 are normal liver

stem cell markers, their double positivity in HCC correlates with the differentiation ability of the cells, colony formation ability and resistance to chemo-therapy⁵⁶. Another study has shown the increase in tumorigenicity *in vivo* upon transplantation of CD133+/ALDH+ cells compared to their CD133-/ALDH+ or CD133-/ALDH- counterparts⁵⁸. Several models were proposed to describe tumor initiation model for HCC. Following scheme encompasses these models (Figure 1.4)

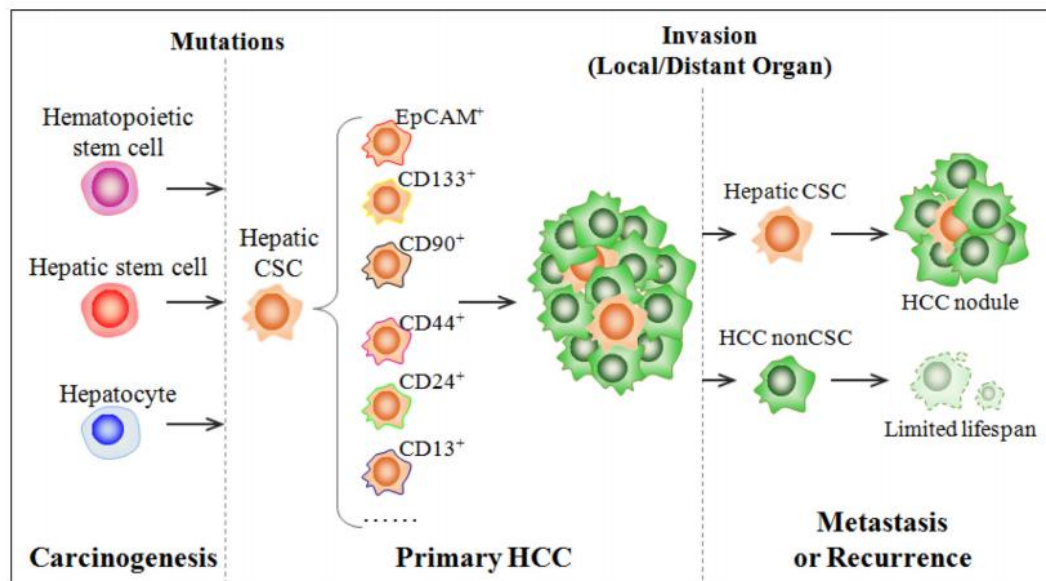


Figure 1.3: CSC based tumor initiation model in HCC (adapted from Ji & Wang 2013⁶⁶)

1.2.4 Signaling Pathways in CSCs and HCC

Multiple efforts were consumed on defining the signaling pathways that differentially regulated during CSC reprogramming. The need for plenty of additional studies to separate mingled pathways is inevitable, yet existence of a complex cross-talk of pathways was observed in the process of CSC formation⁶⁷. Numerous signaling pathways involving i)Wnt, ii)Notch, iii)AKT, iv)transforming growth factor beta (TGF-beta) and v)STAT3, were shown to be altered compared to their natural states. Such a diverse variety may be linked to the alternative origins of CSCs⁶⁶.

Wnt/ β -catenin pathway has significant roles during development, stem cell maintenance and its involvement in CSC metabolism, and currently under the lights

of new findings, it is gaining more and more importance. There are studies showing the activity of Wnt/ β -catenin signaling in hepatic CSCs maintenance⁵⁴. What's more, is activation of Wnt signaling was detected to be increasing EpCAM+CSC levels in HCC^{68,69}.

Altered TGF- β signaling through activation of IL-6/STAT3 system in hepatic progenitor cells lead to misguided differentiation and progress into HCC⁷⁰. In terms of CSC population, TGF- β pathway was indicated to control CD133+ cell population in HCC, additionally, chemo-resistant character of EpCAM+ CSCs was affected by IL-6/STAT3 activity^{71,72}.

AKT signaling activation was found to be attributing to the chemo-resistant nature of CSCs. Hypoxia-inducible factor1- α (HIF1 α) production was observed under hypoxia conditions by the activity of Akt signaling, which later forms a positive feedback mechanism. Following HIF1 α expression, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) upregulation, both of which are known to be effective in chemo-resistance, in addition, Akt signaling trigger a set of mechanisms which eventually lead to increased expression of ABCG2 (a drug efflux transporter)^{71,73}. Interaction between AKT, TGFB, IL6/STAT3 and WNT/B-Catenin axis is summarized in Figure 1.5. Combined intricate actions of these pathways leads to i) self-renewal, ii) differentiation, iii) tumorigenicity, iv) chemo-resistance thereby culminating into increased v) cell survival.

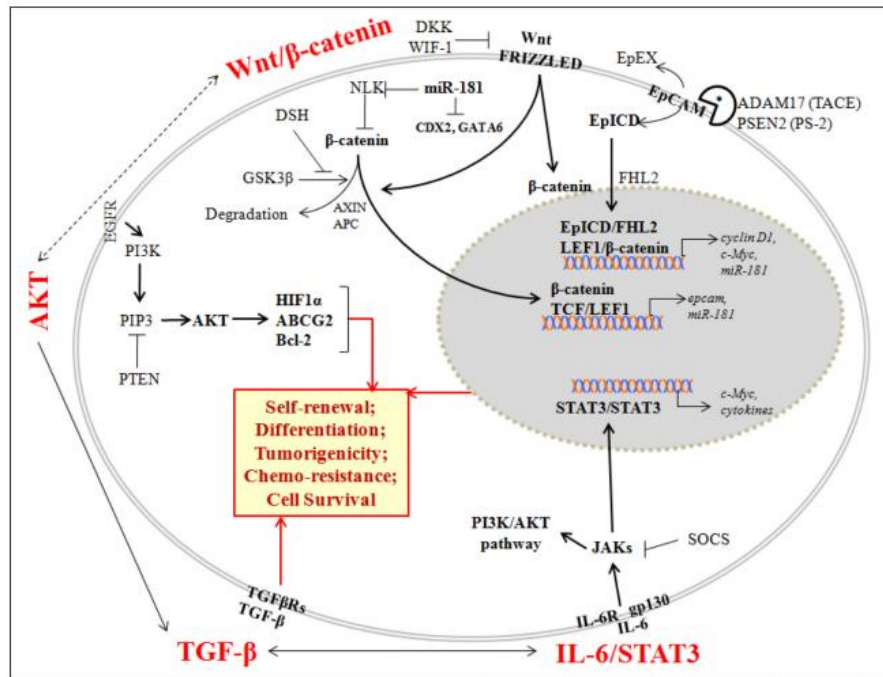


Figure 1.4: Signaling pathways involved in hepatic cancer stem cells (adapted from Ji & Wang 2013⁶⁶).

1.3 Notch signaling pathway

Knowledge on Notch signaling pathway lay down its roots back in 1900s. Although having been worked on almost a century now, there is plenty of questions yet to be answered on involvement of Notch signaling in multiple biological events. Notch gene was discovered, originally, in fruit fly, *Drosophila melanogaster*, in a mutated form it leads to a nicked wing margin phenotype, and inherited as a sex-linked mutation^{74,75}. Notch receptor family involves 4 members (Notch1-4) in mammals, all of which are produced in precursor form and cleaved through furin-like convertase, also known as, S1 cleavage, to become mature receptor⁷⁶. Mature form of the receptor is a combination of its two subunits, one is facing the extracellular domain (ECD), and the other has a trans-membrane, as well as an intracellular domain (ICD, later will be referred as Notch ICD (NICD)). ECD possess several repetitive regions including epidermal growth factor (EGF) -like repeats and LIN2 and Notch repeats (LNRs), enables signaling only in the presence of the ligand, NICD has PEST domain and is the activated form⁷⁷, (Figure 1.6).

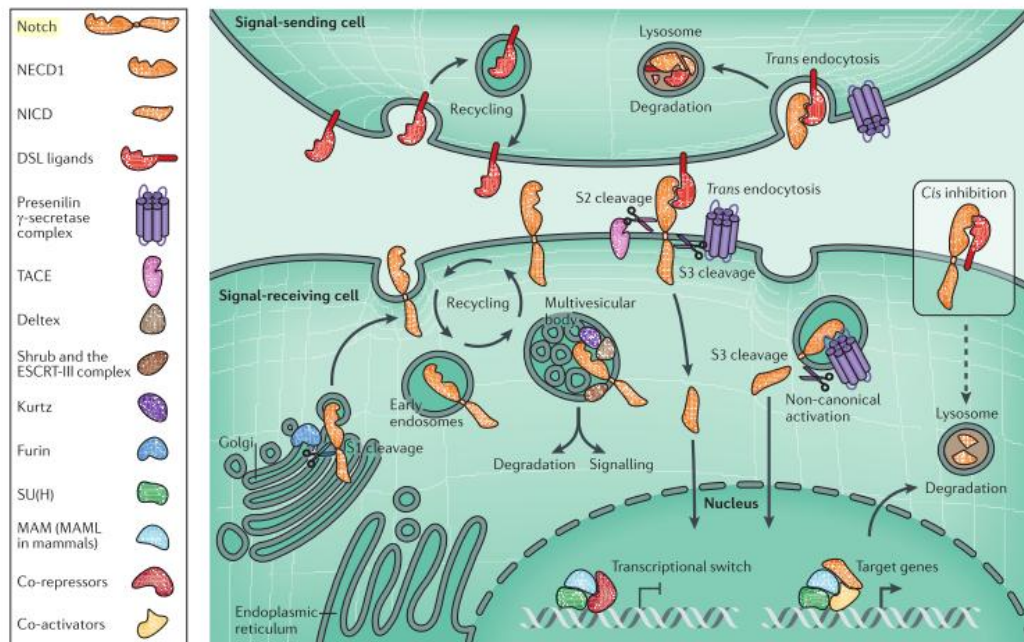


Figure 1.5: Notch signaling pathway (adapted from Guruharsha et al., 2012⁷⁸)

A specific feature of Notch signaling is its activation only in the presence of a physical cell-to-cell contact through the ligands of the neighboring cell. These ligands are known as DSL (Delta-Serrate-LAG2) proteins and the members of DSL family of proteins in mammalian cells are jagged-1(JAG1), jagged-2(JAG2), Delta-like-1(DLL1), DLL2 and DLL3⁷⁶. Binding of the receptor to the ligand ends up with a conformational twist, which makes the ECD susceptible to metalloproteinase tumor necrosis factor alpha converting enzyme (TACE or ADAM17) and results in S2 cleavage. This cleavage triggers the next cleavage and subsequent release of NICD into the cytoplasm. S3 cleavage is mediated by presenilin- γ -secretase complex sheltering nicastrin (NCSTN), presenilin 1 and 2 (PSEN1, PSEN2), anterior pharynx defective 1 (APH1) and presenilin enhancer 2 (PEN2)^{77,78}.

Following S3 cleavage NICD translocates to nucleus and form a complex with CBF1-Su(H)-LAG1 (CSL) and replaces its repressor activity into a transcriptional activator by recruiting Mastermind-like 1 (MAML1). This activation leads to sequential events leading to expression of cyclin D1, Notch ligands and receptors and many more⁷⁹. In case of a turnover of the Notch receptor, it is phosphorylated at its PEST domain by ubiquitin E3 ligases such as SEL10 (FBXW7) to be directed to proteasomal degradation⁸⁰.

Notch signaling has pivotal roles during development, such as; i) cell-fate determination, ii) proliferation and iii) apoptosis, studies has shown the context dependent nature of Notch signaling ⁷⁷. Along with controlling stem cell maintenance, it directs neurogenesis by contact-inhibition, additionally has a crucial role during angiogenesis ^{81,82}. Involvement of Notch signaling in CSC reprogramming and HCC is also an essential part and will be touched upon in the following sections.

1.3.1 Notch signaling in CSCs and HCC

Integrative role of Notch signaling in the maintenance of the stemness state of the stem cells has been known from earlier studies (Figure 1.7). One of such kind of regulation is seen in neural cells, in control of HIF1-alpha Notch acts as a guard for the precursor form of the neural stem cells, keeping them at an undifferentiated state, in case of Notch loss medullablastoma tendency was observed ⁷⁹. Expectedly, upon halting of Notch signaling in these cells, precursor cells were observed to be differentiating, supporting the action of Notch in the maintenance of stemness ⁸⁰.

An alternative study has shown the role for the stem cell programming ability of Notch in which activation of Notch signaling by various ligands exerted its effects as an increase in mammary stem cells and a ten-fold boost mammosphere formation ⁸¹. These different illustrations of Notch activity in potential CSC like ability formation strengthens the view of involvement of Notch signaling in cancer stem cell reprogramming. There are some studies showing the aberration or in some cases inhibition of Notch signaling in HCC and other cancer types but not much is still known to discover the role of Notch in HCC ⁸².

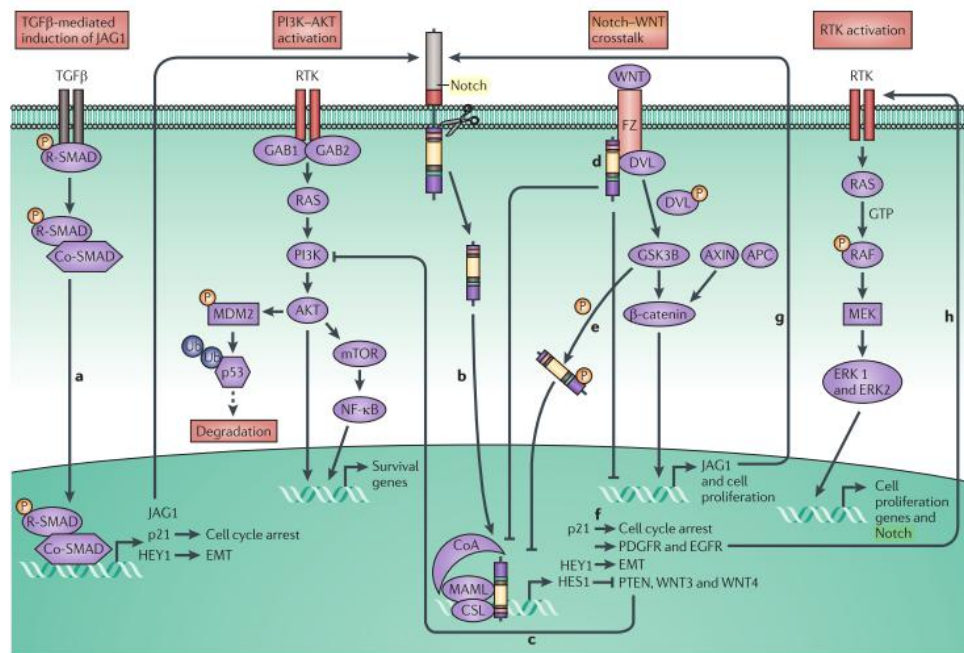


Figure 1.6: Notch signaling crosstalk with other signaling pathways in carcinogenesis (adapted from Ranganathan et al., 2011⁸³).

1.4 Involvement of Toll-like receptors in HCC

In consideration of humans, there are known ten types of toll-like receptors (TLR), which are known to be involved in the identification of pathogen associated molecular patterns (PAMPs), and this renders them to possess the name pathogen recognition receptors (PRRs)⁸⁴. TLRs can be simply categorized into two, one is formed by the cell-membrane located receptors that recognize modified lipids or protein based ligands (TLR1,2,4,5,6,10,11 and 12), and the second type is located at the endosomal compartments (TLR3, 7/8 and 9) and recognize mostly foreign nucleic acids⁸⁵. TLRs contain three major domains one is required for ligand binding another is for single membrane-passing and the third domain is cytosolic Toll/IL-1R (TIR) domain. Signal transfer occurs through two main downstream ways, first is called a Myddosome, which involves an adaptor protein dimerization with the receptor and is called myeloid differentiation primary response protein 88 (MyD88) that activates nuclear factor-kappa B (NF-κB). Another complex is Trifosome that involves interferon (IFN)-regulatory factors (IRFs) which bind to TIR domain containing adaptor protein inducing IFN-beta (TRIF)⁸⁵. Each TLR is induced by a

different agent, TLR2 heterodimerizes with TLR1 and TLR6 and recognizes triacyl or diacyllipoproteins^{86,87}. TLR5 homodimerize upon bacterial flagellin presence⁸⁸ and TLR4 is activated by lipopolysaccharide (LPS)⁸⁹. TLR7 TLR8 and TLR9 are located on endosomes, and although TLR7 and TLR8 are activated by single-stranded RNAs (ssRNAs), TLR9 responds to unmethylated CpG oligonucleotides and finally TLR3 respond to double-stranded RNA⁹⁰⁻⁹³. Although there are not many studies involving TLRs and hepatocellular carcinoma, some studies suggest that TLR4 induction, for instance, in HCV bearing mice, upon alcohol exposure led to HCC, in which the stemness have found to be increased when CD133 levels were investigated⁵⁴. A very recent study shows the potential involvement of TLR7 and TLR9 on their ability of HCC tumor formation in mice⁹⁴. Yet, the effects of TLRs on cancer microenvironment in HCC or tumor therapy is yet to be investigated.

1.5 Cancer-immunotherapy

Molecular and individual identity of a tumor is unique, and through making use of this concept immuno-therapies are trying to be developed against multiple cancer types. These so-called "cancer vaccines" are aimed to direct the fighters of the immune system to prevent tumors from expanding and growing. There are several advantages of the usage of cancer vaccines, accumulating knowledge on viral based vaccines showed the world how immune system works to treat the malignancies within itself, and this strategy supplied a blueprint for the immunization for cancer antigens⁹⁵. There are challenges, of course, within this strategy, antigen promoting must be achieved by dendritic cells, immune suppression by the tumor microenvironment must be overcome with, and protective T cell responses should be activated⁹⁶ along with NK cell activity in cases missing self is an issue.

TLR agonist usage in cancer immunotherapy is recently becoming a preferred topic. TLR agonists lead to immunostimulatory effects, which could be activation of co-stimulatory molecules (CD86, CD40 and CD80) that are located on dendritic cells (DCs). Moreover, they induce the inflammatory cytokine (TNF α and IL-12) secretion and polarize T cells to assume Th1 fate⁹⁷. By using these immunostimulatory effects of vaccines can be developed with the addition of desired

cocktails to treat the tumor masses. This technique is called "Combination immunotherapy" and it makes use of gathering different agents such a signaling pathway inhibitors, immunostimulatory molecules, or cell based therapies together. By making use of the immunostimulatory potential of small molecules in combination with TLR ligands, a better approach to overcome the immunosuppressive environment existing at the tumor niche could be made possible⁹⁸.

1.6 Aim of the Study

Cancer stem cells are shown to be present in solid tumors of various cancer types⁹⁹. Although forming only a small subpopulation in a tumor, cancer stem cells are known to be responsible for chemo-and-radio-therapy resistant disposition of cancer, along with being in charge of the recurrences upon surgical removal of a tumor mass¹⁰⁰. Existence of CSCs in HCC is also known, which can be explanatory for the chemotherapy and radiotherapy resistant nature of HCC tumors⁶⁶.

Notch signaling aberrations are detected to be present in HCC. Involvement of Notch signaling in stem cell regulation, mostly in favor of the maintenance of stemness during embryogenesis and also abnormal deregulation of Notch activity in CSCs of several cancer types, directed us to interrogate the activity of Notch signaling in HCC cancer stem cells⁷⁸.

Importance of TLRs in the therapy of solid tumors are gaining more attention with the piled up evidence of recent studies⁹⁷. Immunostimulatory effects of TLR ligands will be analyzed to eradicate large tumor mass. In this study, expression levels of TLRs in HCC cell lines were investigated to decide on the potential applicability of TLR ligand therapy *in vivo* by checking the effects of TLR ligands on expression levels of TLRs in cell lines.

Upon completion of this work, existence of the CSCs in certain HCC cell lines and the role of Notch signaling on cancer stem cell population will be characterized in order to propose a solution to treat HCC. The ultimate goal in this study was to establish a cancer immunotherapy method, depending on the *in vitro* data acquired from Notch experiments on cancer stem cells and the data showing the possible

candidate TLRs to be upregulated in cell lines upon stimulation, to achieve tumor shrinkage or even eradication.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. General Laboratory Reagents

The reagents highly used in the experiments including Ethanol, Bradford reagent and methanol, haematoxylin were analytical grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA). ECL Prime western blotting detection kit and Hybond nitrocellulose western blot membranes were purchased from Amersham Pharmacia Biotech Company. DMSO and Ponceau S were bought from Applied Biochemia (Darmstadt, Germany). Fixative Medium A was purchased from Invitrogen (GAS003, Carlsbad, CA, USA).

2.1.2. Tissue Culture Reagents and Materials

Media used in cell culture including Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium and OptiMEM were purchased from GIBCO (Invitrogen, Carlsbad, CA, USA). Supplemental reagents such as Penicillin/streptomycin antibiotics, L-glutamine, Non essential amino acids (NEAA), fetal calf serum (FCS) and Trypsin-EDTA were also purchased from GIBCO. All of the plastic materials used in cell culture like flasks, Petri dishes, multi-well-plates, cryotubes were purchased from Corning Life Sciences Inc. (USA). All sterile serological pipettes were purchased from Costar Corporation (Cambridge, UK).

2.1.3. Polymerase Chain Reaction (PCR)

OneTaqMM Quick-Load 2XMM w/Std buffer was used as a ready-to-load master mix.

2.1.4. Primers

Primers used throughout this study were designed by using Primer Designer 3.0 Program (<http://frodo.wi.mit.edu/primer3/input.htm>). All the primer pairs were blasted against the human genome (<http://www.ncbi.nlm.nih.gov/BLAST/>)

Table 2.1.: Primers pairs involved in this study are enlisted as depicted below.

Gene name	Forward Primer	Reverse Primer
hTLR1	5'-CATAACTCTGCTGATCGTCACC-3'	5'-TGCTAGGAATGGAGTACTGCG-3'
hTLR2	5'-GATGACTCTACCAGATGCCTCC-3'	5'-CAGAAGAATGAGAATGGCAGC-3'
hTLR3	5'-CTCAGAAGATTACCAGCCGC-3'	5'-TTCTAGTTGTGGAAGCCAAGC-3'
hTLR4	5'-TTACCTGTGTGACTCTCCATCC-3'	5'-CAGAAGAATGAGAATGGCAGC-3'
hTLR5	5'-CCTTGACTATTGACAAGGAGGC-3'	5'-TTGTAGGCAAGGTCAGAAC-3'
hTLR6	5'-TCTCATGACGAAGGATATGCC-3'	5'-CGATCAGCAGAGTTATGTTGC-3'
hTLR7	5'-ACGAACACCACGAACCTCAC-3'	5'-GGCACATGCTGAAGAGAGTTAC-3'
hTLR8	5'-TGGCTTGAATATCACAGACGG-3'	5'-ACCAGGCAGCATTAACTCTCC-3'
hTLR9	5'-CAACAACCTCACTGTGGTGC-3'	5'-GAGTGAGCGGAAGAAGATGC-3'
hTLR10	5'-GAACTGATGACCACCTGCTCC-3'	5'-GAAGTCTTGATTCCATCACGC-3'
hGAPDH	5'-ACCACCATGGAGAAGGCTGG-3'	5'-CTCAGTGTAGCCCAAGGATGC-3'

2.1.5. Agarose Gel Electrophoresis

Horizontal gel electrophoresis apparatus was Thermo EC Midicell Primo EC330. The power supply and electrophoresis gel system were EC250-90, they were both from Thermo Scientific (MA, USA). Alternative power supplies used in experiments were Power-PAC300 and Power-PAC200, both from Bio-Rad Laboratories (CA, USA).

2.1.6. Spectrophotometry

Protein concentration measurements of Bradford assay were done by using Beckman Du640 spectrophotometer from Beckman Instruments Inc. (CA, USA).

2.1.7. Determination of Gene Expression

2.1.7.1. Total RNA Isolation

Phenol-Chloroform extraction method was followed for RNA isolation from cultured-cells.

2.1.7.2. First Strand cDNA Synthesis

ProtoScript M-MuLV First Strand cDNA synthesis kit from New England Biolabs, Inc. (MA, USA) was used for the first strand cDNA synthesis.

2.1.8. Antibodies

Antibodies, both primary and secondary, were purchased from a variety of incorporations. Information denoting the catalog number, working conditions, and company name were listed in Table 3.

Table 2.2: Listed version of the antibodies used in this study

Antibody Name	Company and catalog number	Western Blot Dilution	Immunostaining Working Dilution
Calnexin	Sigma-Aldrich, C4731	1:5000	

α -tubulin	Calbiochem, CP06	1:5000	
Anti-mouse-HRP	Sigma-Aldrich, A0168	1:5000	
Anti-goat-HRP	Abcam, Ab6741	1:5000	
Anti-rabbit-HRP	Sigma-Aldrich, 6154	1:5000	
β - Actin	Sigma, A5441	1:10000	
Anti-mouse/rabbit Alexa Fluor 488	Invitrogen, A11034		1:750 (IF)
Anti-mouse/rabbit Alexa Fluor 586	Invitrogen, A11034		1:750 (IF)
Sox9	Millipore, AB5535	1:1000	1:1500 (IF)
HNF4 α	Santa Cruz Biotechnologies, sc6556	1:300	1:150 (IF)
CD133 (pure)	Miltenyi, 130-090-422		1:100 (IF/IP)
CD133-APC	Miltenyi, 130-090-826		1:30 (Flow Cytometry)
EpCAM-FITC	Miltenyi, 130-080-301		1:30 (Flow Cytometry)
Annexin V - FITC	BD Pharmingen, 556419		1:20 (Flow Cytometry)
Propidium Iodide Solution	BioLegend, 421301		1:10 (Flow Cytometry)

2.1.9 Immunoperoxidase Assay Reagents

DAKO Envision + system was purchased from DAKO (Glostrup, Denmark).

2.1.10 TLR Ligands

TLR ligands used in this study were supplied from several companies. Lipopolysaccharide (LPS) (isolated from *E.coli*; Sigma, USA), peptidoglycan (PGN) (isolated from *B.subtilis*; Fluka, Switzerland), pI:C (Amersham, UK), R848 (Invivogen, USA), LTA (Invivogen, USA), Resiquimod (Invivogen), K23 (Alpha DNA, Canada), Flagellin (Sigma, USA).

Sequence for K23 is as follows:

K23 (12mer) : 5'-TCGAGCGTTCTC-3', phosphoryl(thio) manufactured.

2.2. Solutions and Media

2.2.1. General solutions

50X Tris-Acetate-EDTA Buffer	242 g Trisma base, plus 100 mL of 0.5 M EDTA (pH: 8.0), plus 28.55 mL of Glacial Acetic Acid were combined and overall completed to 1L with ddH ₂ O. Working dilution: 1X
10X Tris buffered saline (TBS)	12.9g Trisma base and 87.76g NaCl combined in 1 L ddH ₂ O. pH adjustment : 8.0. Working dilution: 1X. 12.1 g of Trisma was dissolved in 100mL of ddH ₂ O. pH adjustment : 6.8

1,5M Tris HCl	18.1 g Trisma base was dissolved in 100 mL of ddH ₂ O.
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pH adjustment: 8.8.

10X Phosphate Buffered Saline (PBS)	80g NaCl, 14.4g Na ₂ HPO ₄ , 2g KCl, 2.4g KH ₂ PO ₄ were combined in 1 liter of ddH ₂ O.
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Working dilution: 1X.

2.2.2. Tissue culture solutions

DMEM/RPMI media	In order to prepare complete medium 1% Non-essential amino acids (NEAA), 1% Penicillin/Streptomycin, 10% Fetal Calf Serum (FCS) all added to supplied commercial medium. Keep at 4°C.
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10X Phosphate buffered saline (PBS)	80g NaCl, 14.4g Na ₂ HPO ₄ , 2.4g KH ₂ PO ₄ , 2g KCl were all dissolved in 1 L ddH ₂ O.
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Working dilution: 1X.

Storage Temperature: 4°C.

2.2.3. Immunofluorescence Staining Solutions

4% Paraformaldehyde	4g paraformaldehyde was dissolved in 100mL ddH ₂ O, solution heated at 130°C for 1 hour. Let cool down before use.
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4% Formaldehyde	100mL of 40% Formaldehyde was dissolved in 1L ddH ₂ O.
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Blocking solution	0.2% PBS Tween-20 with 10% FCS
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Washing buffer	0.2% Tween-20 dissolved in 1XPBS.
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DAPI (4',6-diamidino-2-phenylindole) Stain	Prepared as 1µg/ml solution in ddH ₂ O.
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2.2.4. Immunoperoxidase Staining Solutions

Fixation solution	1:1 ratio solution of Acetone: Methanol, mixed and stored at -20°C
Blocking solution	10%FCS and 0.3% TritonX-100 solution prepared in 1XPBS.
Washing solution	0.3% TritonX-100 dissolved in 1XPBS
3% H ₂ O ₂ solution	Prepared as 6mL 30% H ₂ O ₂ added to 20mL methanol and 34mL ddH ₂ O solution.
DAB reagent	Chromogen and substrate were mixed as described in DAKO Envision + Dual Link System-HRP kit.

2.2.5. Sodium Deodecyl Sulphate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblotting Solutions

10X Tris buffered saline (TBS)	12.9g Trisma base + 87.76g NaCl in 1L of ddH ₂ O. pH adjustment: 8.0 Working dilution: 1X.
10X SDS Running buffer	144g Glycine + 30g Trisma base and 50mL of 10% SDS were mixed in 1L ddH ₂ O. Working dilution: 1X.

10X Transfer buffer	72g Glycine + 58g Trisma base + 2mL of 10% SDS dissolved in 1L ddH ₂ O. Working dilution is 1X, and this solution includes 10-20% Methanol (depending on the experiment setup).
TBS-Tween 20	0.2% Tween 20 dissolved in 1X TBS.
Ponceau S	0.1% (w/v) Ponceau S and 5% (v/v) acetic acid prepared in ddH ₂ O.
Blocking solution	0.2% TBS-Tween 20 was prepared. Non-fat dry milk or bovine serum albumin (BSA) was added to the solution to get 5% (w/v) final ratio. (Usage of dry milk or bovine serum is determined according to the antibodies.)

2.2.6. Preparation of DAPT and rhJagged-1

DAPT was purchased from Sigma-Aldrich Chemie GmbH (MO, USA). Recombinant human Jagged-1 Fc Chimera was purchased from R&D Systems (MN, USA). Recombinant human Jagged-1 was reconstituted at 200µg/mL in sterile PBS as described in manufacturer's protocol. Working concentration for rhJagged-1 was 50ng/ml. DAPT was reconstituted at 18mg/mL in sterile DMSO. Working concentration was 50uM.

2.3. Methods

2.3.1 Tissue Culture Methods

2.3.1.1 Cell Lines and Growth Conditions of Cells

Various hepatocellular carcinoma cell lines were studied throughout this work. Some of these cell lines including FLC4, FOCUS, Hep3B, Hep40, HepG2, Huh7,

PLC/PRF/5, Mahlavu, and SK-HEP-1 were cultured in DMEM. Rest of the cell lines SNU182, SNU387, SNU398, SNU423, SNU449, and SNU475 were kept in RPMI medium. Supplemental media including 10% fetal calf serum, 1XNEAA, 2mM L-Glutamine and 100 units of Penicillin/Streptomycin were added to DMEM and RPMI to acquire their complete forms. Cultured cells were incubated at 37°C with 5% CO₂ maintenance. Before usage, all media were pre-heated to warm up until 37°C.

2.3.1.2 Passaging Cells

Adherent HCC cell lines were passaged before reaching complete confluency. Following the transfer of plates from incubator to hood, the old media was aspirated and washed once with sterile 1XPBS. Having been washed with PBS, and the consequent aspiration of PBS, Trypsin-EDTA was dropped equally over the cells to make them detach from the surface. With the addition of Trypsin-EDTA plates were put back into the incubator, which was set for 37°C with 5% CO₂ maintenance. Upon detachment of the cells from the bottom of the plates, fresh sterile media was added onto the cells to halt the action of trypsin (meanwhile, it is important to keep cells in Trypsin as short as possible to prevent any damaging to cell surface). With pipetting, cells were distributed as single cells and divided into different dilutions according to the experimental requirements.

2.3.1.3 Thawing and Cryopreservation of Cells

Thawing of cells required important amount of caution, upon removal of cells from liquid nitrogen, cells were placed on ice to prevent their contact from warm air. Cryotubes were immediately put in water bath, which was pre-heated to 37°C. After the ice in the vials melted, cells were transferred into a 15mL falcon tube having 5mL of the preferred media. By pipetting the cells were carefully gotten rid of DMSO, which is toxic to cells. Then, the falcon was centrifuged at 1600rpm for 4 minutes. After centrifuge supernatant was aspirated and the pellet was re-suspended with fresh complete media, and put in petri dish. The dish was then placed into the incubator, which was set for 37°C with 5% CO₂ maintenance.

Cells reaching around 60-80% confluency were counted as “freezable”. For this process, old media was aspirated and washed once with 1XPBS. Upon aspiration of PBS, Trypsin-EDTA solution was dropped over cells, and plates were incubated at 37°C for 5 min. After collecting trypsinized cells in a falcon, cells were centrifuged at 1500 rpm for 4 mins and the supernatant was aspirated. In the meantime, a “freezing media” containing 70% of the preferred medium, 20% FCS and 10% DMSO was prepared. This media was placed on the cells and following re-suspension cells were transferred into a cryovial labeled beforehand. The cryovial was placed in -20°C for 45-60 min. The vials were then transferred to -80°C fridges. Within 7-10 days cells were placed into liquid nitrogen in order to store them for longer periods of time.

2.3.1.4 Treatment of Cells

Treatment of the cells has taken place in differential conditions from serum free media to complete media. For the marker assays, either 24 or 72 hours after cell seeding the complete media was removed, washed once with 1XPBS and replaced with media containing 0.001% FCS with 1mM Na₂SeO₃. For Jagged-1 and DAPT treatments, after preparing the components in desired concentrations, cells were treated after 24 or 72 hours following cell seeding, with their controls such as PBS or DMSO. In case of TLR ligands experiments, cells were seeded, waited overnight and treated with different TLR ligands, 4 hours following treatments, cells were trypsinized and prepared for RNA isolation.

2.3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze the products of PCR analysis. For this process, Agarose was weighed to be 1.5% (w/v) and dissolved in 1X TAE buffer by melting in microwave. Having gone through the melting step, EtBr (10µg/mL) was mixed with the agarose gel, and poured into the tray; appropriate comb was chosen and placed to the tray, as well. The gel was left for cooling under fume hood until it solidifies. Following solidification, the comb was removed and the tray is placed in the gel tank previously filled with fresh 1X TAE. Since the PCR products

were already ready-to-load products, samples were loaded into the gel in desired order. A DNA ladder was used as a marker to understand the band pattern. Following loading the tank lid was closed and run with 100V until the desired band separation was achieved. With the end of running, the gel was taken under visualization device, which uses Chemi-Capt software for image acquisition (Vilber Lourmat, Paris, France).

2.3.3 Total RNA Extraction from Cultured Cells

Before isolation cells were scraped and pipetted with cold media, and then cells were transferred into a 15mL falcon tube. The tube was centrifuged at 2500rpm for 5 min. at 4°C. The supernatant was aspirated and 1mL Trizol was added on top of the pellet. Up-down the cells thoroughly, at this stage cells could be stored at -80°C for 1 week, if desired. Upon the addition of Trizol cells were mixed and transferred to a 1.5 mL eppendorf tube, 200µL of chloroform was added to the mixture. Shaken vigorously for 15sec, and left at room temperature for 3min. Next phase-separation step takes place, for this tubes were centrifuged at 13200rpm for 17min. at 4°C. Next was an important step, only the top phase was taken without disturbance to any other layers, which would result in DNA, and cellular content contaminations. The top-phase was transferred into a new eppendorf and 500µL isopropanol was added and mixed gently, left to sit at RT for 10min. Then, the tubes were centrifuged at 14000rpm for 10 min at 4°C. Following centrifugation, isopropanol was removed and 1mL 75% EtOH was added to the tube, vortex shortly, until the white flake moved from its place. The tube was centrifuged at 8000rpm for 8min. at 4°C. Next, 75% EtOH was removed and 99.8% 1mL EtOH was added to the tube and vortexed shortly and put in centrifuge at 8000rpm for 8 min. at 4°C. Having gotten rid of the EtOH pellet was put in a container and placed under fume hood to remove remaining EtOH. After 10-15min. the pellet was dissolved in 20-30µL of Nuclease-free ddH₂O. RNA samples were checked under Nanodrop 2000 for their purity and contamination scores and transferred to -80°C for storage.

2.3.4 First Strand cDNA Synthesis

For cDNA synthesis, ProtoScript M-MuLV First Strand cDNA synthesis kit from New England BioLabs, Inc. (MA, USA), was used by following manufacturer's protocol. For this procedure, 1µg of total RNA was mixed with 2µL of Oligo d(T)₂₃VN primer and the total volume was completed to 8 µL by Nuclease-free H₂O. Tubes were spun down for 3-5 sec. and put in the machine for pre-denaturation program which was set to 70°C for 5 min. After program ending, a mixture of 10µL M-MuLV Reaction Mix and 2µL M-MuLV Enzyme Mix were added onto the tubes, spun down for 3-5sec and put in the machine with cDNA program, which was set as 42°C for 60min., 80°C for 5min., and 4°C for 10min. which made a cycle 35 times. After synthesis cDNAs were stored at -20°C for short-term store.

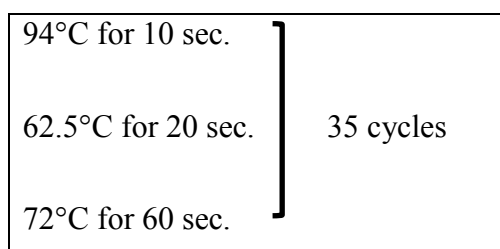
2.3.5 PCR

For PCR OneTaq^{MM} Quick-Load 2XMM w/Std Buffer from New England BioLabs, Inc. (MA, USA) was used. The protocol for PCR sample preparation is shown in Table 2.3.

Table 2.3: Reagents used in PCR preparation.

Reagent	Volume
2xDyNAzyme II Master Mix (F-508)	12,5 µL
Nuclease-free H ₂ O	9.5 µL
10pmol Forward Primer	1 µL
10pmol Reverse Primer	1 µL
cDNA	1 µL
Total Volume	25 µL

Human TLR primer Running conditions are:



After PCR samples were loaded into agarose gels as desired.

2.3.6 Total Protein Extraction from Cultured Cells

Cultured cells were trypsinized, and centrifuged at 1600rpm for 4 min. Following centrifuge the pellet was dissolved in 1X PBS and tubes were placed on ice. RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH was adjusted to 8.0. Buffer should be prepared freshly and just before use, 1X Protease inhibitor (PI) cocktail must be added to the buffer) was added on top of the cells in a volume of 2:1 (RIPA: pellet volume) and the tubes were vortexed. Cells were left in ice for 30 min to be lysed and tubes were vortexed ever 5 min. When lysis was over samples were sonicated for 10 sec. at 4°C and transferred into 1.5mL eppendorf tubes and centrifuged at 13000rpm for 45-60 min. at 4°C. At the end of centrifugation supernatant of the tubes were taken into new 1.5mL eppendorf tubes. The samples were labeled and stored at -20°C for short term or -80°C for longer storage.

2.3.7 Western Blotting

Before start a western blot, the concentration of the proteins to be used must be determined and quantified. For this matter, Bradford assay was used to detect the total protein concentrations. Dependent on the need but, usually 30-40µg of protein was loaded to the gel generally prepared in 20-30µL, including 20X DTT and 5X loading dye. A mixture of these ingredients were prepared and put in 1.5mL eppendorf tubes, and consecutively placed in heater, which was pre-heated to 100°C and incubated for 10 min. At the end of 10 min. samples were placed on ice for 1 min. and just before loading they were spun down for 5-10 sec.

The concentration of a gel depended on the molecular weight of the proteins to be used, in this study most of the proteins had molecular weight between 50-90kDA,

heretofore 10-12% gels were used, though Stacking gel was 5%. The ingredients for 10% and 12% gels were as follows:

Solution Components	10% Gel (10mL)	12% Gel (10mL)
ddH ₂ O	4 mL	3.3 mL
30% acrylamide mix (pH:8.8)	3.3 mL	4 mL
1.5M Tris (pH : 8.8)	2.5 mL	2.5 mL
10% SDS	0.1 mL	0.1 mL
10% Ammonium persulfate	0.1 mL	0.1 mL
TEMED	0.01 mL	0.01 mL

For the preparation and running procedures, Bio-Rad Mini PROTEAN Tetra Cell system was used. Completing the preparation of the gels, they were transferred into mini tank, and upon transfer, the tank was filled with 1X running buffer, after which the comb was removed. Previously prepared samples were loaded into gel, and the power-supply was connected and set to 80V until the samples escaped the stacking gel, then the voltage was raised to 120V until the desired time. After running, gels could be either dyed by Coomassie Brilliant Blue staining or they could be put in transfer directly. Transfer procedure, in its very essence, aimed to transfer the proteins stuck in gel to a PVDF or nitrocellulose based membrane for immunodetection. For this, a wet transfer system by using the apparatus of Bio-Rad Mini Transblot Cell, the transfer was performed. Complete transfer buffer contained 1X Transfer buffer, 10-20% Methanol, and completing the rest with ddH₂O. Either nitrocellulose or PVDF membranes were prepared as 6.5X9cm², as well as Whatman papers. Two sponges, Whatman papers, membrane and gel were placed properly into the sandwich apparatus, which was then enclosed and placed into transfer tank filled with complete transfer buffer. Transfer was done at 100V for 90 min. Following transfer; Ponceau staining could be done in order to assess the efficiency of the transfer. Otherwise, blocking step was the next step; in which according to the antibody to be used the content of the blocking solution was changed. Blocking solution was either 5% non-fat dry milk or BSA which was dissolved in 0.2%

Tween-20 in TBS-T, and was performed as 1 hour at RT, though in some cases overnight shaking incubation at 4°C is also plausible. Next step was primary antibody incubation, for this manufacturer's protocols were followed, and if not worked properly, as set of optimization experiments were performed to get the best conditions. After primary antibody incubation, the membranes were washed with 0.2% Tween-20 in TBS-T as twice 5 min, 10 min and following twice 5 min. Then, secondary antibody incubation was performed as primary was done. Another set of washing steps was performed just as the previous one. Secondary antibody incubation was done in RT for 1 hour with gentle shaking. The last two steps were immunodetection of the membrane, and transfer of the signal to an X-ray film. For the preceding step, ECL Prime chemiluminescent detection kit was used and incubated for 5 min at dark, and then the membrane was hybridized with the film with differential time points in a dark room with X-ray developer.

2.3.8 Immunoperoxidase Assay

Immunoperoxidase assay was began with the fixation of the cells with 1:1 Acetone: Methanol fixation medium, incubated at -20°C for 10min. Next, wells were washed with 1X PBS for 5 min. on shaker. In order to halt the endogenous peroxidase activity to prevent it from interfering with the actual signal, 3% H₂O₂ was used and incubated for 10 min at dark at RT. Following incubation wells were washed with 1X PBS three times each for 5 min. with shaking. The latter step was blocking, blocking solution containing 10% FCS and 0.3% Triton X-100 in 1X PBS was put over cells plates were covered with stretch film and incubated at RT for 1 hour at dark. Primary antibody preparation was designed depending on the type of the protein as described in manufacturer's protocols. Primary antibody was prepared in blocking solutions and 100 µL of the solution was dropped over the cover slips and incubated overnight at 4°C. Next day, plates were washed once with 1X PBS-t (0.3% Triton X-100), and secondary antibody incubation was done by putting 80 µL from Dako EnVision + Dual Link system – HRP for 1 hour at dark. Then wells were washed with 1X PBS-t (0.3% Triton X-100) twice for 2 min each. Finally, DAB solution was put over the slides as 80 µL and waited until the color change. Wells were washed with distilled H₂O to stop the reaction. The last step was haematoxylin staining, which was also

known as counter staining. In this staining haematoxylin was put into each well and waited for 5min at RT, wells were washed three times with tap water. After washing steps were completed, slides were taken and in each, a drop of 90% glycerol was dripped, and coverslips were placed on these slides, then slides were visualized under light microscope to assess the signal.

2.3.9 Immunofluorescence Assay

Plates were taken out and the media was aspirated, wells washed once with 1X PBS for 5min with gentle shaking. 4% Formaldehyde solution was used for fixation, and put in wells and incubated at RT for 10 min. Wells were rinsed with distilled H₂O for once and washed with 1X PBS for 3min on shaker. Permeabilization step was the following step, in which cells were washed with 1X PBS/ 0.5% Saponine/ 0.3% Triton X-100 solution for three times each for 5min. with shaking. Washing of the cells was performed by 1X PBS for 3 min on shaker. In blocking step, the blocking solution was prepared as a mixture of 1X PBS/ 10% FCS/ 0.3% Triton X-100, and this solution was applied to cells plates were incubated for 1 hour at 37°C. After blocking wells were washed once with PBS and incubation of primary antibodies was taken place according to the preferred antibody concentrations. Incubation lasted for 1-4 hours at RT. Then, cell were washed with 0.3% Triton X-100 PBS three times for 5 min with shaking. For secondary incubations different fluorescently tagged antibodies were used such as anti-goat, anti-mouse, anti-rabbit. Secondary antibody incubation was done by incubating cells in dark at RT for 1 hour. Cells were washed three times with 0.3% Triton X-100 PBS. DAPI stain was prepared in distilled H₂O, and cells were incubated for 1 min at dark. After DAPI staining cells were washed once with ddH₂O. Fluorescent mounting medium was put over slides and cover slips were placed over the slides. Slides must be kept at dark, and then could be visualized under fluorescent microscope.

2.3.10 Flow Cytometry

Cells were trypsinized and collected in 15mL falcon tubes, centrifuged at 1600rpm for 5min. Following aspiration, cells were washed twice with 1X PBS, and centrifuged at 1600rpm for 5min in each washing step. After aspirating PBS clearly,

Fixation medium A was mixed with cell pellet and vortexed for 10 sec and incubated at RT for 15 min. When fixation finished, cells were washed once with PBS-Na Azide-BSA solution in order to get rid of excess fixation medium, and were centrifuged at 1800rpm for 5min. Next step was the antibody incubation step in which fluorescent signal-tagged antibodies were prepared in PBS-BSA-Na Azide solution and incubated with cells for 10 min at 4°C. Then, 2mL PBS-BSA-Na Azide solution was added on top of the cells and centrifuged at 1800rpm for 5 min. Finally, the supernatant was aspirated and cells were re-suspended in 1X PBS. The prepared cells were finally read and analyzed by BD CSampler machine.

2.3.11 Immunotherapy of Hepatocellular Carcinoma in Athymic mice Xenografts

For tumor immunotherapy experiments of mice, Huh7 cells (5×10^6 cells/animal in 100ul media) were injected sub-cutaneously into 20 athymic nude mice. Tumor sizes were followed and upon reaching around 400-500 mm³ combination therapy was initiated as shown below. Tumor bearing animals were divided into 4 treatment groups (see below). The injection plans were as follows:

Subcutaneous injection:

Mice Group	Day -21	Day 0
Group 1	Huh7 only	PBS only
Group 2	Huh7 only	DAPT (100 ug/mice)
Group 3	Huh7 only	pl:C (100 ug) + LTA (10ug)/mice
Group 4	Huh7 only	pl:C (100 ug) + LTA (10ug)/mice

Peritumoral injection #1

Mice Group	Day 1
Group 1	PBS only
Group 2	DAPT (50ug/mice in 100 ul)
Group 3	R848(25ug) + pl:C(50ug)+ K23(25ug)
Group 4	R848(25ug) + pl:C(50ug)+ K23(25ug)(100ul/mice) + DAPT (50ug/mice)

Peritumoral injection #2

Mice Group	Day 6
Group 1	PBS only
Group 2	DAPT (100ug/mice in 100ul)
Group 3	R848(25ug) + pl:C(50ug)+ K23(25ug)(100ul/mice)
Group 4	R848(25ug) + pl:C(50ug)+ K23(25ug)(mice) + DAPT (100ug/mice)

2.3.12 Statistical Analysis

Statistical significance between control and experimental groups was calculated using Student`s T- test. $p < 0.05$ was considered as significant.

Chapter 3

Results

The aim of this thesis was to identify the CSC sub-population among several established HCC cell lines. Our initial efforts were directed to understand the CSC levels as evidenced by CD133 expression.

3.1 Investigation of CD133 Positivity Levels in Hepatocellular Carcinoma Cell Lines as a Biomarker

Examination of a cell population requires a marker, to begin with, in this regard, since our aim was to understand the biology of CSCs in HCC, determination on a specific and strong marker is an essence. In this study, analyses were mostly done by CD133, which is a penta-span membrane protein of prominin family of proteins. As well as being used as one of the prime candidate stem cell markers, CD133 levels were investigated to assess CSC status of different type of cancers including human colon carcinoma, prostate tumors, pancreatic adenocarcinoma, neural tumor, renal tumors, and focus of this study hepatocellular carcinoma tumors⁵⁷. High preference of CD133 as a cancer stem cell marker, as well as the quality of the articles using this markers led us to prefer it as a way to begin our research.

3.1.1 Panel for Basal CD133 Abundance of Hepatocellular Carcinoma Cell Lines

Seventeen HCC cell lines were used in this study, some of which are known to be well-differentiated and some known as poorly-differentiated. These cells lines are HepG2, HepG2-2215, Hep3B, Hep3B-TR, Hep40, Huh7, FLC4, FOCUS, Mahlavu, PLC/PRF/5, SK-HEP-1, SNU182, SNU387, SNU398, SNU423, SNU499 and SNU475. Basal CD133 levels of these cell lines were established as a panel to pick up our candidate cell lines to be used in further studies. Immunoperoxidase method

was used for these initial screening studies, cells were seeded on cover slips in complete media and 48 hours later were fixed and checked for CD133 levels. The results of this study revealed that off 17 cell lines at hand, 11 HCC cell lines were found to be negative for CD133 expression, (Figure 3.1) and 6 HCC cell lines were found to be positive for CD133 expression (Figure 3.2).

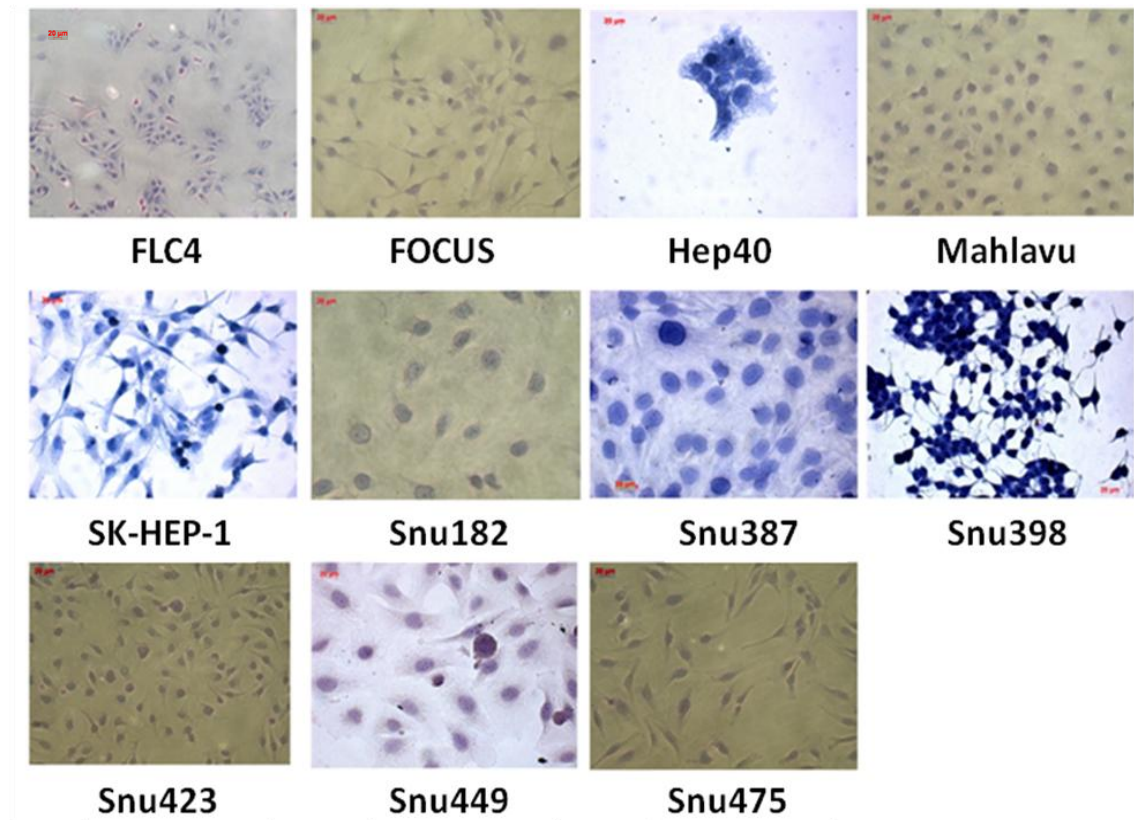


Figure 3.1: Eleven hepatocellular carcinoma cell lines that were detected to be negative for CD133 expression. CD133 antibody hybridized slides were stained by immunoperoxidase method and images were taken by brightfield microscope at 40X magnification. Absence of brown staining indicates low or no CD133 expression.

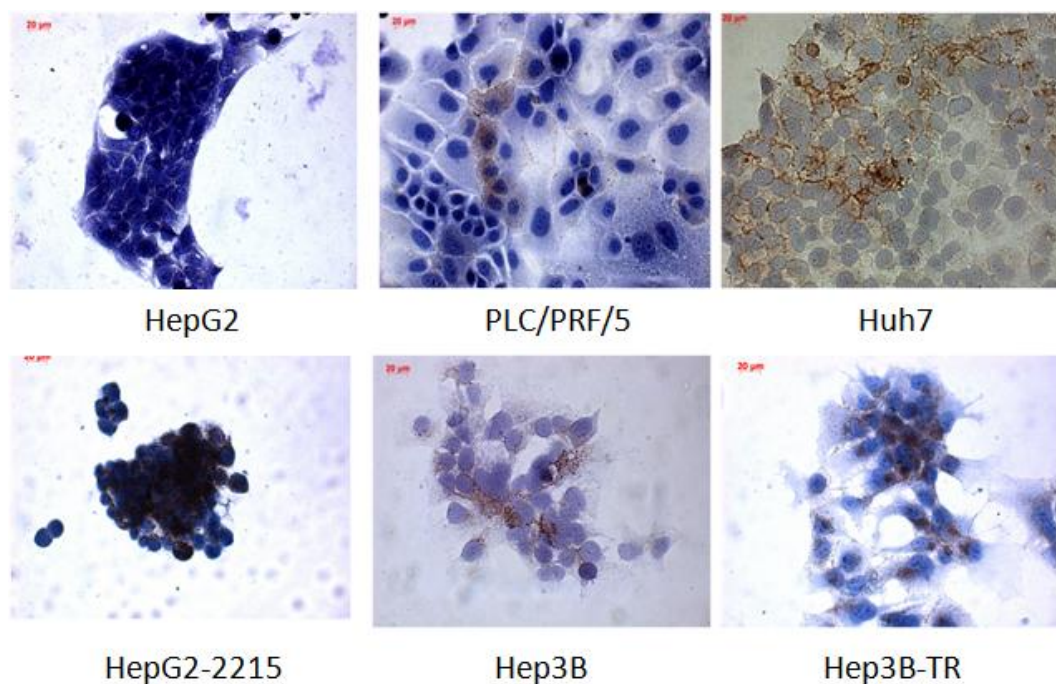


Figure 3.2: Six HCC cell lines were detected to be positive for CD133 expression. CD133 antibody hybridized slides were stained by immunoperoxidase method, and images were taken by brightfield microscope at 40X magnification. Cell lines were ranked based on the brown staining intensities representing increasing CD133 positivity levels.

3.1.2 Checking CD133 Positivity Panel Results by Flow Cytometry

Having been determined the initial qualitative states of CD133 in HCC cell lines, presence of a potential cancer stem cell population was indicative. Yet, a quantitative method has to be developed before convincingly reach a conclusion. For this very reason, we repeated the cell line screening once again using flow cytometry method, which is a highly trusted and quantitative method widely used in similar studies^{50,51,53,56}. While performing the staining APC conjugated CD133 antibody was used as a single marker. Similar to what was done during immunoperoxidase staining, we repeated FACS analyses with all 17 HCC cell lines (Figures 3.3 and 3.4). Here, we set a threshold for accepting a cell line positive or negative for CD133 expression. Any read-out that was determined to be less than 10% CD133 positivity we would consider that cell line as negative.

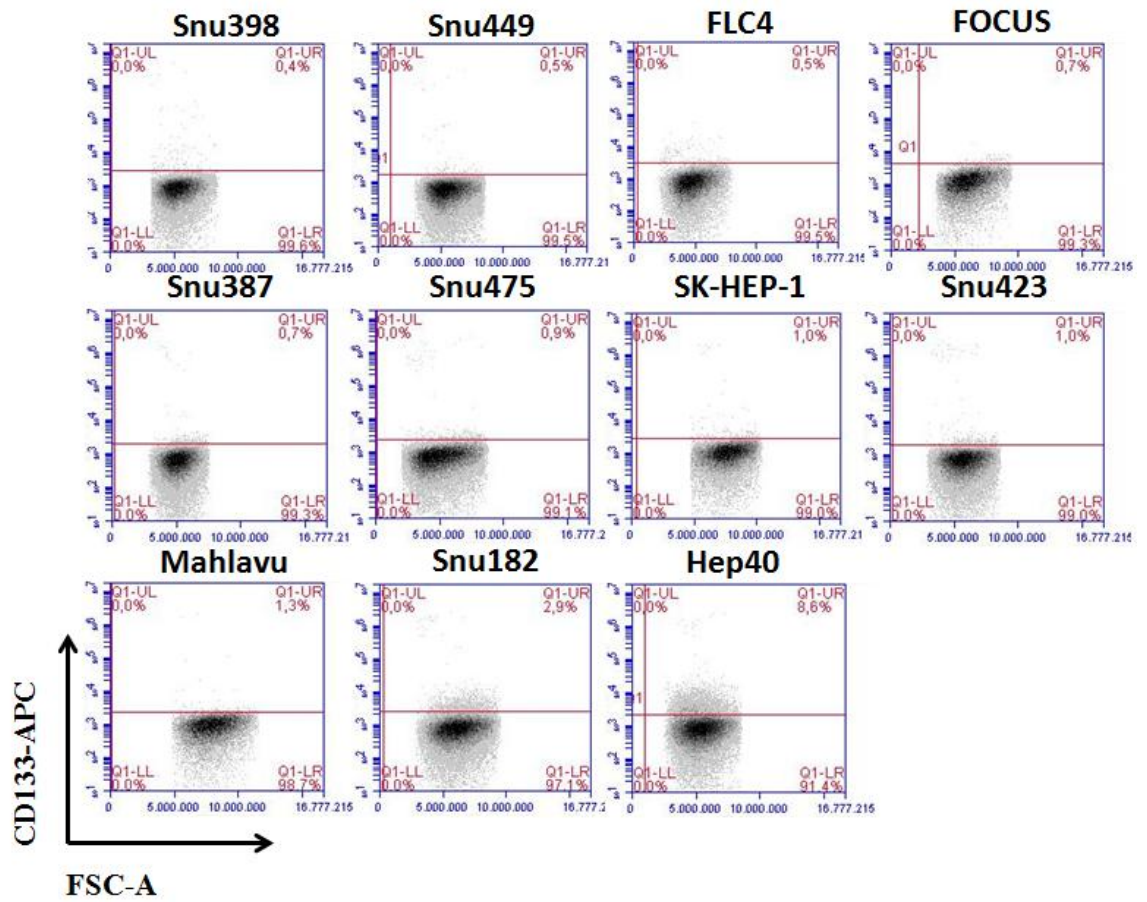


Figure 3.3: Representative dot plots of eleven HCC cell lines lacking CD133 expression. Flow cytometric analyses was conducted by using anti-CD133-APC conjugated antibody.

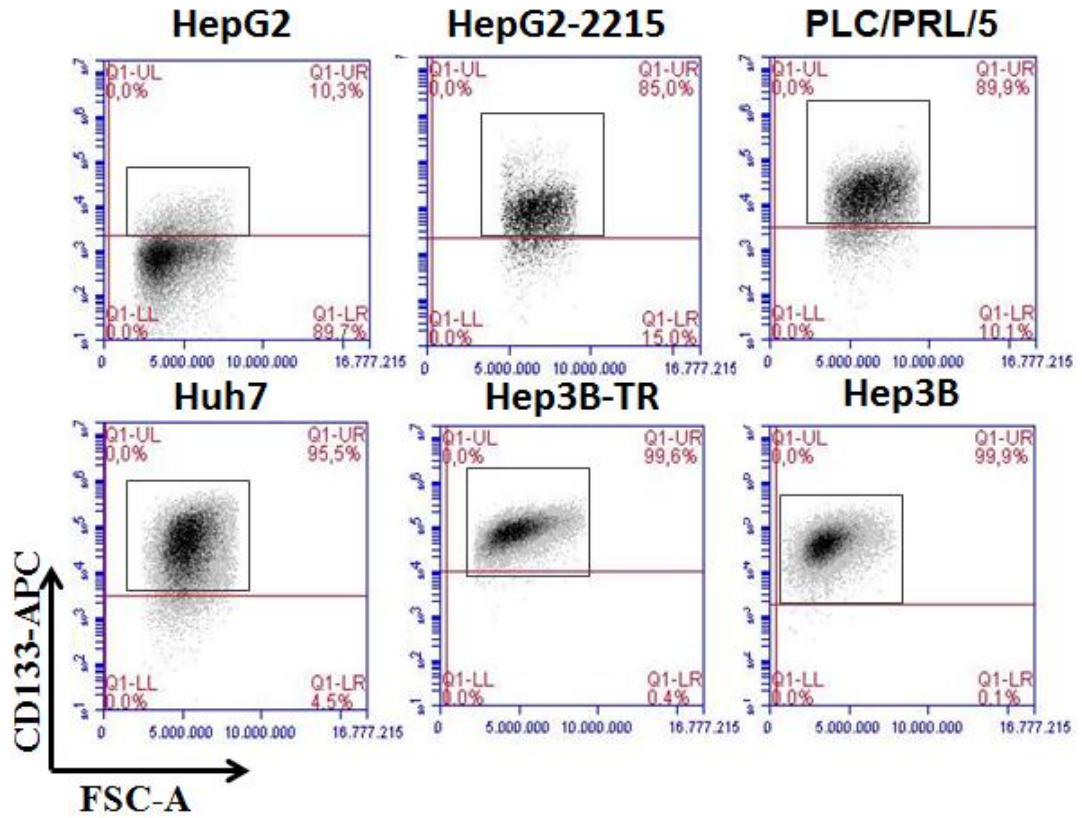


Figure 3.4: Representative dot plots of HCC cell lines positive for CD133 expression. Flow cytometric analyses was conducted by using anti-CD133-APC conjugated antibody.

Overall analyses with flow cytometry reproduced what we initially observed with immunoperoxidase staining. Here, a similar pattern was obtained, 6 cell lines, HepG2, HepG2-2215, PLC/PRF/5, Huh7, Hep3B-TR, Hep3B were detected to be positive (Figure 3.4) compared to the remaining 11 cell lines, Snu182, Snu387, Snu398, Snu423, Snu475, Snu449, FOCUS, FLC4, Mahlavu, SK-HEP-1 and Hep40, (Figure 3.3). The expression profiles of these cell lines based on CD133 positivity is presented on Figure 3.5.

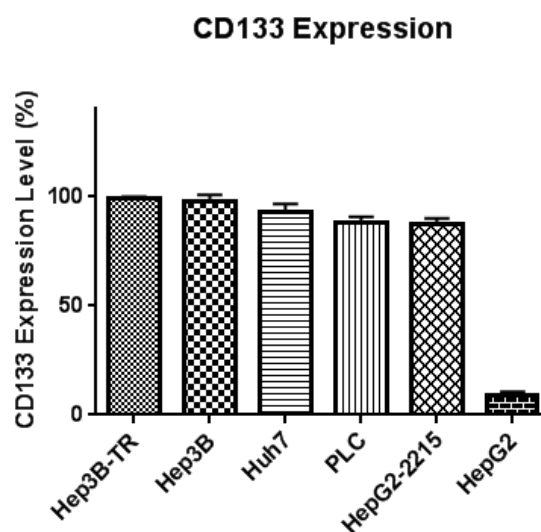


Figure 3.5: Graphical representation of the flow cytometry data showing the CD133 positivity levels of 6 HCC cell lines. Experiments were done in duplicates, and the results were plotted after two independent repetition results.

3.2 Serum Starvation Model to Assess Cancer Stem Cell Sub-Populations in Hepatocellular Carcinoma Cell lines

Until this step, cells were grown in complete media, having all the necessary components for them to grow freely. Yet, in order to understand the true nature and level of existing CSCs within the HCC population and to enrich the ones that are CSCs, we adapted a stress condition onto these cells. After three days in complete media, we cultured these cells in serum starvation media. The ultimate logic behind this approach was the following, non-CSC cells need nutrients in order to continue to grow in the culture dish, and if the supplement was discontinued, they will enter apoptosis, yet the cells that have the potential of being CSC remain alive because of their resistance to harsh conditions. Therefore, this model was chosen to force the CSCs to be the sole survivors, and eliminate the ones that are less stem-like.

3.2.1 Effects of Serum Starvation on CD133⁺/EpCAM⁺ Cancer Stem Cell Population

Having a marker, CD133, was an acceptable approach to assess CSCs, yet in similar studies it is more convincing to include another marker. We chose to include another common CSC marker, EpCAM for these studies. For the rest of the study, double staining protocol will be employed to designate CSC sub-populations.

To confirm that we do detect correct CSCs in our analyses, three cell lines, one with low CSC population (HepG2), one with a medium CSC level (Huh7), and one from high-expresser candidate cell lines (Hep3B-TR) were chosen. To begin with, the basal CD133/EpCAM double positive subpopulations in these three cell lines were detected (Figure 3.6). Following that adjustment, cells were seeded in parallel plates and at Day 1, they were fixed, stained with FITC conjugated EpCAM antibody and APC conjugated CD133 antibody, and analyzed by flow cytometry. On the parallel plates, cell were kept at complete media for 72hours and changed to serum starved media, and left in serum starved media for 72 hours as well, then cells were collected, fixed, stained and analyzed with flow cytometry to detect any substantial variances (Figure 3.6).

Upon analysis of the results, CD133⁺/EpCAM⁺ sub-population in HepG2 shows a 25% shift when left in serum starved media for 72 hours (Figure 3.6). A resembling increase was also seen in Huh7, nearly 23% increase in CD133⁺/EpCAM⁺ cells was detected (Figure 3.6). Conversely, in case with Hep3B-TR, a sister cell line Hep3B but desensitized form that does not respond to TGF- β 1, no significant change in CD133⁺/EpCAM⁺ sub-population levels were assessed (Figure 3.6).

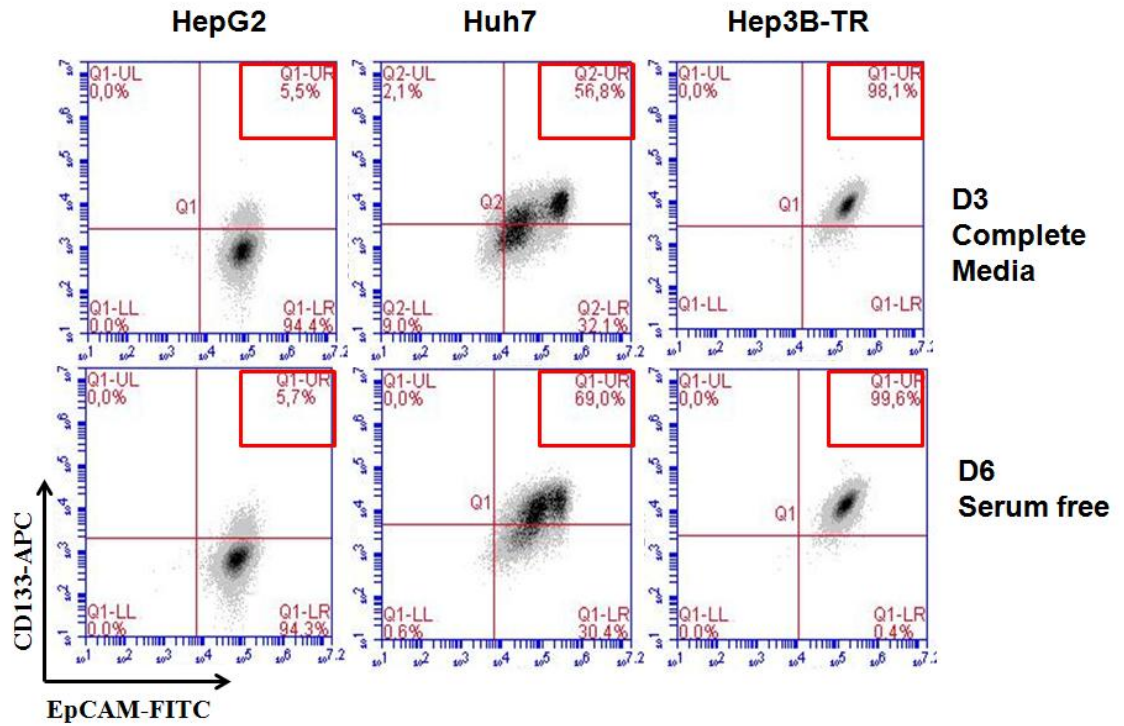


Figure 3.6: Effects of serum starvation on CD133⁺/EpCAM⁺ subpopulation of CSCs in selected HCC cell lines. D3 represents cells incubated for 3 days in complete media whereas D6 represent cells incubated in starvation media for 3 days.

3.2.2 Time-dependent alteration of CD133⁺/EpCAM⁺ Cancer Stem Cell Fraction in Serum Starvation

Experiments with 72 hours starvation led us to dissect this phenomenon in greater detail. There was an apparent change, yet how this trend shifted over time needed extra evaluation. To focus more on time dependency, daily differences in CD133⁺/EpCAM⁺ sub-population within first 72 hours in complete media was checked. Cells were plated and parallel plates were designed and fixed at 24 hours, 48 hours and/or 72 hours after seeding. Fixed cells were stained and analyzed by flow cytometry to assess CD133⁺/EpCAM⁺ CSC populations. Analyses for the first 72 hours in complete media revealed that there was no significant change in the CD133⁺/EpCAM⁺ subpopulations as expected (Figure 3.7).

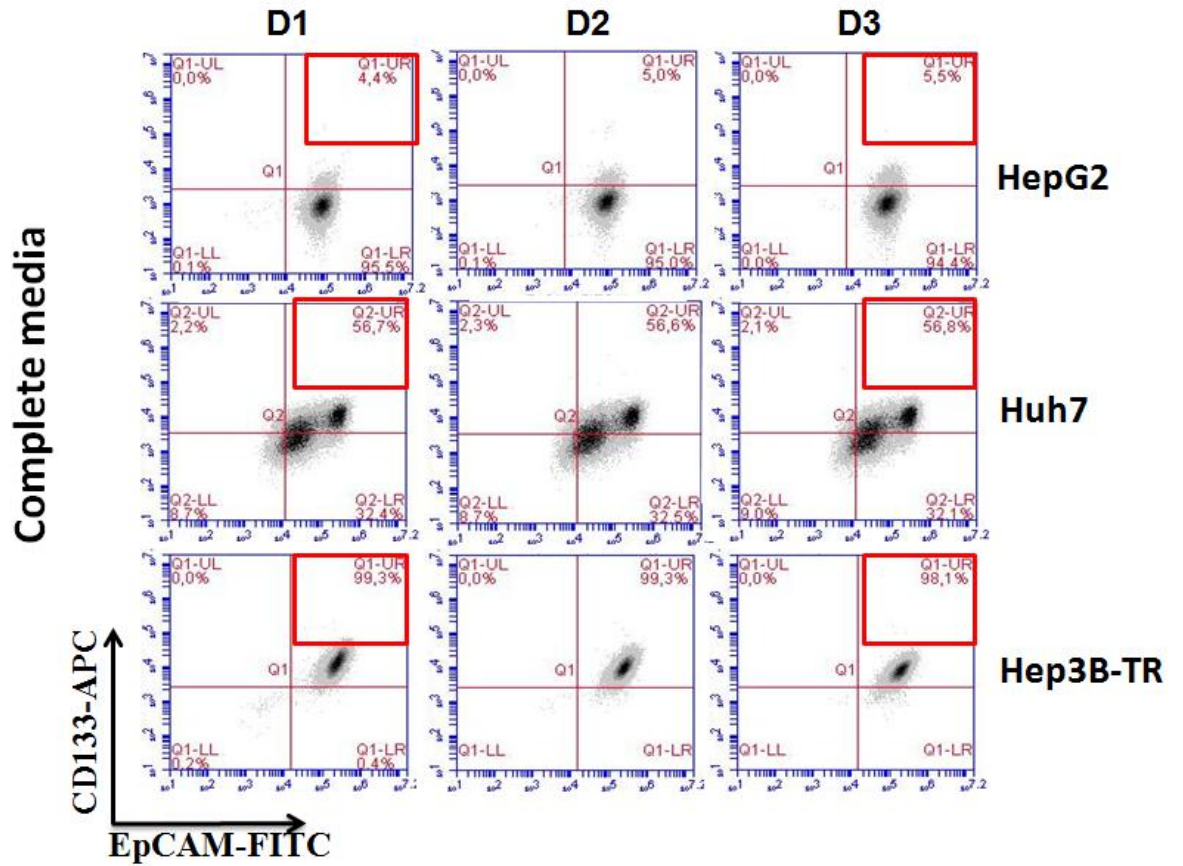


Figure 3.7: Dot plots showing the time-course change in CD133⁺/EpCAM⁺ subpopulation within three different HCC cell lines. D# represents incubation number of days in complete media.

Next, three different cell lines expressing low (HepG2), medium (Huh7) and high (Hep3B-TR) CD133⁺/EpCAM⁺ were subjected to serum starvation for 72 hours, following 72 hours incubation in complete media. As done previously, cells were fixed and checked at different time points 24 hour, 48 hour or 72 hour serum deprivation for any shifts in CD133⁺/EpCAM⁺ levels to determine the time course effect of serum deprivation on CD133⁺/EpCAM⁺ subpopulation (Figures 3.8-3.10).

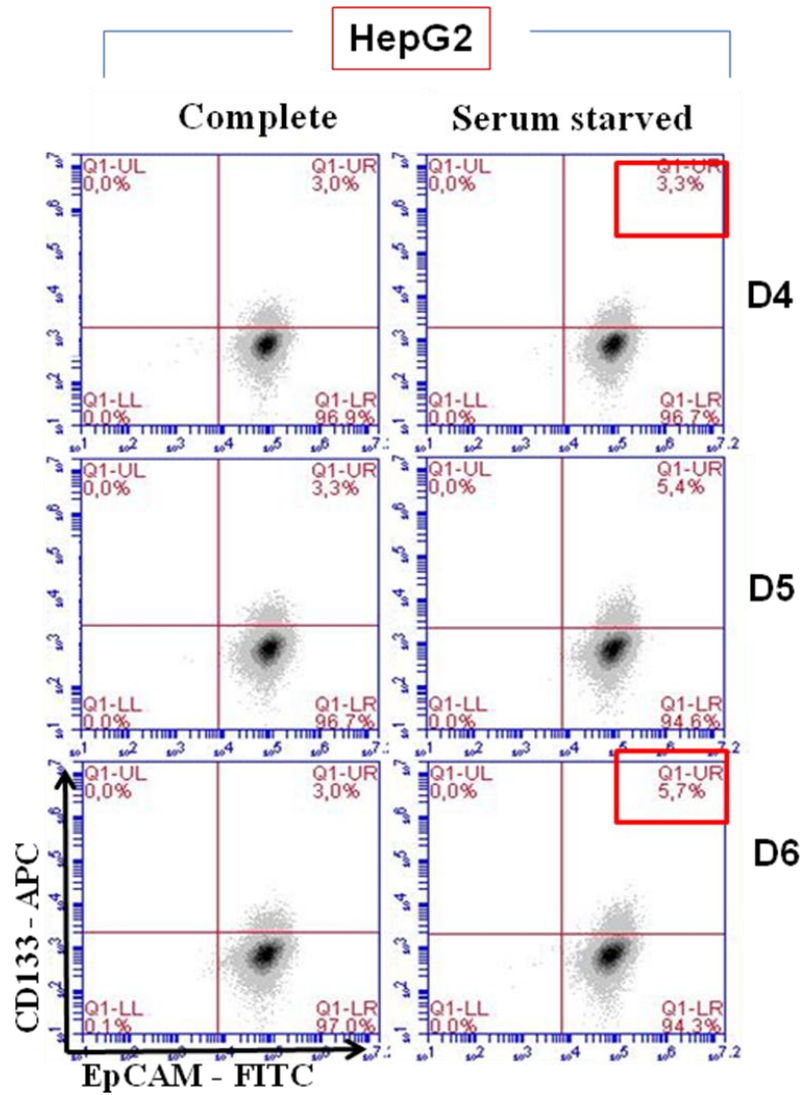


Figure 3.8: Flow cytometry analyses showing the daily changes occurring in $CD133^+/EpCAM^+$ subpopulation within serum deprived media for HepG2. For the top row D# indicates the number of days spent in complete media, for the bottom row D4 serum starvation represents 3 days in complete media plus 1 day in serum starved media, for D5 2 days in serum starved media and D6 3 days in serum starved media.

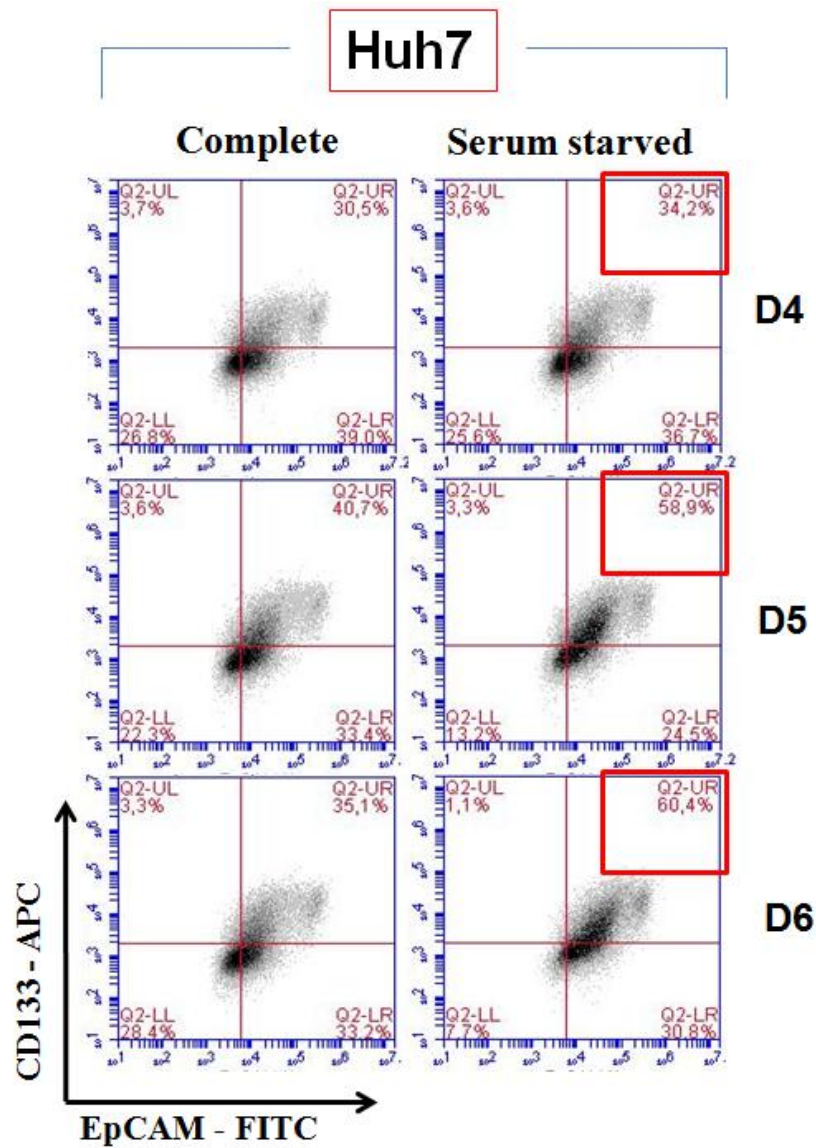


Figure 3.9: Flow cytometry analyses showing the daily changes occurring in $CD133^+/EpCAM^+$ subpopulation within serum deprived media for Huh7. For the top row D# indicates the number of days spent in complete media, for the bottom row D4 serum starvation represents 3 days in complete media plus 1 day in serum starved media, for D5 2 days in serum starved media and D6 3 days in serum starved media.

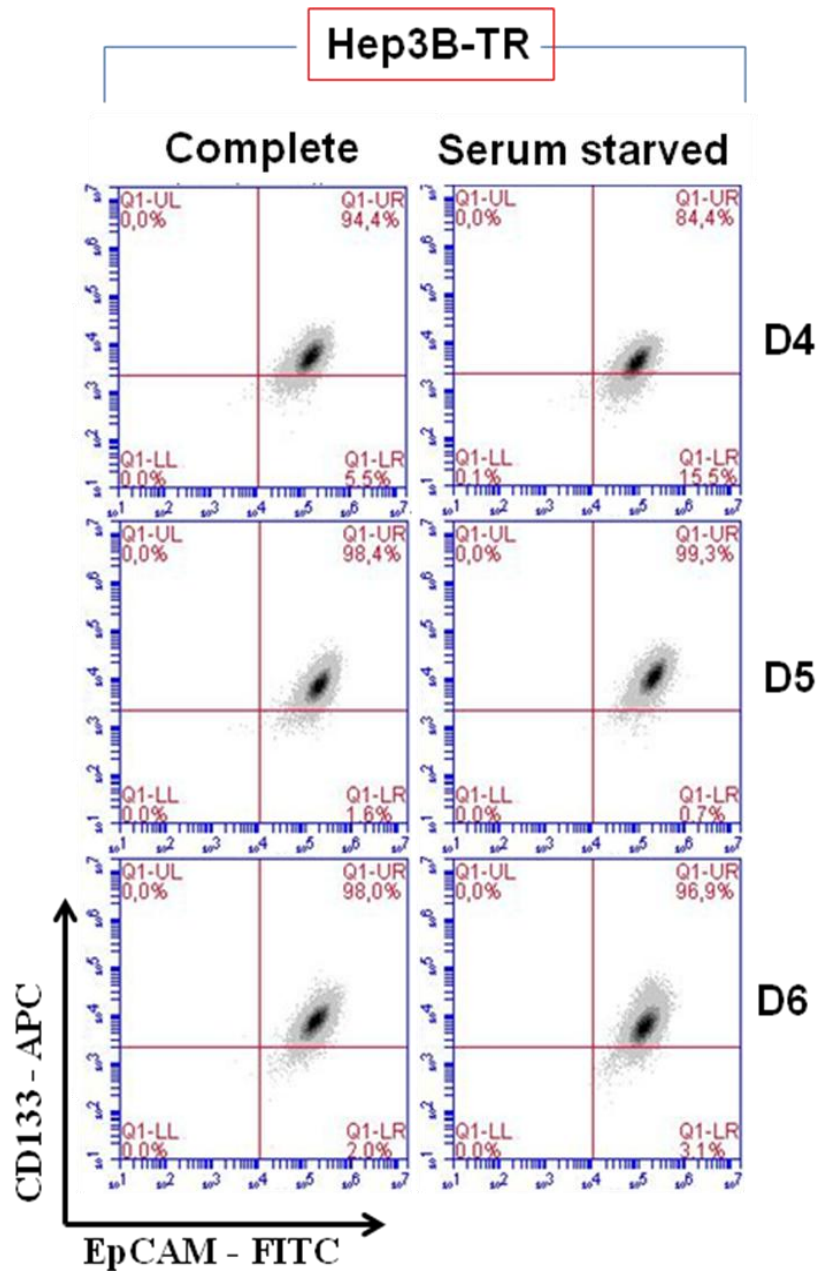


Figure 3.10: Flow cytometry analyses showing the daily changes occurring in $CD133^+/EpCAM^+$ subpopulation within serum deprived media for Hep3B-TR. For the top row D# indicates the number of days spent in complete media, for the bottom row D4 serum starvation represents 3 days in complete media plus 1 day in serum starved media, for D5 2 days in serum starved media and D6 3 days in serum starved media.

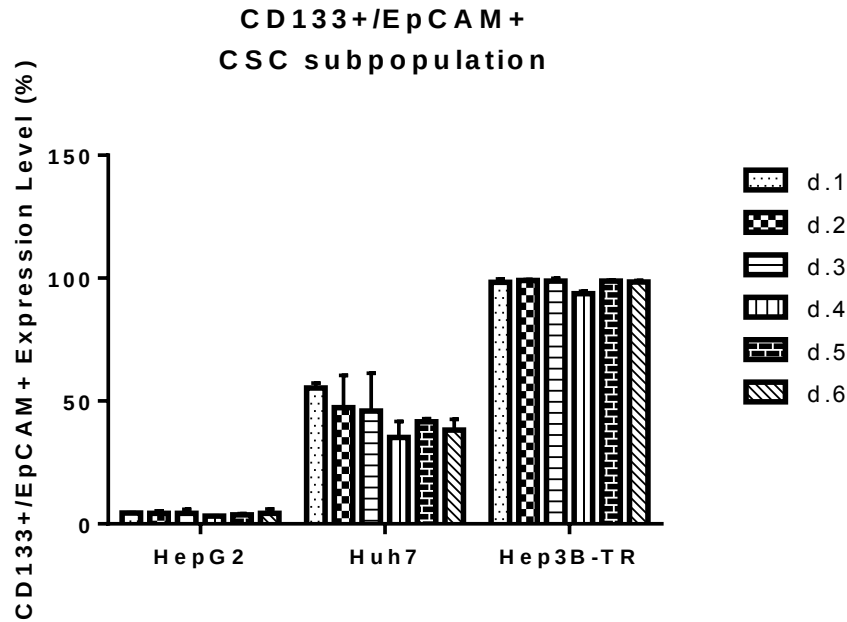


Figure 3.11: Graphical representation of the changes occurring in CD133⁺/EpCAM⁺ subpopulation on a daily basis for 6 days in three different HCC cell lines. Experiments were done in duplicates, and the results were plotted after two independent repetition results.

Upon comparison of the effect of time spent in complete media on CD133⁺/EpCAM⁺ fraction, different trends of the cell lines can be detected. For HepG2 and Huh7, there seems to be a gradual drop in CD133⁺/EpCAM⁺ population levels after Day 3, same is true for Huh7 as well, yet Hep3B-TR acts through a completely different response, almost no shifts in CD133⁺/EpCAM⁺ population level (compare Figures 3.9-3.11 and 3.12).

Exposure to serum starved media exerts its effects in favor of CD133⁺/EpCAM⁺ population in both HepG2 and Huh7, from Day4 until Day 6. However, no significant change in Hep3B-TR was observed in response to serum deprivation (Figure 3.12).

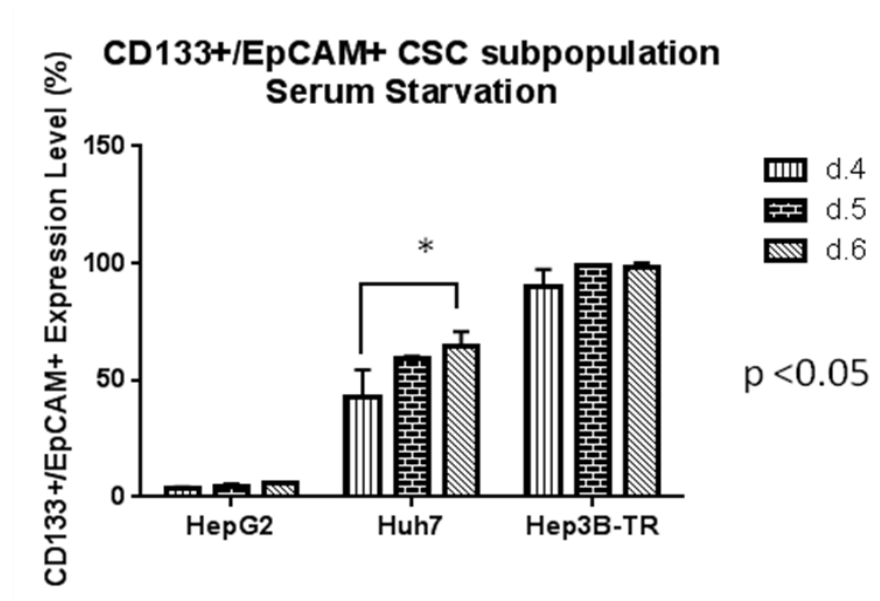


Figure 3.12: Graphical representation of the changes occurring in $CD133^+/EpCAM^+$ subpopulation on a daily basis in serum starvation for 3 days in three different HCC cell lines. Experiments were done in duplicates, and the results were plotted after two independent repetition results.

Analyses of all three cell lines revealed that $CD133^+/EpCAM^+$ population is affected by serum starvation and levels were increased in both HepG2 and Huh7 on a daily basis, but not in Hep3B-TR (Figure 3.13-3.15).

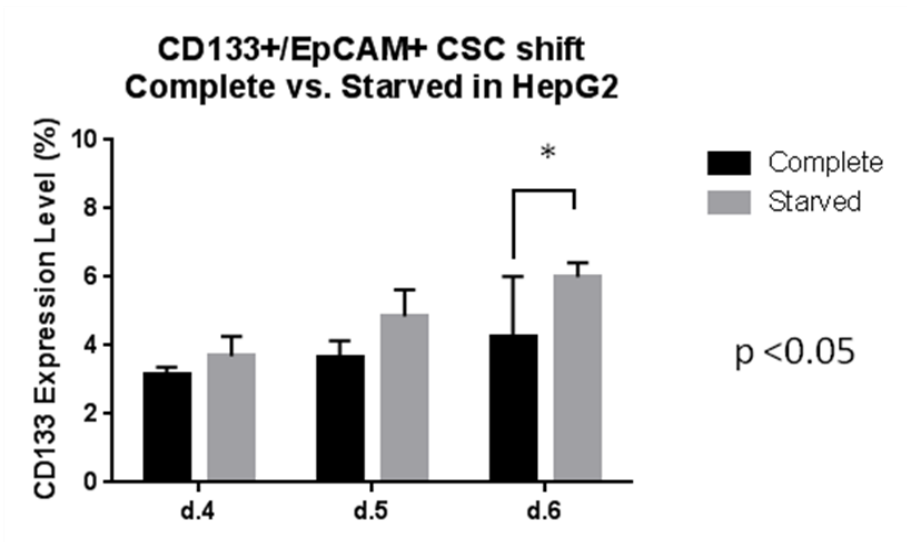


Figure 3.13: Graphical representation of the changes occurring in $CD133^+/EpCAM^+$ subpopulation in complete vs. serum-starved media for HepG2.

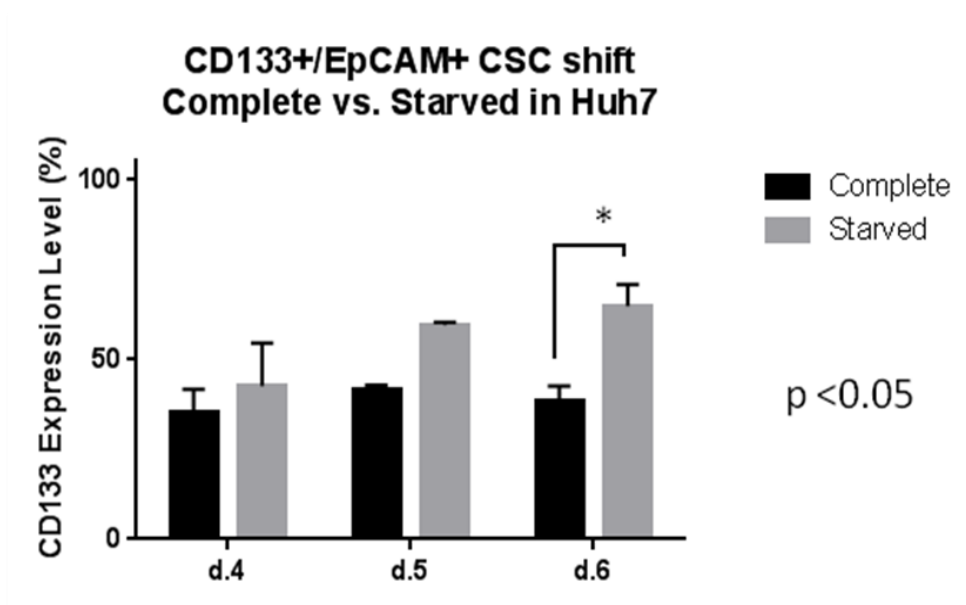


Figure 3.14: Graphical representation of the changes occurring in $CD133^+/EpCAM^+$ subpopulation in complete vs. serum starved media for Huh7.

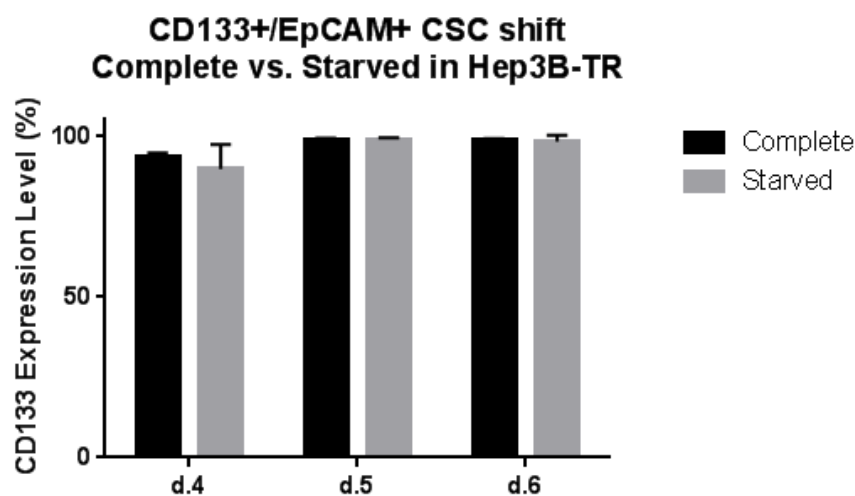


Figure 3.15: Graphical representation of lack of the changes occurring in $CD133^+/EpCAM^+$ subpopulation in complete vs. serum starved media for Hep3B-TR.

Taken together, our findings revealed that HepG2 and Huh7 CSC levels rose by ~2x when incubated in serum starved condition (for 3 days) compared to complete media incubation. As expected, Hep3B-TR did not display any alteration in CSC positivity irrespective of stress condition.

3.3 Notch Signaling Involvement in Regulation of Cancer Stem Cell Population in Hepatocellular Carcinoma

Notch signaling pathway is one of the fundamental pathways necessary during embryogenesis, development and adulthood in maintenance of stemness, regulation of differentiation and fate switching^{81,83,101}. In immature mice hepatocytes, Notch activation triggers switch from hepatocytes to an oval cell like structure and then to biliary epithelial cells (BECs)¹⁰¹. It is known to be deregulated in numerous cancer types. Notch activation was found to be leading to an increase in mammosphere formation, and also reported to trigger angiogenesis during solid tumor formation⁸³. Therefore, Notch signaling pathway is one of the prime candidates to be responsible to regulate in CSC reprogramming which is the very reason to be investigated in this study, as well.

3.3.1 Altered Notch Signaling Affects CD133+ Cancer Stem Cell Fraction in Huh7

To unravel the role of Notch in CSC population, effects of inhibition as well as activation of the pathway was tried. The contribution of Notch on CD133+ sub-population of HCC cell lines was surveyed. Jagged-1 is a soluble ligand that is specific for Notch-1 receptor, and leads to activation of Notch signaling. DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), on the other hand, is a gamma-secretase inhibitor and block the presenilin complex activity, which is naturally in charge of NICD cleavage. Having been blocked by DAPT, gamma-secretase complex cannot work on NICD, therefore release of NICD cannot occur, which will render Notch signaling to be inactive. In this part of the work, Jagged-1 and DAPT was used to understand the effects of Notch interruption/activation on CD133⁺ CSC population.

For the initial experiments, cells were plated in very diluted amounts to form colonies so that they could be forced into a clonal expansion, and at this state we could observe any changes during colony formation. Cells were left in complete medium for 72 hours and at the end of this duration, medium was changed to serum starved media and cells were treated with either DAPT or Jagged-1 (3.16).

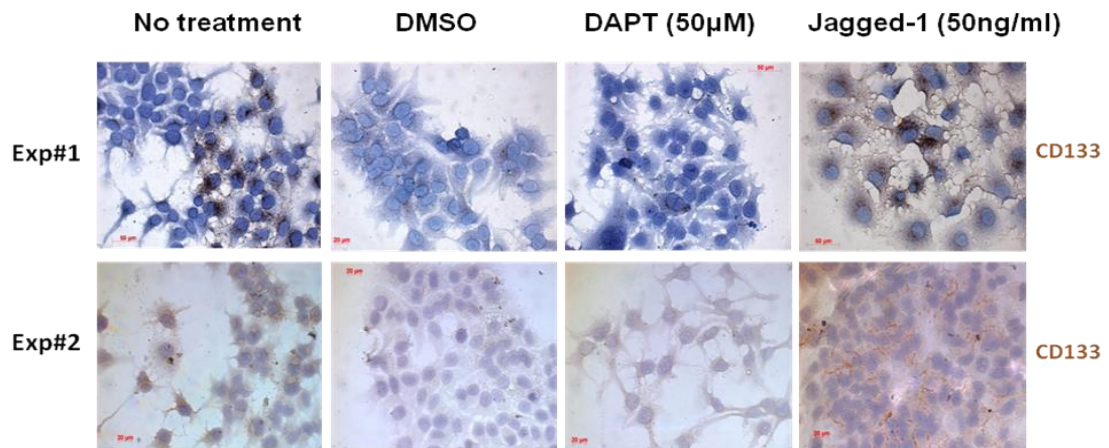


Figure 3.16: Immunoperoxidase assay results showing colony formation experiment with Notch agonists and antagonist to understand their effects CD133 positivity. Cells were stained with anti-CD133 antibody as primary Ab, DAKO Envision + system was used for HRP-conjugated secondary Ab incubation.

Immunoperoxidase experiment results were chosen to be representative images that are in accordance with the results seen in Figure 3.16. Jagged-1 treatment leads to a two-fold increase in the levels of CD133 positive colony count. Yet DAPT is responsible for a drop in the number of CD133 positive colony counts. DMSO seems to be having no effect at all (Figure 3.16, Figure 3.14).

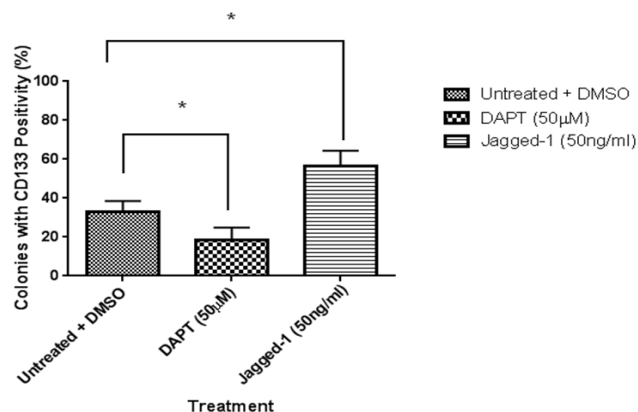


Figure 3.17: Quantitative representation of the colonies having CD133 expression higher than 20% within themselves upon Notch ligand and inhibitor treatment of Huh7 cells. Statistical analysis was done by Student's t-test and $*p < 0.05$ between DAPT and control - Jagged-1 and control.

3.3.2 Dose-dependent Effects of Notch Alteration on CD133⁺/EpCAM⁺ Cancer Stem Cell Population

Through confirmation of differential Notch effect on CD133 positivity of Huh7 CSC sup-population from Figure 3.17, we next aimed to understand the effective concentrations of Jagged-1 and DAPT to eliminate any toxic effects on the cells. In order to do so, the cell lines were treated with different doses of Jagged-1 as 2ng/ml, 10ng/ml and 50ng/ml and with DAPT 6.6μM, 20μM and 60μM respectively. Cells were first plated and left in complete media for 3 days and at day 4 replaced with serum-free media supplemented either with Notch ligand or the inhibitor DAPT, and analyzed at day6 by flow cytometry. Instead of single staining this time a double staining with CD133 and EpCAM was performed.

Analyses of the data related to dose-dependent Notch related treatment of the cells yielded slightly different outcome compared to what was seen during immunoperoxidase data (compare 3.17 with 3.18-3.20). Jagged-1 does not seem to be having a positive effect on CD133⁺/EpCAM⁺ population also the p-value between Jagged-1 treatment and untreated samples was non-significant ($p > 0.05$) (Figure 3.18, and Figure 3.19). In case for Hep3B-TR no Jagged-1 induction was used since it does already have a quite high percentage of CD133⁺/EpCAM⁺ cells. The most important data, in these set of experiments, was obtained from DAPT treatments (Figure 3.18, Figure 3.19 and Figure 3.20). Both in HepG2 and Huh7, DAPT treatment seems to be downregulating CD133⁺/EpCAM⁺ population as 60% and 50%, respectively. However, again, DAPT does not necessarily alter CD133⁺/EpCAM⁺ fraction in Hep3B-TR (Figure 3.21). Hep3B results can be found at Appendix A1.

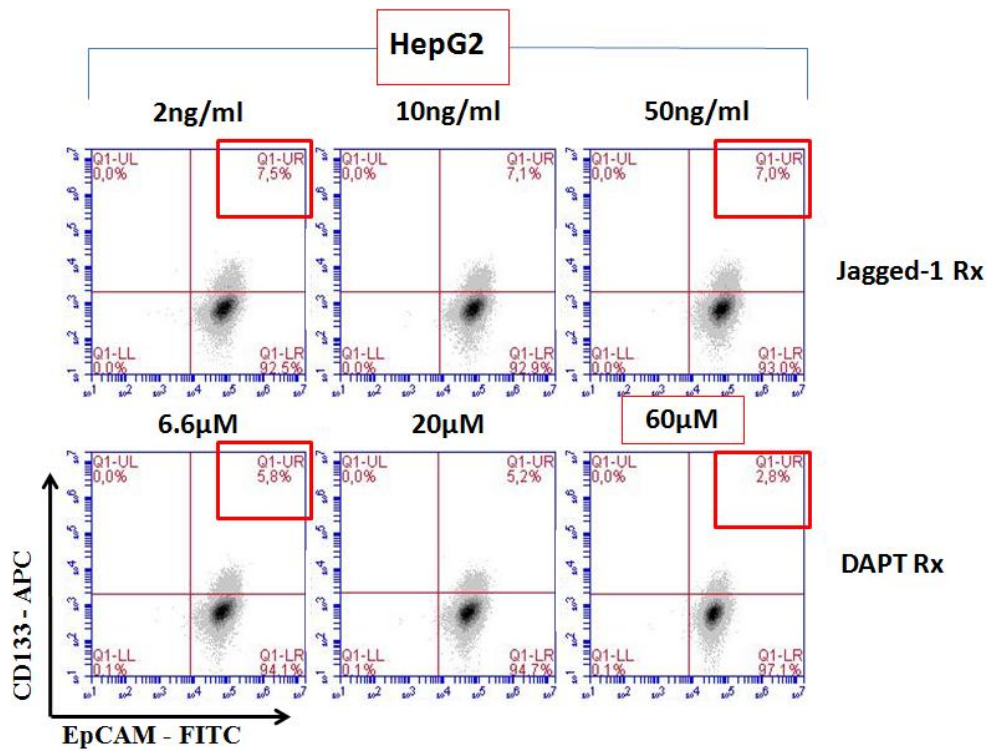


Figure 3.18: Dot-plot analyses showing the dose-dependent effects of Jagged-1 and DAPT on CD133⁺/EpCAM⁺ subpopulation shifts in HepG2 at day 6.

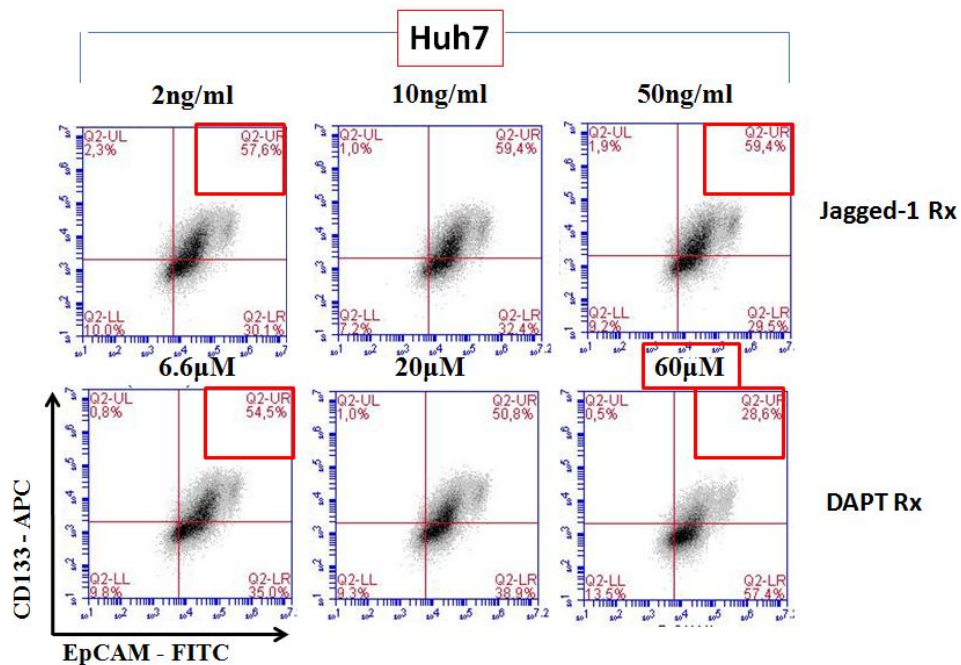


Figure 3.19: Flow cytometry analyses showing the dose-dependent effects of Jagged-1 and DAPT on CD133⁺/EpCAM⁺ subpopulation shifts in Huh7.

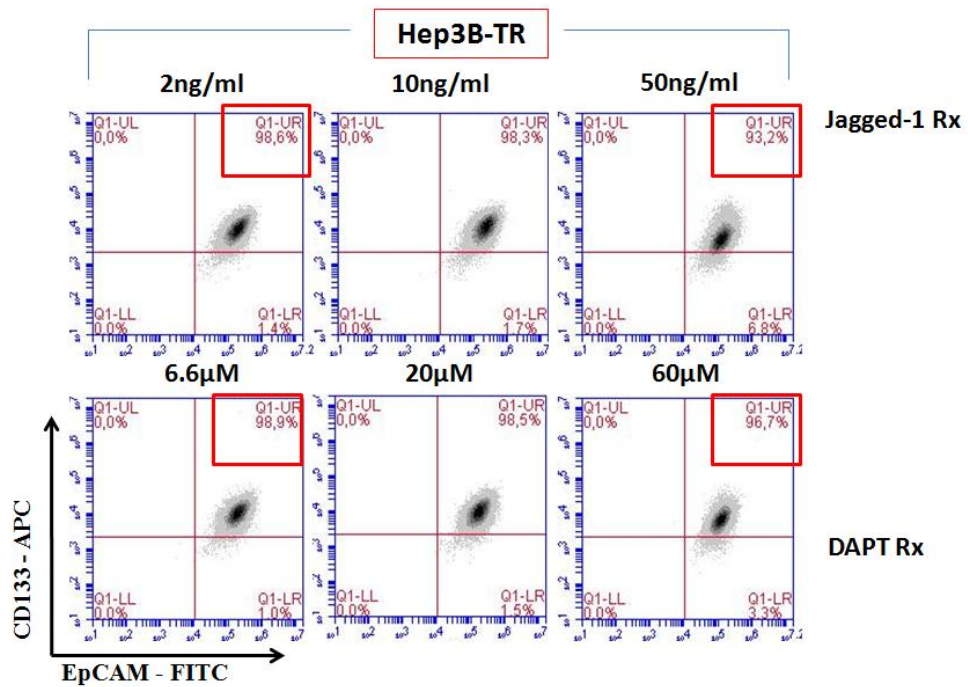


Figure 3.20: Flow cytometry analyses showing the dose-dependent effects of Jagged-1 and DAPT on CD133⁺/EpCAM⁺ subpopulation shifts in Hep3B-TR.

When this set of findings are taken together, it is fair to conclude that while the positive effect of Jagged-1 on CSC upregulation is inconclusive, DAPT treatment leads to significant reduction of CSC sub-population in HepG2 and Huh7 (Figure 3.21). This finding is critical for developing an effective immunotherapeutic approach against certain HCC conditions. It deserves to test the validity with xenograft experiments in athymic mice.

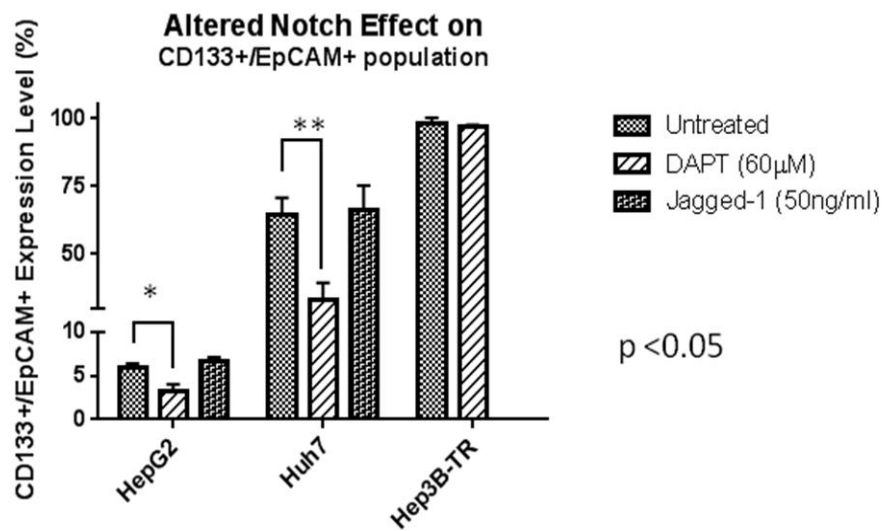


Figure 3.21: Graphical representation showing the effects of Jagged-1 and DAPT on CD133⁺/EpCAM⁺ subpopulation shifts in HepG2, Huh7 and Hep3B-TR. Results were analyzed by Student's t-test and p-values for both HepG2 and Huh7 $p < 0.05$.

3.3.3 Time-dependent Effects of Notch Alteration on CD133⁺/EpCAM⁺ Cancer Stem Cell Population

Previous experiments demonstrated that there is a reduction of CD133⁺/EpCAM⁺ population by DAPT treatment, but at 50 ng/ml Jagged-1 there was no improvement in CD133⁺/EpCAM⁺. In the first set of experiments samples from 72 hours serum starvation were checked. To assess at time-dependent changes of CSC subpopulation by DAPT, and Jagged (with higher doses though) cells were collected and analyzed at 24 hours, 48 hours and 72 hours after 60 μM DAPT treatments (Figures 3.22-25). In these set of experiments while we increased Jagged-1 from 50 to 200 ng/ml, we kept DAPT at 60 μM.

As seen in the following Figures, Jagged-1 even at 200 ng/ml had no positive effect on CD133⁺/EpCAM⁺ ^{sup}populations of the tested cell lines. Similar to previous observations DAPT treatment reduced CSC level by ~ 2X for all the cell lines tested over the course of three days. The only exception that DAPT was ineffective was on Hep3B-TR (Figure 3.24, 3.25).

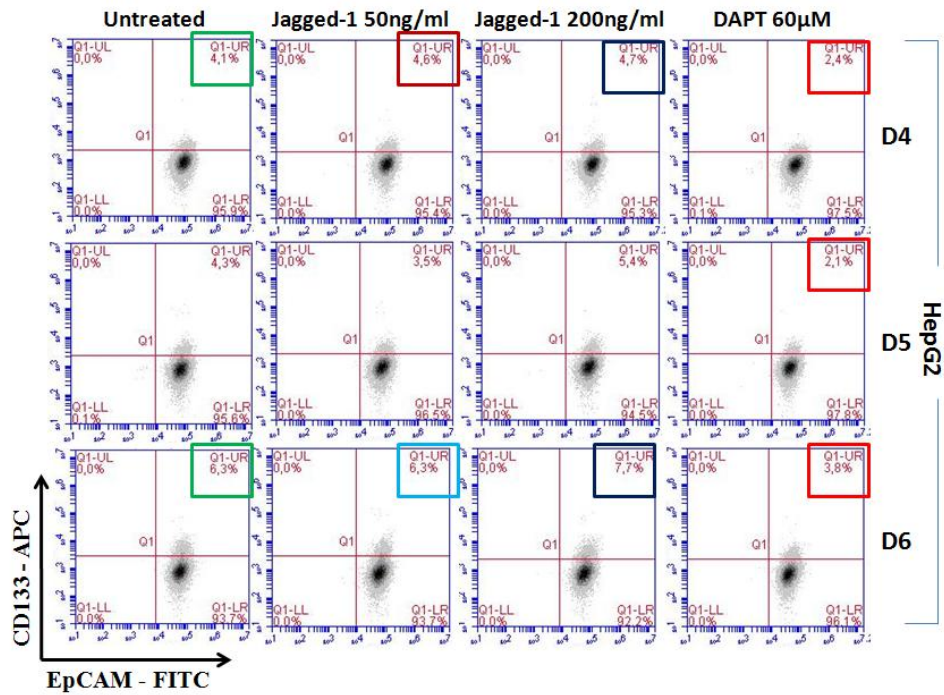


Figure 3.22: Flow cytometry analyses showing the time dependent changes of CD133+/EpCAM+ fraction upon DAPT and Jagged-1 treatments in HepG2.

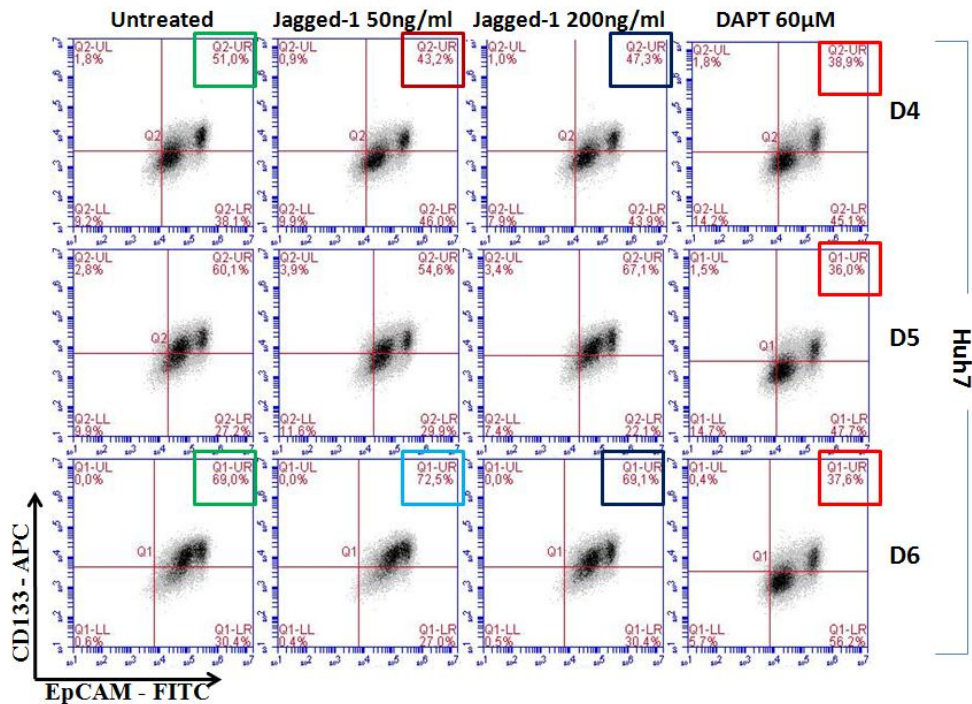


Figure 3.23: Flow cytometry analyses showing the time dependent changes of CD133+/EpCAM+ fraction upon DAPT and Jagged-1 treatments in Huh7.

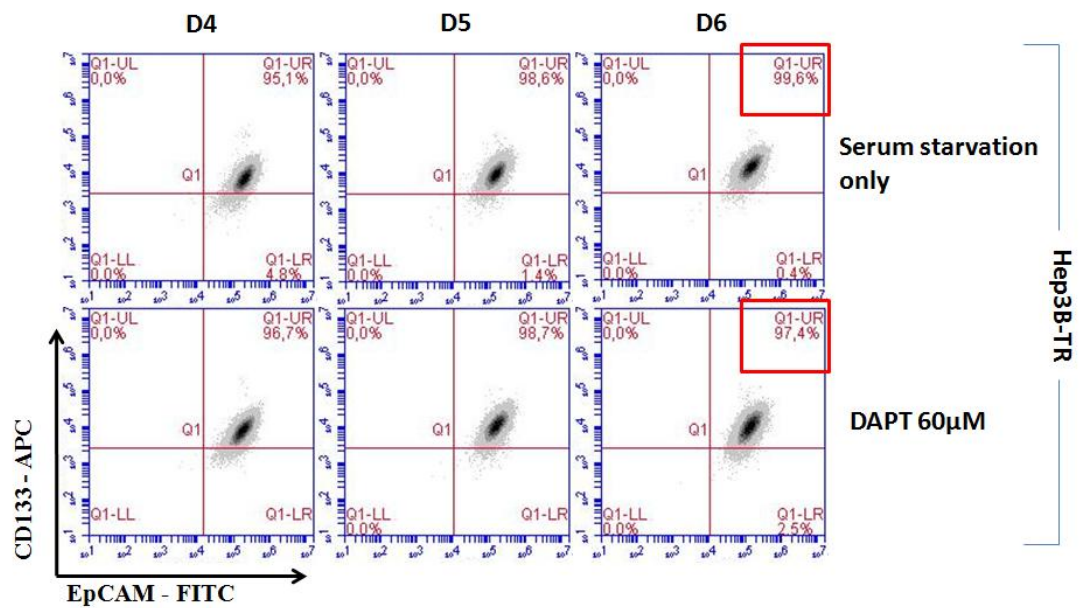


Figure 3.24: Flow cytometry analyses showing the time dependent changes of CD133⁺/EpCAM⁺ fraction upon DAPT and Jagged-1 treatments in Hep3B-TR.

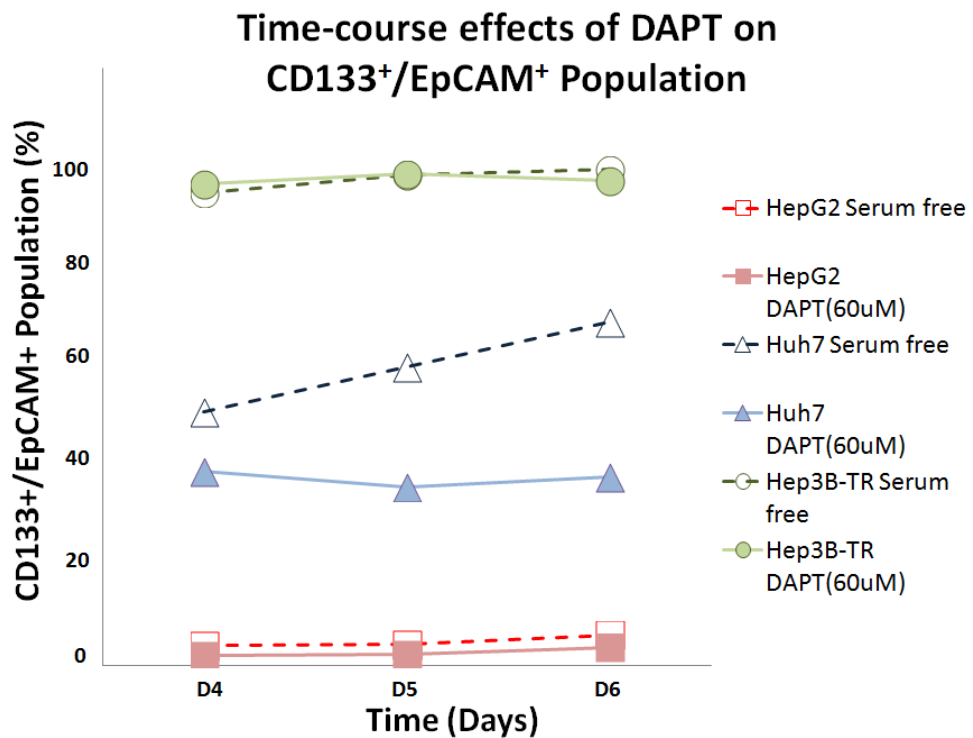


Figure 3.25: Effects of DAPT on CD133⁺/EpCAM⁺ population in a time-dependent manner.

As we have mentioned earlier, Hep3B-TR, is a desensitized for TGFB1 receptor expression. The findings presented in Figure 3.24 are not unexpected, since inhibition of Notch by DAPT will have little or no effect on TGFB1 receptor levels.

3.4 Investigation of Oval Cell-like Population in Hepatocellular Carcinoma Cell Lines

Oval cells are named also as the stem cells of the liver, in case of an injury this population of cells, since possessing a bipotential character, differentiates into hepatocytes or cholangiocytes (cells forming the bile ducts), which are the two major types of epithelial cells resident in the liver ¹⁰². There are studies showing that upon Notch signaling activation in new born mice, immature hepatocytes go through a fate switch and differentiates into biliary epithelial cells ¹⁰¹. In another study, it was shown that HNF4 α + hepatocytes in adult mice liver upon ectopic Notch activation or during biliary injury undergoes a fate switch and become HNF4 α + / SOX9+ hepatocytes. Later these cells assume a following switch and become only SOX9+ cells. These intermediate bi-phenotypic stage cells show morphological and marker-based characteristics of oval cells ¹⁰³.

In this part of the study it was hypothesized that, since Notch signaling activation seems to be interfering with CSCs, and this bi-phenotypic cells resemble oval cells, presence of these double positive cells could be included as a contaminant in our CSC sup-population. In order to check this hypothesis, HNF4 α and SOX9 protein levels in the cell lines previously detected to be positive for CD133 expression was examined. Western blot analysis was done in 6 HCC cell lines positive for CD133, these were HepG2, HepG2-2215, Hep3B, Hep3B-TR, Huh7 and PLC. A CD133 negative cell line FLC4, was also used to assess the HNF4 α + / Sox9+ status (Figure 3.26).

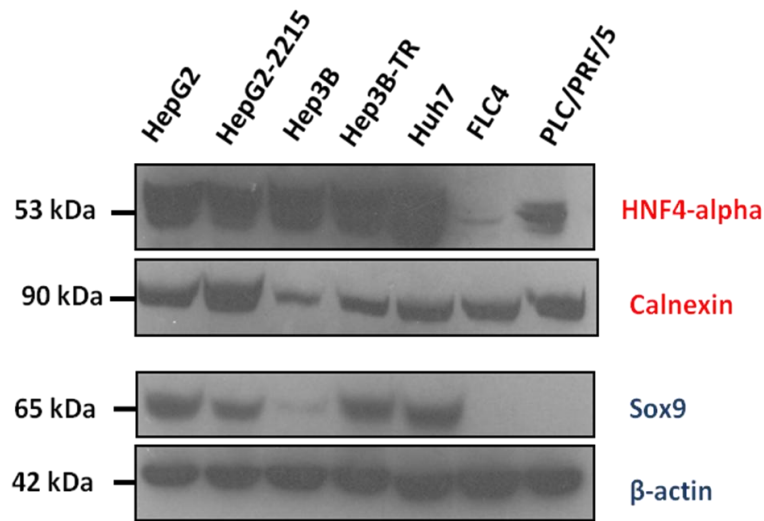


Figure 3.26: Western blot analysis images showing the heterogeneous expression of *HNF4 α* ⁺ and *Sox9*⁺ oval cell markers in HCC cell lines.

After the western blot of CD133 expressing cell lines, 5 HCC cell lines (HepG2, HepG2-2215, Hep3B, Hep3B-TR) were detected to be expressing both HNF4 α and SOX9. Detection of co-expression of these markers has evoked the question of whether this expression is present within individual cells, since presence of such cells will be a sign of potent stem cell or oval-like cells in a cancer cell line. This may further relate to the biology of CSCs, in terms of their resemblance of oval-like cells. Immunofluorescence analyses was done to further both confirm the results from western blot analyses and in order to point out individual cells showing a HNF4 α ⁺ and/or SOX9⁺ phenotype (Figure 3.27-3.30).

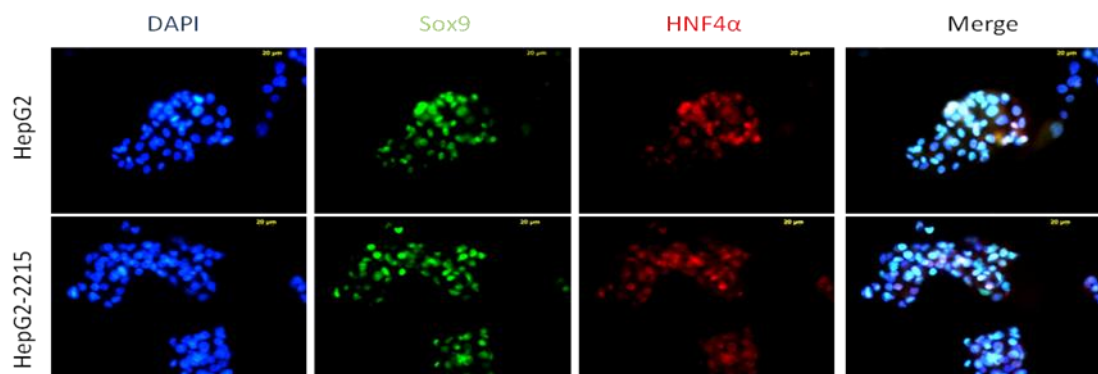


Figure 3.27: Immunofluorescence images showing presence of co-expression of HNF4 α and SOX9 in HepG2 and HepG2-2215 cell lines. Antibodies were Sox9-Alexa 488 and HNF4 α -Alexa 568.

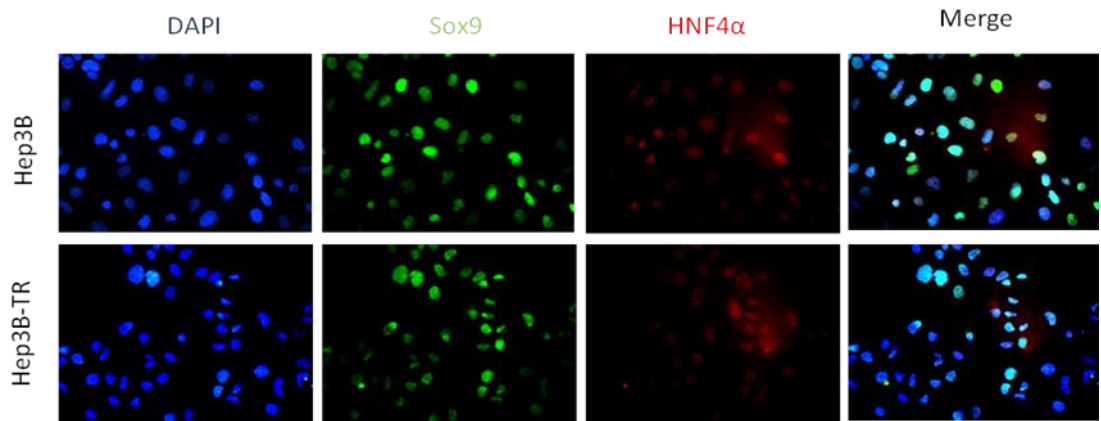


Figure 3.28: Immunofluorescence images showing presence of co-expression of HNF4 α and SOX9 in Hep3B and Hep3B-TR cell lines. Antibodies were Sox9-Alexa 488 and HNF4 α -Alexa 568.

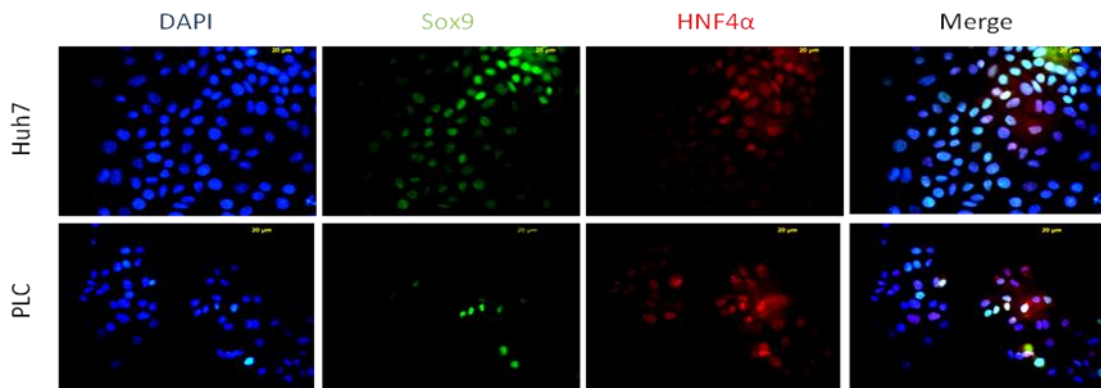


Figure 3.29: Immunofluorescence images showing presence of co-expression of HNF4 α and SOX9 in Huh7 and PLC cell lines. Antibodies were Sox9-Alexa 488 and HNF4 α -Alexa 568.

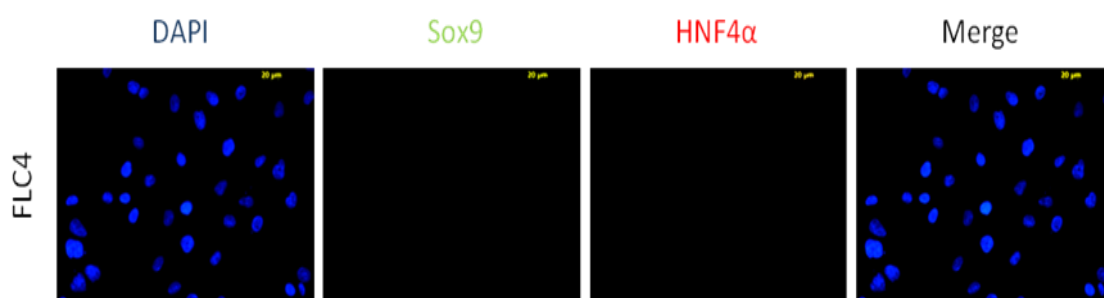


Figure 3.30: Immunofluorescence images showing absence of co-expression of HNF4 α and SOX9 in FLC4. Antibodies were Sox9-Alexa 488 and HNF4 α -Alexa 568.

After immunofluorescence analysis of these different HCC cell lines, we detected that both HNF4 α + / Sox9 + double positive cells co-existed within CD133+ / EpCAM+ cell sub-populations. These intriguing results suggest the following possibilities. i) These markers could be used to identify potential CSC sub-populations in a cell line. ii) These cells could attain these extra markers and comprise what was known as an oval cell. Further analysis, needs to be done to analyze these possibilities in the future. The regulatory role of Notch signaling on these marker expressions also deserves detailed investigation.

3.5 Determination of Toll-like Receptor Activity in Hepatocellular Carcinoma

Lately, tumor microenvironment and cancer-immunotherapy has become hotter topics. Usage of TLRs as a way of cancer-immunotherapy was applied for a while now. However, TLR therapy on HCC was not a topic worked on very often previously. Yet, in order to construct a hypothesis, our team went through a screening set of experiments to detect the presence of TLR expressions if any.

3.5.1 Basal Toll-like Receptor Expression in Hepatocellular Carcinoma Cell Lines

To assess the basal TLR levels in 6 HCC cell lines, which were found to be positive for CSC markers CD133 and EpCAM in variable percentages. A PCR based analysis method was used for this cells were seeded and left overnight, next day cells were

collected, total RNA was isolated and cDNA was synthesized. PCR was then performed to construct a panel for TLRs in HCC.

As seen from Figure 3.30, there is a differential TLR expression in different cell lines, yet most of them express TLR5. HepG2 seems to have TLR4, TLR5 and TLR6 expression, yet HepG2-2215 has TLR4 and TLR5 expression.

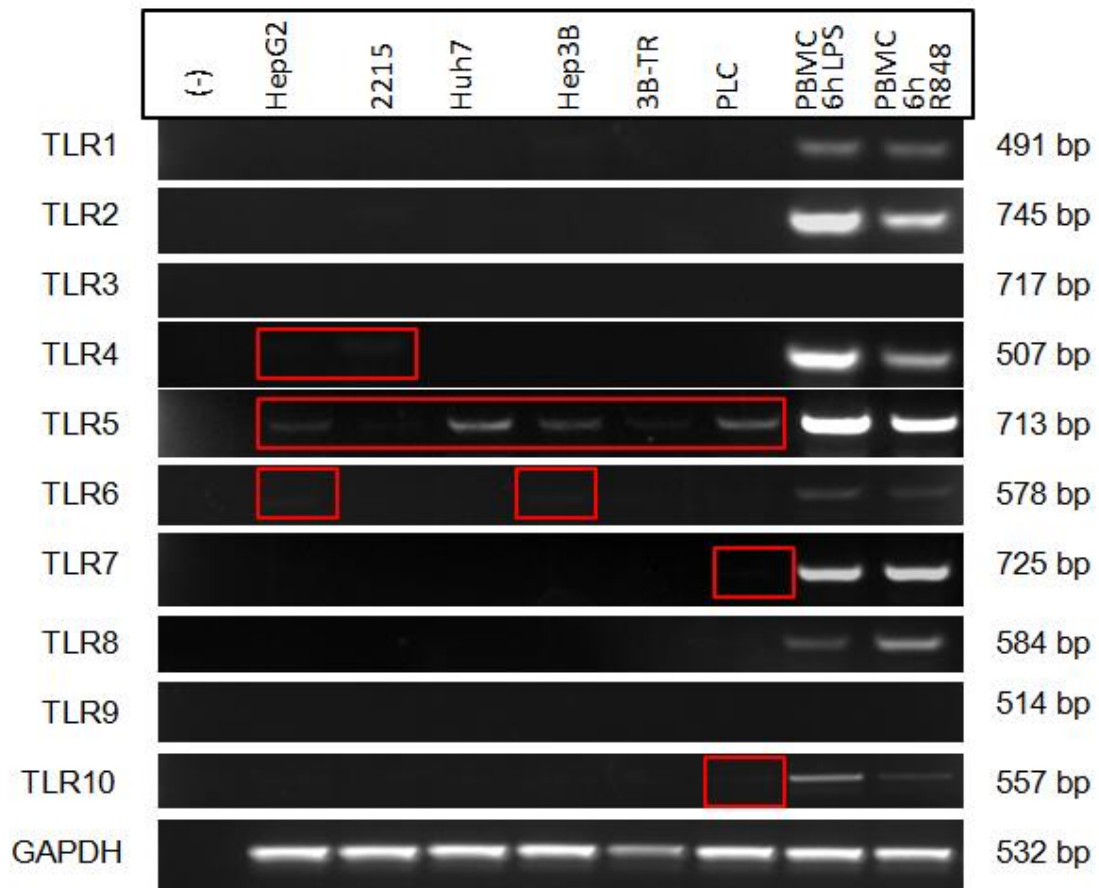


Figure 3.31: Gel image showing PCR product bands for basal TLR levels in 6 HCC cell lines. Red box: upregulation.

Huh7 shelters only TLR5 expression, whereas, Hep3B has TLR5 and TLR6 expression. Hep3B-TR has only TLR5 expression but PLC has TLR5, TLR6 and a very slight TLR10 expression. 6 hour LPS treated PBMCs and 6hour R848 treated PBMC RNAs were used as positive controls.

3.5.2 Effects of TLR ligand treatments on the expression levels of TLRs in HCC cell lines.

In this part, we aimed for inducing the TLR receptors by using different TLR ligands, including lipoteichoic acid (LTA), peptidoglycan (PGN), lipopolysaccharide (LPS), polyinosinic: polycytidilic acid (Poly(I:C)), flagellin, R848, resiquimod and K23 on different HCC cell lines. Cells were seeded the day before, and settled overnight, next day, cells were induced by different concentrations of TLR ligands, after 4 hours of induction total RNA was isolated from the cultured cells. These analyses were conducted on 4 cell lines HepG2, Huh7, Hep3B, and Hep3B-TR. Hep3B results can be found in Appendix A2.

For HepG2 compared to the untreated group, LTA, PGN, LPS, poly(I:C), R848 and K23 seem to be upregulation of TLR5, only PGN causes a slight increase in TLR6 levels. LTA, PGN, LPS and K23 increase TLR7 levels, and TLR8 is induced by LTA, PGN and K23. TLR9 is only induced by K23, whereas TLR10 upregulated by induction of LTA, PGN, poly(I:C), flagellin, R848 and K23 (Figure 3.32).

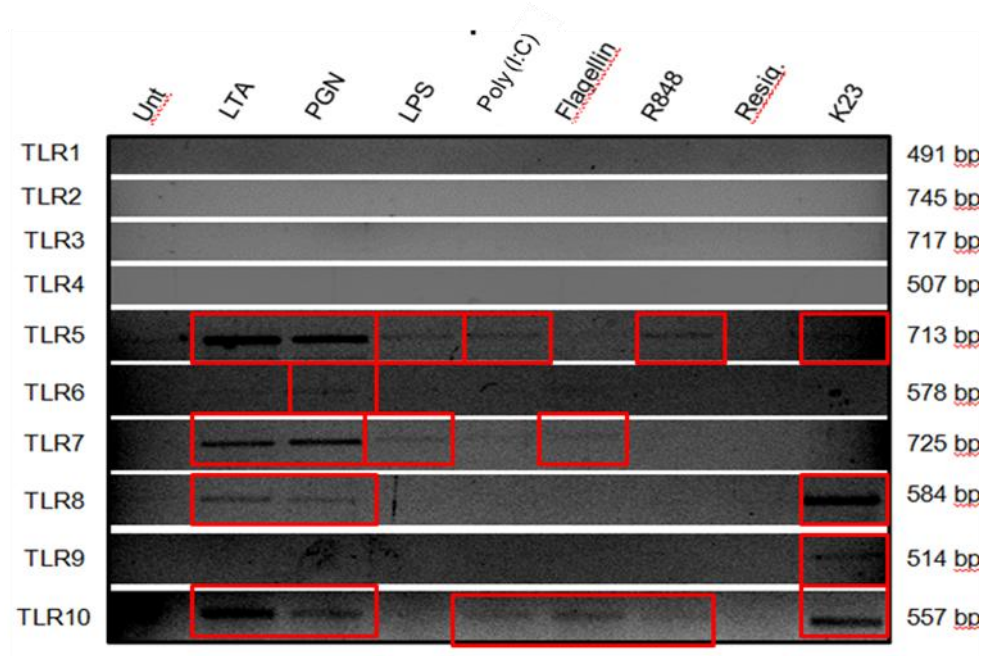


Figure 3.32: Gel image showing PCR product bands of TLR levels in HepG2 upon under treatment of different TLR ligands. Red box: upregulation.

For Huh7 expression changes, TLR5 is induced by LTA, LPS and especially upregulated by Poly (I:C), but down-regulated by flagellin. There seems to be an effect of Resiquimod on TLR7 induction but actual impact comes from K23. TLR10 is induced by K23, as well (Figure 3.33).

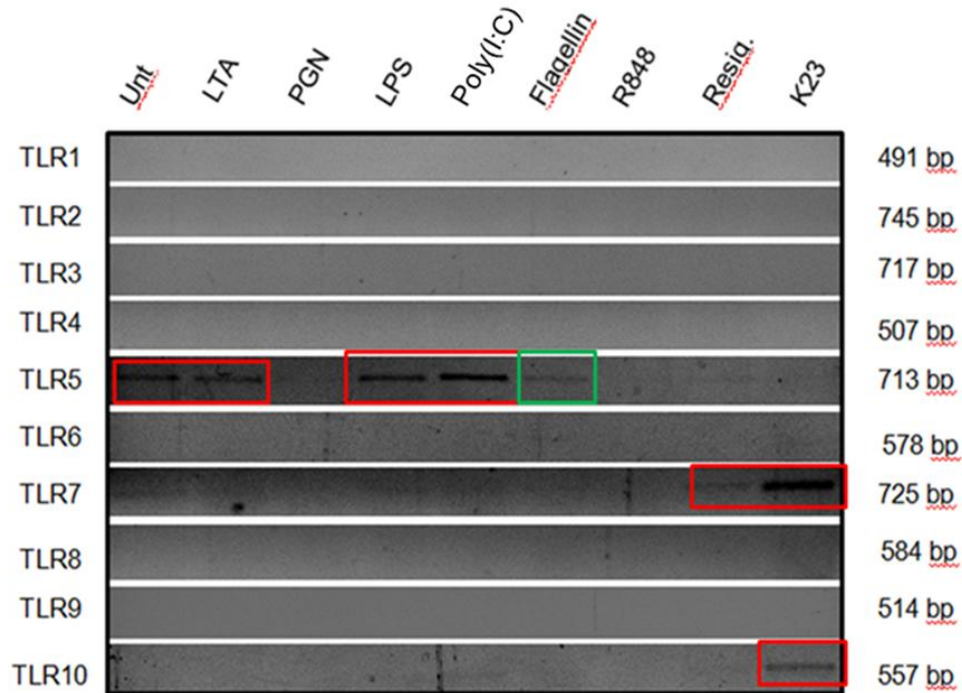


Figure 3.33: Gel image showing PCR product bands of TLR levels in Huh7 upon treatment with different TLR ligands. Red box: upregulation, green box: down regulation.

For Hep3B-TR, LTA leads to a strong induction in TLR2 levels, as well as TLR4. PGN, LPS and Poly(I:C) cause a decrease in TLR4 levels. TLR5 levels, this time surprisingly lowered by LTA, LPS, Poly(I:C), flagellin and K23 induction. TLR was induced by LPS. TLR10 signal was lowered by LTA, Poly(I:C) and R848, but was increased by LPS, Resiquimod and K23 induction (Figure 3.34).

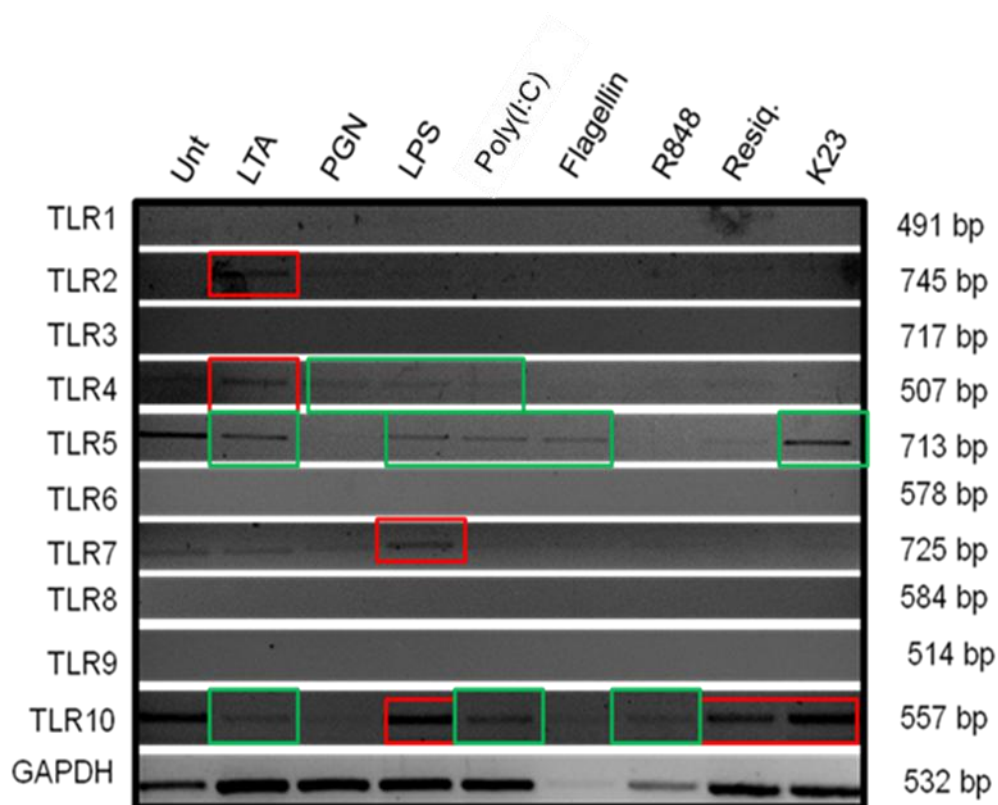


Figure 3.34: Gel image showing PCR product bands of TLR levels in Hep3B-TR upon treatment with different TLR ligands. Red box: upregulation, green box: down regulation.

3.6 Strategies for effective Immunotherapy of Hepatocellular Carcinoma in Athymic mice Xenografts

For the immunotherapy for Huh7 mediated established HCC in nude mice, following large tumor formation different treatment strategies were conducted for each group. It is important to note that only two injections (6 days apart) were performed during immunotherapy.

We hypothesized that TLR ligands mediated immune activation plus Notch inhibitor effect will induce a synergistic and combined tumor cell elimination from these mice. As seen in Figures 3. 34 and 3.35, inclusion of both therapeutic agents in Group #4, reduced tumor burden up to 87.5 % (Group #1 average volume: $2650 \pm 1350 \text{ mm}^3$ vs Group #4 average volume: $330 \pm 357 \text{ mm}^3$) at the end of day 9 post treatment. Although Group #2 and Group #3 substantially reduced tumor burden, their

combination induced more pronounced tumor clearance (compare Groups #2 vs #3 vs #4; $397 \pm 223 \text{ mm}^3$ vs $730 \pm 710 \text{ mm}^3$ vs $330 \pm 357 \text{ mm}^3$, respectively).

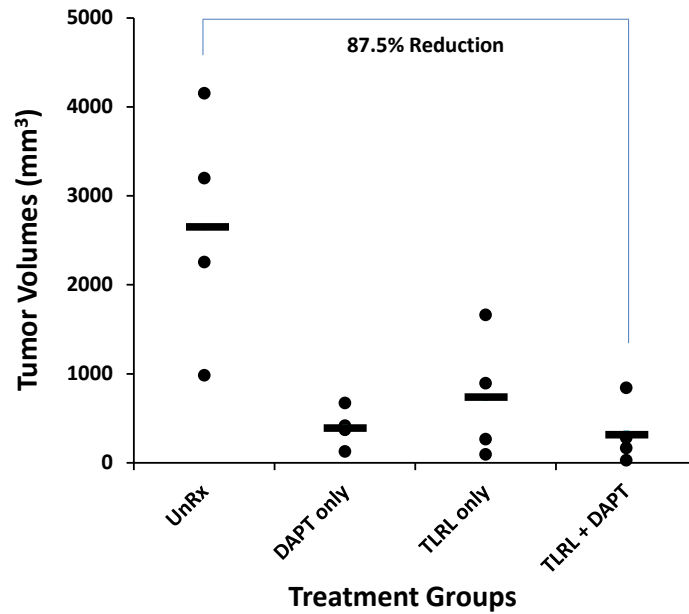


Figure 3.35: HCC bearing mice (N=4) tumor burden plots. Animals were xenotransplanted with 4×10^6 Huh7 cells, tumor formation was followed until they reached 300-400mm³ in size and peritumoral injections of the indicated treatments were done on day 1 and days 6. Tumor volumes were followed by daily measurements (TLRL: TLR3, TLR7, and TLR9 ligand combinations; DAPT: a gamma-secretase inhibitor).

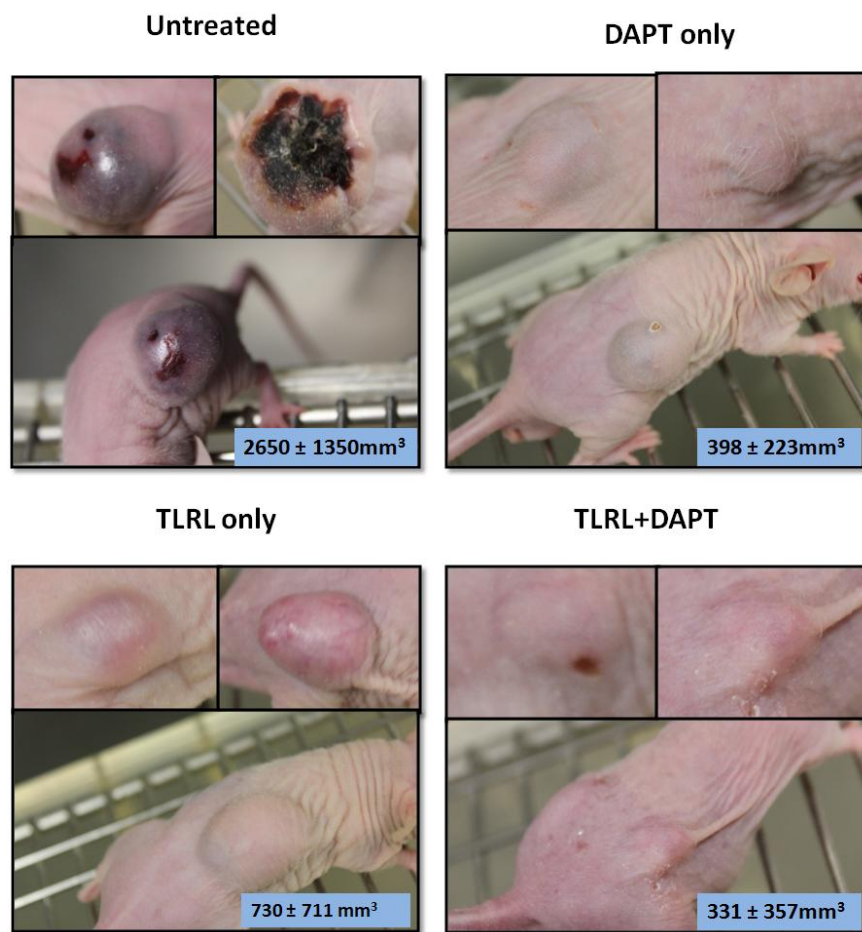


Figure 3.36: Photomicrographs showing tumors of different mice at day 9 post therapy.

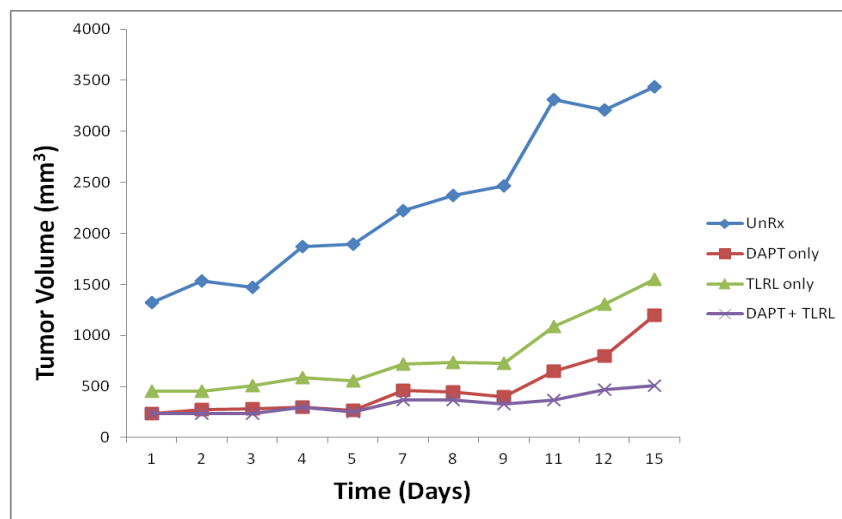


Figure 3.37: Time course tumor growth kinetics of untreated and immunotherapy applied test groups.

Chapter 4

DISCUSSION

There were several questions to tackle throughout this thesis. First, we wanted to differentiate the CSC subpopulation rates among HCC cell lines upon incubating them in sustained stress conditions. Furthermore, another aim was to identify the effect of Notch signaling modulation and its effect on CSC alteration. Within the scope of this thesis was to develop an approach that would help to eradicate established large HCC tumors in athymic mice. For that, we wanted to take advantage of TLR signaling along with CSC reduction mechanism. In the lights of our observation, we achieved this by using sequential administration of several TLR ligands along with a Notch inhibitor, namely DAPT. Eventually, our in vivo data revealed that coupling a notch inhibitor along with nucleic acid TLR sensors (i.e. TLR3, TLR7/8 and TLR9) synergistically contribute to eliminate HCC tumors in mice even after just two injections six days apart.

4.1 Investigation of cancer stem cell subpopulation in hepatocellular carcinoma cell lines.

Cancer stem cells possess self-renewal ability as well as giving rise to differentiated progeny of cells as normal stem cells. Chemotherapy and radiotherapy resistant nature of cancer, and recurrences after surgical removal, or even metastatic capability of a tumor are suggested to be orchestrated by CSCs ⁴⁷. Different cancer tumor types were shown to be expressing intersecting markers with others or sometimes specific markers for themselves ¹⁰⁴. Leukemia-initiating cells were, CD34⁺/CD38⁻, whereas breast cancer initiating cells were CD44⁺/CD24^{-low}. In determination of cancer stem cells in HCC, there were several cardinal markers as listed in Table 1.1. Two of these markers, however, were highly preferred, by many investigators working on this

topic, these are; CD133 (Prominin-1) and EpCAM^{56,57,61,63,67,69}. Hepatocellular carcinoma is the most commonly seen subtype of HCC with 80%, and is known to be highly resistant to chemo-and-radio therapy. Several studies demonstrated the existence of CSC in HCC^{53,69}. Therefore, in addition to already existing therapies it is critical to understand CSC involvement and offer better targeting strategies therapeutically. Following CSC screening of 17 HCC derived cell lines either by flow cytometry or immunoperoxidase analyses (Figure 3.1-3.4), we established that 6 cell lines (35%) were positive for CD133⁺/EpCAM⁺ expression.

Following initial determination of CD133⁺ subpopulation bearing HCC derived cell lines, next step was to strengthen these outcomes with a second CSC marker, EpCAM. We selected 3 cell lines to work thoroughly for the rest of the study. One with low-CD133⁺/EpCAM⁺ subpopulation (HepG2), another with mediocre level of CD133⁺/EpCAM⁺ (Huh7), and the last one with highest CD133⁺/EpCAM⁺ subpopulation level (Hep3B-TR) (Figure 3.6). Correlation between CD133⁺. Cancer cells, normally do need nutrients from the environment to grow on, in the absence of nutrient supplement they start dying, however, stem cells are more resistant to harsh conditions than differentiated cancer cells. By assuming a similar role for CSCs, we have applied varying serum starvation experiments for our selected cell lines. Initially, cells were seeded and left in complete media for three day, and then changed into serum starved media for another 3 days. At the end of the sixth day, there was around 20% increase in CD133⁺/EpCAM⁺ subpopulation in both HepG2 and Huh7, but that was not the case for Hep3B-TR, which is desensitized for TGF-beta-1 receptor (Figure 3.6). Yet, also, the reason for no increase is that Hep3B-TR already has around 96% CD133⁺/EpCAM⁺ subpopulation, therefore, additional markers might be used to further analyze the shifts occurring in this cell line upon serum starvation. An expression analysis study between the 3rd day in complete media vs. 3rd day in serum starved media might give enlightening results on the set of genes regulating this increase in stemness to launch an idea.

Daily follow up of the CD133⁺/EpCAM⁺ levels in selected cell lines did not show any difference during first three days in complete media (Figure 3.7). However, when the results were inspected at day 4, day 5 and day 6 all of which were in serum

free media, we have detected a gradual increase, which was finalized around 40% increase in CD133⁺/EpCAM⁺ subpopulation in HepG2, and furthermore detected around 20% increase in Huh7 levels.

4.2 Effects of Notch signaling pathway on CD133⁺/EpCAM⁺ subpopulation in selected HCC derived cell lines

Notch signaling pathway is one of the key signaling pathways during development. It is involved in stemness maintenance in embryonic stages, differentiation and fate decision control, as well as angiogenesis during development^{64,80,82}. Aberrations in Notch signaling was detected in cancer stem cells of numerous cancer types including breast cancer, leukemia, brain tumors and glioma¹⁰⁵. However, information on how Notch signaling acts on CSCs of HCC or on anti-tumoral effects of Notch signaling inhibition is not clear and needs further investigation. In this study to understand the effects of Notch signaling, a selected cell line, Huh7 was treated with definite Jagged-1 and DAPT concentrations to see the changes in CD133⁺ population. Because of this colony formation assay, colonies with 20% CD133 positivity was increased by two fold in Jagged-1 treated cells, whereas upon DAPT treatment CD133 positivity levels of the colonies were decreased by half. These results may suggest a potential role of Notch signaling in the regulation of CD133⁺ CSC subpopulation in Huh7. Yet, this is only qualitative observation. To interrogate the data further, three of the selected cell lines were treated with Jagged-1 and DAPT at varying concentrations with serum starvation. After three days in complete media and three days in serum starved media with treatments, a significant decrease (by half) in CD133⁺/EpCAM⁺ subpopulation subpopulation was detected in HepG2 and Huh7 upon DAPT(60μM) treatment. However, with Jagged-1 treatment no significant changes were observed. These results may refer a potential role for Notch signaling effect on CD133⁺/EpCAM⁺ subpopulation, yet downstream molecules of Notch signaling needs further investigation to further understand the detailed mechanism. A study has shown that in colon cancer model EpCAM cleavage by gamma-secretase (PS2), which coordinates S3 cleavage of NICD, along with TACE, coordinating S2 cleavage of Notch. After that intracellular domain of

EpCAM, EpICD, translocates into the nucleus where it binds to beta-catenin to activate cell proliferation ¹⁰⁶. Such a discrepancy was not investigated in HCC before, therefore, Notch or signaling elements might be somehow involved in the regulation of EpCAM, our data supports that it does decrease the CD133⁺/EpCAM⁺ subpopulation. This needs to be further investigated.

Previously, it is known that in immature mice Notch signaling activation promotes the conversion of HNF4-alpha positive hepatocytes into biliary epithelial cells that are lining the bile ducts and biliary tree ¹⁰¹. Recently, the study of Yanger et al. suggested that upon toxic substance damage or ectopic Notch activation in the liver of mature mice, HNF4-alpha positive hepatocytes were directed into a reprogramming process, in which they were turned into HNF4 α ⁺/Sox9⁺ cells that resemble the features of oval cells, which were known to be the stem cells of the liver ¹⁰³. This phenomenon led us to question the probability of the presence of such a population in HCC derived cell lines, especially the ones that are CD133⁺/EpCAM⁺. Initial screening was done by western blot, and apart from FLC4 and PLC, all the other 5 cell lines were found to be positive for both HNF4 α and Sox9 (Figure 3.25). These results were extremely surprising for us because this suggest the probability of oval cells being the source of somehow involved at an intermediate step through reprogramming into CSCs. To further make sure that individual cells co-express these two markers, we have performed immunofluorescence analysis, and the results were as expected, individual cells in 5 cell lines, Hep3B, Hep3B-TR, HepG2, HepG2-2215 and Huh7 were HNF4 α ⁺/Sox9⁺, but PLC showed only mild Sox9 co-expression with HNF4 α , which could be expected since very-low expression of proteins may not be detected by western blotting. FLC4 was negative for both HNF4 α and Sox9 (Figure 3.26-3.29). These results, however, need further support to show the co-expression of HNF4 α and Sox9 with CD133 and EpCAM, as well. Moreover, the biological precedence of the markers should be settled down to understand whether these cells become first HNF4 α ⁺/Sox9⁺, then CD133⁺/EpCAM⁺ or vice versa. This will make us to reach one-step further in understanding the biological origins of liver cancer stem cells.

4.3 TLR expression in HCC and cancer-immunotherapy by TLR ligands and Notch inhibition

Tumor cells surprisingly have developed themselves to create an immune suppressive environment. They do this by mediating parallel actions such as secretion of TGF- β , adenosine or VEGF-A. Some of these molecules lead to dendritic cell suppression, and some inhibit T-cell penetration into the tumor bed. In case of hypoxia, for instance, tumor cells secrete adenosine, which will trigger T-cell activation suppression⁹⁸. There are several approaches to overcome this immunosuppressive environment of the tumor bed, one of which is to make use of the immunostimulatory effects of toll-like receptor activation through TLR ligands. In this model, we use nude mice whose ultimate deficiency in tumor therapy is the lack of cytotoxic (CD8+) T cells (CTLs), which specifically target tumor cells. In nude mice' however, the major group of cells responsible for the defense against tumors are natural killer cells (NK cells). Previous usage of TLR agonists in HCC tumor in nude mice was done in only a couple of studies, which were not resulted in very efficient tumor shrinkage, unfortunately. Moreover, there were no hits detected upon TLR agonist therapy in combination with Notch inhibitor DAPT. There were studies showing that upon ectopic activation of NK cells which constitutively produce and respond to IFN- α , they become more activated in an efficient way to attack on tumor cells in nude mice¹⁰⁷. An in vitro study on TLR3 agonist treatment in HCC has shown that, upon dsRNA treatment to activate TLR3 in HCC cell lines, cells were shown to be entering into apoptosis, migration abilities were inhibited¹⁰⁸. Throughout the last part of this study, we have detected upregulation of four TLRs mainly in Huh7, TLR5, TLR7 and TLR9 and TLR10. Yet, in this study since involvement of TLR3 was also shown before, we have used TLR agonists for TLR3, TLR7 and TLR9. Along with their immunostimulatory effects by combining this therapy with DAPT, it was aimed to both strengthen the immune attack on tumor cells as well as eradicating the CSC population to prevent any follow-up recurrences. The combination therapy treated mice shown the highest success, although DAPT alone and combination therapy resulted in similar reduction in tumor sizes, tumors treated with only DAPT started to rebound, whereas combination

therapy applied mice tumors were still stable in volumes. Such an effect may imply that two-hit method we developed result in better response against tumor relapse (Figure 3.37).

There are still some further research must be done in order to understand the involvement of CSCs in HCC initiation, progression, maintenance or therapy. To begin with, the immune cells infiltrated into tumor or the periphery must be controlled to determine activity of the NK cells and macrophages (in nude mice) compared to the immunosuppressive activity of myeloid derived suppressor cells (MDSCs). The aim in study is to come up with a therapy method at the end, to eradicate the tumor or to keep the tumor size under control, and for such a case the toxic effects of DAPT on non-target cells must be cleared to prevent any cytotoxic effects.

Regarding to oval cells, further studies are needed done to be able to gain more insight into whether oval-like cells are responsible for the programming of CSCs, since they are in a progenitor cell state, their upcoming de-differentiation following genetic mutations might be leading to the $CD133^+/EpCAM^+$ CSC subpopulation or vice versa.

In order to extend the amount of the impact of the combination therapy in reduction of the HCC tumors, experiments to check any connection between Notch signaling pathway and TLRs must be investigated. In case of the existence of any relation, depending on the pattern of relation, a therapy alternative to abusing this relation may be administered.

Apart from starting with large tumor sizes, another challenge in this in vivo study was the usage of athymic nude mice. Although having its innate immune cells such a NK cells or macrophages, tumors shrinkages were increased upon treatment with the TLR ligands only. Yet, in the real life scenario, patients will have intact immune system, and within the adaptive immune system, $CD8^+$ cytotoxic T cells are especially involved in targeting of the tumors cells as a defense mechanism. Therefore, repeating these tumor formation and combination therapy experiments is a necessity within mice having intact immune system, this way, reduction in the tumor

sizes may increase, or even the tumor may be completely eradicated. As final remark, DAPT should be administered in other cancer types (e.g. breast cancer) to discover if there is any potential inhibition in cancer immunotherapy.

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Appendices

Appendix A

Appendix A1 Cancer stem cell studies on Hep3B

Serum starvation effects were also investigated on Hep3B cell lines, and it was detected that at the end of 72 hour serum starvation, CD133⁺/EpCAM⁺ population was not affected by serum deprivation (Figure 6.1).

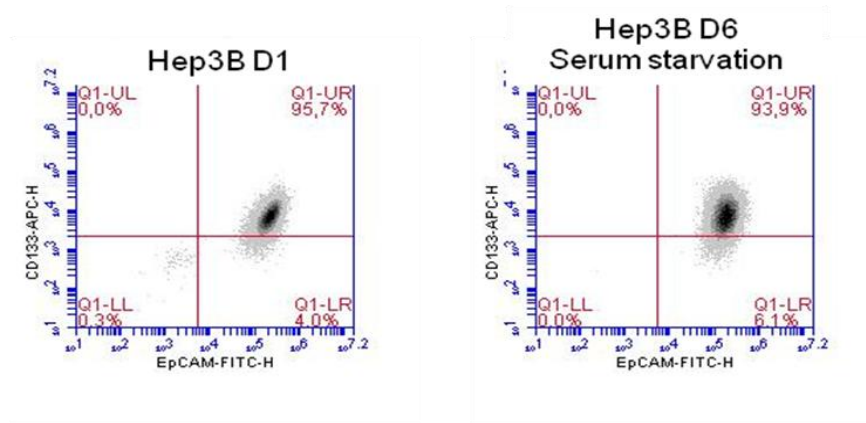


Figure A1: Flow cytometry analysis showing the CD133/EpCAM double positive population changes upon serum starvation.

In order to understand the daily shifts occurring in the level of CD133⁺/EpCAM⁺ population, cells were seeded, and fixed after 24hours, 48 hours and 72 hours after seeding. Analyzed by flow cytometry, during first 72 hours, a significant alteration in of CD133⁺/EpCAM⁺ fraction could not be observed (Figure 6.2).

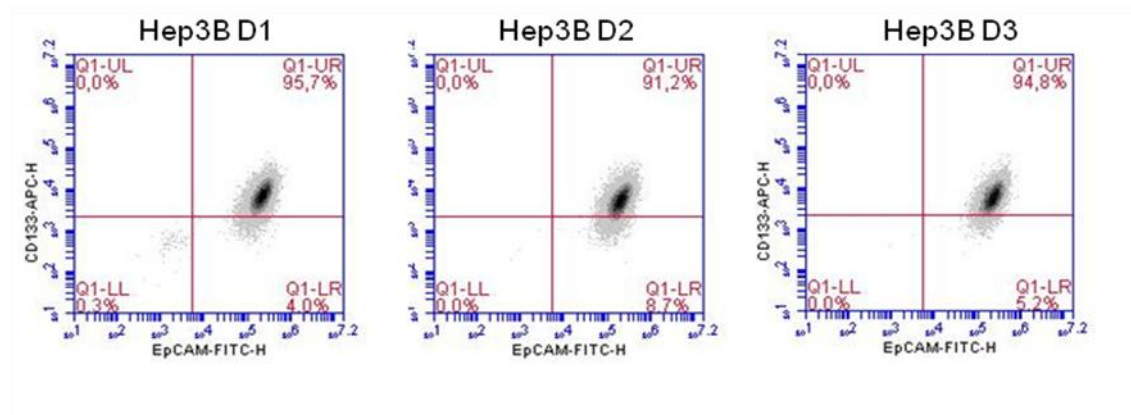


Figure A2: Flow cytometry analysis showing the CD133/EpCAM double positive population changes on a daily basis in complete media.

The next step to be checked was to shifts in of CD133⁺/EpCAM⁺ population upon serum starvation. For this matter, cells were seeded and changed to serum deprived media at the end of 72 hours, then cells were collect 24 hours, 48 hours and 72 hours after media change. Analysis results did not show a significant alteration in of CD133⁺/EpCAM⁺ population upon serum starvation (Figure 6.3).

The final analysis on Hep3B cell line was on the effects of Notch signaling pathway on of CD133⁺/EpCAM⁺ population. For this analysis, cells were seeded and treated with Jagged-1 and DAPT in serum starved media, after 72 hours of plantation, then cells were collected 72 hours after treatment and analyzed. Analysis results revealed that Jagged-1 does not seem to have an effect on of CD133⁺/EpCAM⁺ population, but DAPT leads to a drop on the levels of of CD133⁺/EpCAM⁺ population (Figure 6.4).

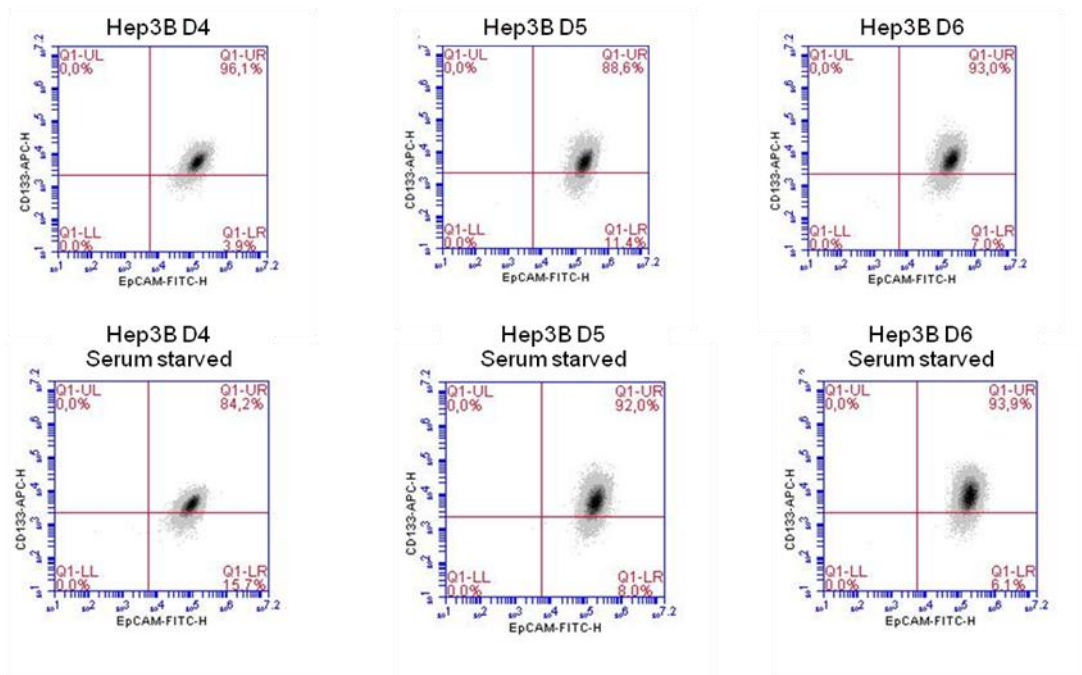


Figure A3: Flow cytometry analysis showing the CD133/EpCAM double positive population changes on a daily basis in complete media vs. serum starved media.

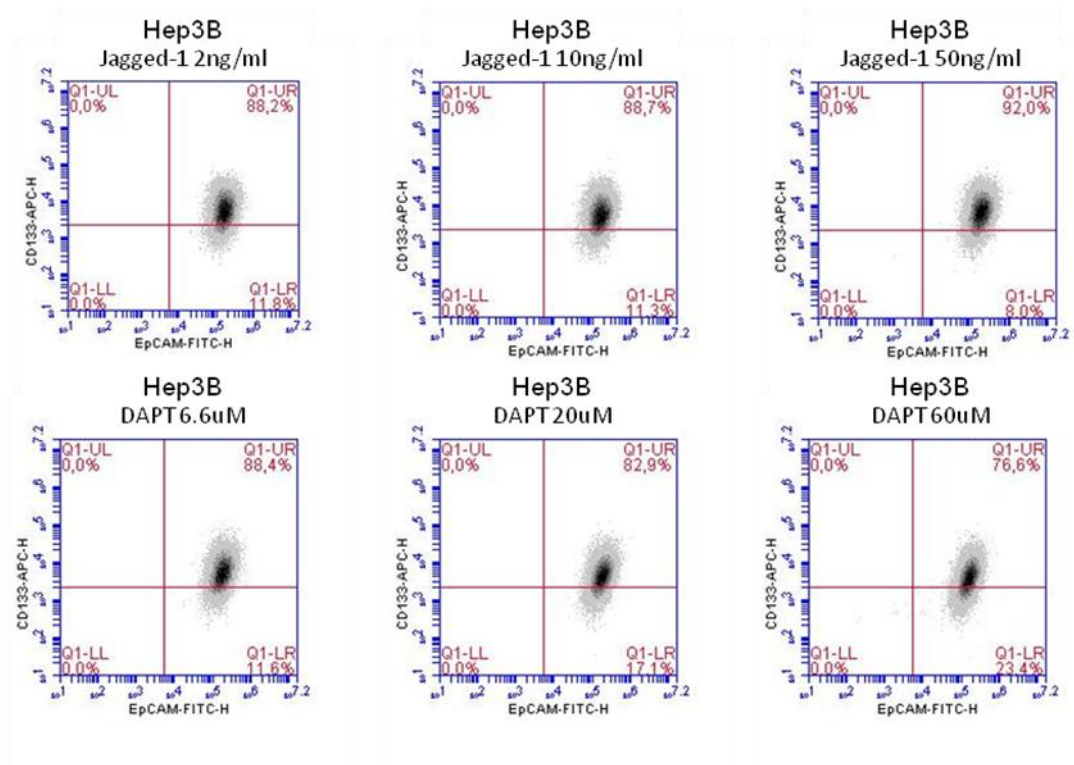


Figure A4: Flow cytometry analysis showing the CD133/EpCAM double positive population changes upon Notch signaling alteration.

Appendix A2 TLR ligand treatment effects on TLR levels in Hep3B

In this part, our team aimed for inducing the TLR receptors by using different TLR ligands including lipoteichoic acid (LTA), peptidoglycan (PGN), lipopolysaccharide (LPS), polyinosinic:polycytidilic acid (Poly(I:C)), flagellin, R848, resiquimod and K23. Cells were seeded the day before, and settled overnight, next day, cells were induced by different concentrations of TLR ligands, after 4 hours of induction total RNA was isolated from the cultured cells.

Hep3B shows a more induced pattern than Huh7 and HepG2. TLR1 was affected by Poly(I:C). TLR4 shown only a very slight banding in response to Poly(I:C), Resiquimod and K23. TLR5 showed induction by LTA, Poly(I:C), flagellin, but decreased expression by PGN, R848, Resiquimod and K23. TLR6 has given expression upon induction by LTA, PGN, Poly(I:C), flagellin, R848 and K23, but these responses are very similar to the untreated one. TLR7 was induced by PGN, Poly(I:C) and flagellin. TLR10 was also upregulated by Poly(I:C) and flagellin, also by K23, but downregulated by R848 and Resiquimod induction Figure 6.5

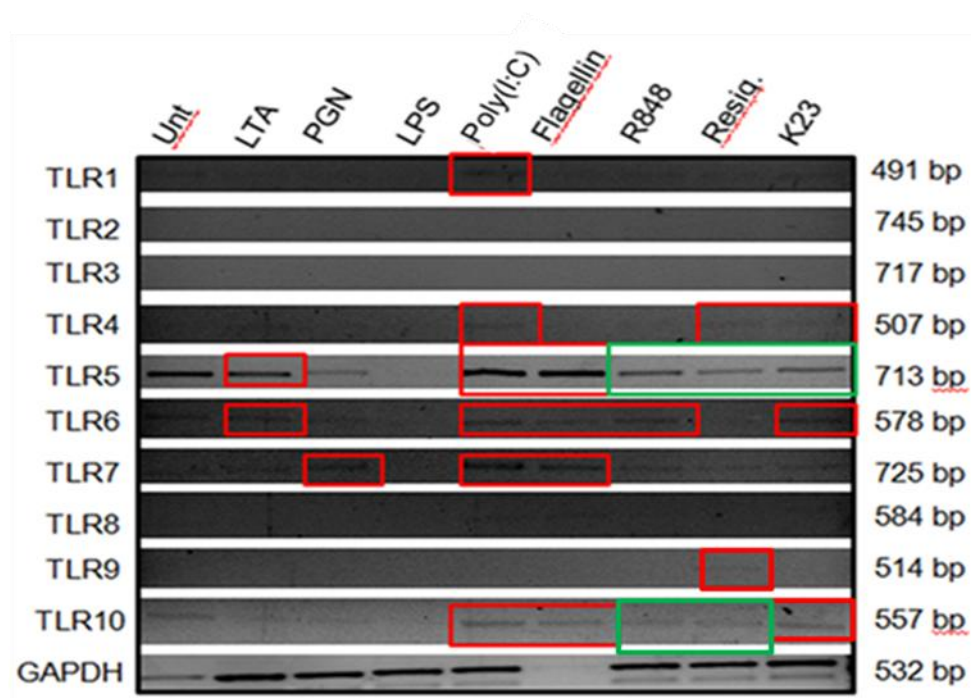


Figure A5: Gel image showing PCR product bands of TLR levels in Hep3B upon treatment with different TLR ligand

Appendix B

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