THE ROLE OF TETRASPANIN-8 IN ASTROCYTE ELEVATED GENE-1 MEDIATED PROGRESSION OF HEPATOCELLULAR CARCINOMA

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Human and Molecular Genetics at Virginia Commonwealth University

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LIST OF ABBREVIATIONS

- **HCC:** Hepatocellular Carcinoma.
- **HBV:** Hepatitis B Virus.
- **DEN:** Diethyl- nitrosamine
- **HCV:** Hepatitis C Virus.
- **NAFLD:** Non-alcoholic fatty liver disease.
- **HRQL:** Health Related quality of Life.
- **ORF:** Open Reading Rrame
- **HBcAg:** Hepatitis B Core Antigen.
- **TERT:** Telomerase Reverse Transcriptase.
- **PDGFRβ:** platelet-derived-growth-factor receptor-β.
- **MAPK1**: Mitogen Activated Protein Kinase 1
- **ER:** Endoplasmic Reticulum.
- **miRNA:** micro Ribonucleic Acid
- **STAT3:** Signal Transducer and Activator of Transcription 3
- **SREBP**: Sterol Regulatory Element-Binding Protein.
- **VEGF:** Vascular Endothelial Growth Factor
- **HIF1α:** Hypoxia-Inducible Factor-1α
- **Wnt pathway:** Wingless-int pathway
- **pRB:** pophorylated Retinoblastoma.
- **PKR:** Protein Kinase R
- **GSK-3:** Glycogen synthase kinase 3
- **AFB1:** Alfatoxin B1

- **HRAS:** Harvey Ras
- **ADH3:** Alcohol dehydrogenase type 3
- **IL6:** Interleukin 6
- **IL1β:** Interleukin 1β
- **SNPs:** Single Nucleotide Polymorphisms
- **BMI:** Body Mass Index
- **EGF:** Epidermal Growth Factor
- **EGFR:** Epidermal Growth Factor Receptor
- **GF:** Growth Factor
- **IGF:** Insulin Growth Factor
- **IGF-R:** IGF- receptors.
- **IGFBP:** Insulin Growth Factor Binding Protein
- **MET:** Mesenchymal-Epithelial Transition factor.
- **mTOR:** mammalian Target Of Rapamycin
- **PI3K:** Phosphatidylinositol 3-kinases
- **COX-2:** cyclooxygenase 2.
- **PGE2:** prostaglandin E2.
- **TNFα:** Tumor Necrosis Factor α
- **BCLC:** Barcelona Clinic Liver Cancer
- **CLIP:** Cancer of the Liver Italian Program
- **TNM:** Tumor, Node and Metastasis
- **JIS:** Japanese Integrated Staging Score
- **AASLD:** American Association for the Study of Liver Diseases

- **ANG-2:** angiopoetin-2
- **MMPs:** Matrix metalloproteinases
- **VEGF:** Vascular endothelial growth factor
- **VE-cadherin:** Vascular Endothelia cadherin.
- **FGF:** Fibroblast Growth Factors
- **PDGF**: Platelet-Derived Growth Factor
- **PAF:** Platelet Activating Factor
- **NO:** Nitric Oxide
- **E-cadherin:** epithelial cadherin
- **EMT:** Epithelial-to-Mesenchymal Transition
- **AEG1:** Astrocyte elevated gene-1
- **HIV-1** Human Immunodeficiency Virus-1
- **PHFA:** Primary Human Fetal Astocytes
- **AIDS:** Acquired Immunodeficiency Syndrome
- **RaSH:** Rapid Subtraction Hybridization
- **MTDH:** Metadherin
- **KD:** Kilo-Dalton
- **FISH:** Fluoresence in situ hybridization
- **qPCR:** quantitative PCR
- **TMD:** transmembrane domain
- **NLS:** Nuclear Localization Signals
- **IM-PHFA:** Immortal Primary Human Fetal Astrocytes
- **FM516-SV:** normal immortal melanocytes

- **CREF:** Cloned Rat Embryonic Fibroblasts
- **DPYD:** dihydropryimidine dehydrogenase
- **TS:** Thymidylate Synthase.
- **MDR1:** multidrug resistance gene 1
- **FGFα:** Fibroblast Growth Factor α
- **PIGF:** Placental Induced Growth Factor
- **EAAT2:** Excitatory Amino Acid Transporter- 2
- **CNS:** Central Nervous System
- **ALS:** Amyotrophic Lateral Sclerosis
- **Rrs1**: ribosome synthesis 1
- **HD:** Huntington Disease
- **Cdk2:** Cycline dependent kinase 2
- **SND1:** Staphylococcal nuclease domain containing 1
- **TSPAN-8:** Tetraspanin-8
- **TM:** Transmembrane
- **TEM:** Tetraspanin Enriched Microdomain
- **GPCR:** G-protein-coupled receptor
- **EPCAM:** Epithelial cell Adhesion Molecule
- **PKC:** Protein Kinas C
- **PLCγ:** phospholipase Cγ
- **CD 9:** Cell Differentiation 9
- **PI4KII:** phosphatidylinositol 4-kinase II
- **ERM:** Erzin-Radixin-Moesin

- **ADAMS:** A Disintegrin And Metalloprotease
- **DIC:** disseminated intravascular coagulation
- **DMEM:** Dulbecco's Modified Eagle Medium
- **FBS:** Fetal Bovine Serum
- **SDS-PAGE:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- **APS:** Ammonium persulfate
- **DMSO:** Dimethyl sulfoxide
- **HUVEC:** Human umbilical vein endothelial cells

ABSTRACT

THE ROLE OF TETRASPANIN-8 IN ASTROCYTE ELEVATED GENE-1 MEDIATED PROGRESSION OF HEPATOCELLULAR CARCINOMA.

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Human Genetics at Virginia Commonwealth University. Virginia Commonwealth University, 2012

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Hepatocellular carcinoma (HCC) is a devastating form of liver cancer that accounts for 80% of liver cancers. HCC has a poor prognosis with five-year survival of less than 12% in the United States. We in previous studies have identified Astrocyte Elevated Gene-1 (AEG-1) as an aberrantly overexpressed gene in many cancers including HCC, regulating tumor progression. Microarray studies identified the small transmembrane protein, tetraspanin8 (TSPAN8) as a downstream of AEG-1. TSPAN8 belongs to the family of

TETRASPANINS with the characteristic of crossing the membrane four times, and regulating a wide range of cellular phenomena. TSPAN8 is implicated in metastasis and is classified as a metastasis promoting tetraspanin. To understand the role of TSPAN8 in the context of AEG-1 regulated tumor progression of HCC, we generated knockdown clones of TSPAN8 in AEG-1-8 cell lines (HepG3 cell lines with stable overexpression of AEG-1), and analyzed cellular events that mediate metastasis such as migration, invasion and in-vivo tumorogenesis. Our in-vitro studies show that knockdown of TSPAN8 in AEG-1 overexpressing cells significantly abrogated migration, matrigel invasion, proliferation and endothelial cell activation. Moreover, we show that knockdown of TSPAN8 significantly inhibited intrahepatic metastasis of orthotopic xenografts in the livers of athymic nude mice. TSPAN8 might be a useful diagnostic marker and potential therapeutic target for HCC. These findings indicate that upregulation of TSPAN8 might be an important event in mediating the oncogenic function of AEG-1.

CHAPTER 1: INTRODUCTION

1.1 Hepatocellular Carcinoma (HCC):

Hepatocellular Carcinoma (HCC) is a term given to tumors that arise from epithelial cells of liver, the hepatocytes. The macroscopic pathology is characterized as scattered, large and multinodular tumors (1). HCC represents more than 80% of liver cancers. It is a serious worldwide life-threatening morbid illness with poor prognosis. Globally, HCC ranks the fifth in cancer prevalence, and the third in cancer reported deaths (2, 3). Gender-wise, HCC frequency is considered to be the fifth in males and the seventh in females (4). The likelihood of developing HCC is four to five times higher in males than females (4). This gender difference in occurrence of HCC is thought to be due to the influence of sex hormones (5, 6).

While androgen is believed to enhance the development of Hepatitis B Virus (HBV) triggered HCC in HBV transgenic male mice (5, 7, 8), estrogen is believed to reduce the occurrence of HCC in female mice upon exposure to the hepatocarcinogen, diethyl-nitrosamine (DEN) (5, 6). Recent findings on the regulatory mechanism showed that ligand dependent activation of androgen receptor mediates hepatocarcinogenesis via upregulation of cell cycle–related kinase (CCRK) in β-catenin dependent manner (9). Androgen pathway was also shown to mediate transcription of micro-RNA 216-a that negatively regulates tumor suppressor in lung cancer-1 gene (TSLC1) messenger RNA (mRNA) (10). However, the regulatory action by which androgen and estrogen orchestrate the disparity between sexes is still largely unexplored (5).

The number of reported new cases of HCC is escalating each year with a less than 12% five years survival rate. The American Cancer Society estimates that in 2012, there will be 28,720

new cases and 20,550 reported deaths in the United States (4, 11). Chronic hepatitis B virus infection constitutes 50% of HCC cases around the world (4).

In contrast, in the United States HCV related HCC is more common than HBV. HCV constitutes 48% of the cases compared to 16% of HBV related HCC cases in the United States (11, 12). Immigrants from countries, where HBV is endemic account for most of the HBV related HCC cases in the United States (12). The remaining percentage of cases is unequally scattered around risk modifying diseases such as alcohol related liver disease and metabolic syndromes (11, 13). However, HCV, alcohol related liver disease and non-alcoholic fatty liver disease (NAFLD) tend to be common risk factors in the United States (4, 11).

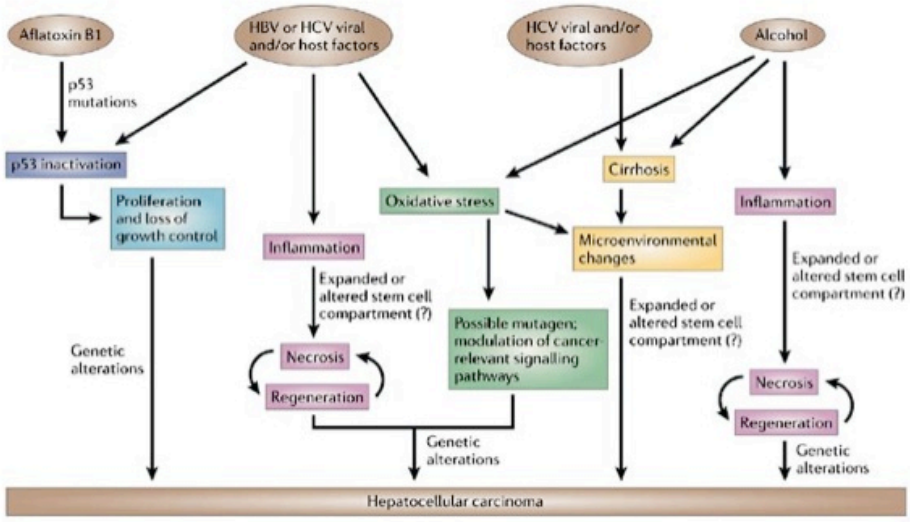
HCC is not a population specific illness. However, recent statistical data on incidence of HCC show that Caucasians, Hispanics and African Americans have a higher incidence than others in the United States, and HCV related HCC accounts for most of the cases (11). The incidence of HCC in the United States has increased from 1.6 to 4.9 (more than 200% increase) cases per 100,000 of U.S. population from 1975 to 2005 (14). Knowing that HCV related HCC manifests mostly at the age of 70 (4), the increase of HCV related HCC in US- citizens might be linked to the time where drug abuse was prevalent (more the 30 years ago), which could be explained by the cohort study of HCV infected patients due to sharing needles for drug abuse (2, 15). HCC is a highly heterogeneous type of cancer, which might be due to the diversity and increased number of etiological factors (16). The majority of risk factors lead to chronic liver disease that later progress to HCC. Patients with HCC have an extremely low health related quality of life (HRQL), which is a marker that was proposed to be considered when assessing prognosis of HCC, compared to chronic liver disease (4, 17).

1.1.1 Risk Factors:

A recent epidemiological review on Hepatocellular Carcinoma (HCC) showed that when geographical location and the population of hepatocellular carcinoma patients are taken into account, we would expect to see diverse and increased sets of risk factors because of the variability in environmental and socio-economic statuses, which might explain the heterogeneity and the complexity seen in HCC (2, 4, 16, 18). For instance, if we compare China to the United States, we would see that HBV and Alfatoxin B1, a mycotoxin produced from *Aspergillus flavus*, are more common risks for HCC in China than the United States (2).

This is due to the fact that HBV is endemic in Asia but not in the United States. The warm weather and the inappropriate storage and cleaning of cereals such as rice creates a favorable environment for growth of molds that secret Alfatoxin B1; a food contaminant the metabolite of which is carcinogenic (4, 19-23). Most risk factors induce chronic liver disease, which lead to fibrosis, cirrhosis and eventually HCC. In fact, more than 80% of HCC cases arise from a cirrhotic microenvironment (11, 15, 19, 24, 25).

According to a recent review published by El-Serag, the five-year cause dependent cumulative risk of HCC in patients with cirrhotic liver is estimated to fall in between 5% to 30% with an increased risk in HCV infected patients (4). Moreover, in the United States the common modifiers of risk for the development of HCC are HCV, HBV, alcoholic liver disease and non-alcoholic fatty liver disease (NAFLD) (4). Other uncommon risks include hereditary hemochromatosis, alpha1-antitrypsin deficiency, auto-immune hepatitis, porphyrias, and Wilson's disease (4). Here, I will describe the common risk factors for HCC (Figure 1).



Farazi and DePinho *Nature Reviews Cancer* **6**, 674–687 (September 2006) | doi:10.1038/nrc1934

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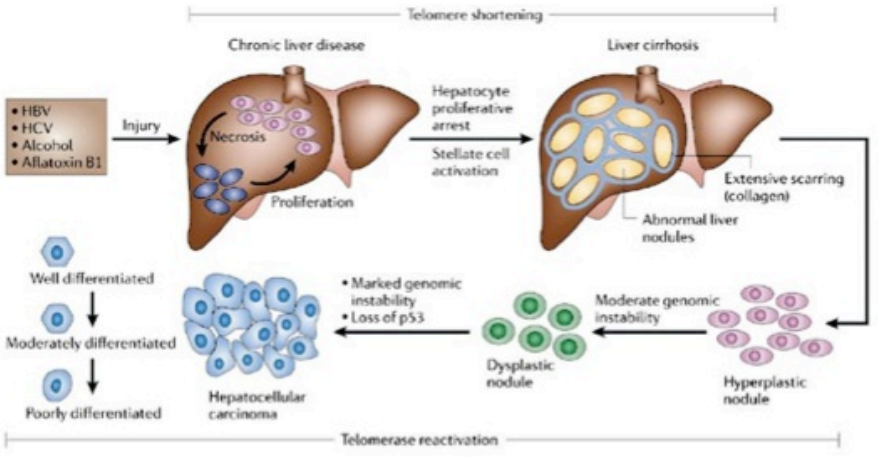
Figure 1:

A schematic diagram, representing common risk factors and possible actions on cell behavior that initiate the development of Hepatocellular Carcinoma (HCC). Identical colors represent same likelihood for the sequence of events.



Farazi and DePinho *Nature Reviews Cancer* **6**, 674–687 (September 2006) | doi:10.1038/nrc1934

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Figure 2:

A schematic representation of a common theme for the histopathological progression of Hepatocellular Carcinoma (HCC). Regardless of the type of risk factor, upon liver injury, an endless cycle of proliferation/necrosis is initiated to repair the damaged tissue. This cycle predisposes a chronic liver disease with inflammation, which leads to cirrhosis, hyperplasia, dysplasia and eventually HCC.

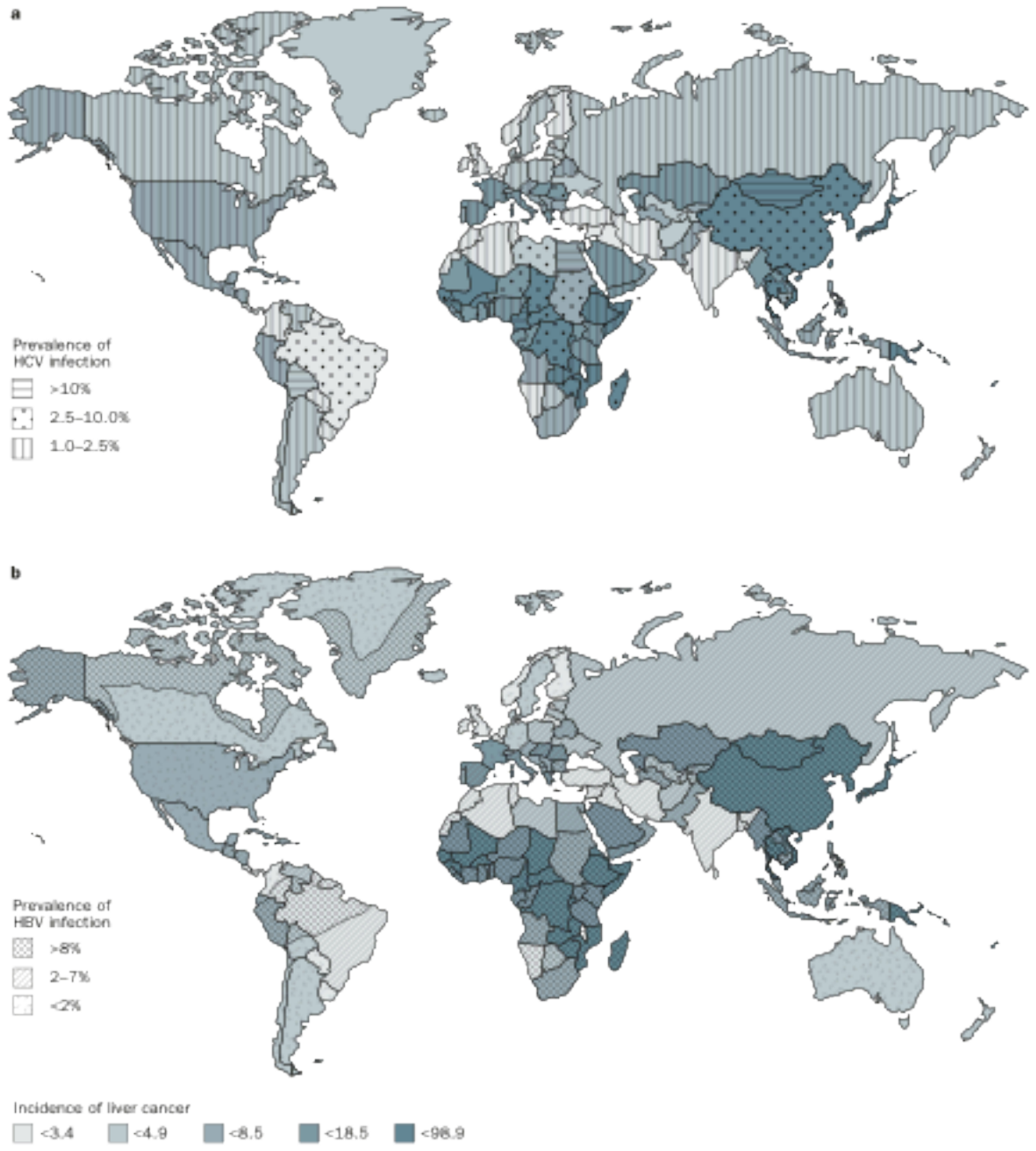

Figure 3:

A world map representing the prevalence of HBV and HCV infection and the age adjusted incidence of liver cancer. The figure was adopted from (14).

1.1.1.1 Hepatitis B Virus (HBV):

Hepatitis B Virus is classified as a member of the family *hepadnaviridae* (16). The virus has a partial double-stranded DNA, which encompass four open reading frames (ORFs) that encode the virus envelope, core protein (HBcAg), virus polymerase, and HBV X protein (26). HBV is the most common cause of HCC in countries that have low health care support systems with an endemic HBV infection such as Eastern Asia (Figure 3).

In Asia, the mother to infant transmission (i.e. vertical) is the most common in HBV infection. On the other hand, in the United States or European countries, HBV is transmitted by unsafe exchange of body fluids such as unprotected sexual intercourse and sharing needles (14). The infection of HBV tends to be acute in most cases; however, in a number of patients the virus doesn't get cleared out of circulation due to its ability to integrate in the genome. Another possibility is that the virus can evade the host immune response as a consequence of mutations that occur during the virus life cycle. As a result, the persistent integration into the host promotes the initiation of a chronic state where inflammation is thought to play a major role in facilitating the environment for HCC development. This was shown to be plausible due to host-viral interaction.

The infection of HBV triggers the immune response to recruit T-lymphocytes. Consequently, this might cause hepatocyte necrosis, inflammation and hepatocyte regeneration. The endless cycle of necrosis/regeneration to restore the tissue architecture of the liver is thought to initiate dysfunctional telomeres and genomic instability which in turn will give rise to dysplastic lesion that progress to tumorigenic lesions (16). The available evidence in the literature shows that HBV participate in the transformation of hepatocyte to HCC by regulating and interacting with a number of genes and signaling pathways that control cell survival, apoptosis and the degradation

of various proteins through interaction with components of the ubiquitin-proteasome system (27). For instance, the integration of HBV into the genome has been linked with host microdeletion of genes (i.e. tumor suppressor genes). Similarly, the viral enhancer elements can lead to upregulation of genes that favor the development of limitless proliferation potential such as telomerase reverse transcriptase (TERT) (Figure 2), platelet-derived-growth-factor receptor-β (PDGFRβ), PDGFβ and mitogen activated protein kinase 1 (MAPK1) (16).

Another possible way is that when the virus interacts with the Endoplasmic Reticulum (ER) this leads to activation of ER stress and generation of free radicals that cause further mutations (Figure 1). Moreover, it has been shown that HBx, a viral protein with yet unclear function, can perturb the expression of a number of genes, which include growth-control genes, such as Src tyrosine kinases, Ras, Raf, MAPK, ERK, JNK and a number of other genes (16, 28). Moreover, HBx can bind and nullify p53 function, a tumor suppressor gene with multifaceted actions (29). In addition it has been shown recently that HBx alters the centrosome replication, which leads to rearrangement of chromosomes with micronuclei (28).

Interestingly, HBx was also implicated to enhance the generation of cancer stem cells that will propagate to supply endless number of cells, further strengthening the cell of origin and cancer stem cell hypothesis in HCC that might explain the recurrence of HCC due to the inherent resistance of cancer stem cells to chemotherapy (30, 31). It also has been proposed that HBV might cause epigenetic perturbations through altering histone modifications (32). Through all these genetic and epigenetic interactions with different genes and proteins that regulate the behavior of the cell, HBV is believed to trigger a cascade of events which include chronic inflammation and activation of stellate cells, quiescent cells that when activated provoke proliferation and secrete collagen to restore the structural architecture of the liver. The excessive

secretion of collagen leads to fibrosis which then progress to cirrhosis and eventually HCC (Figure 2) (16).

1.1.1.2 Hepatitis C Virus (HCV):

Hepatitis C virus is an enveloped; positive single-stranded RNA virus of the *Flaviviridae* family. (33). The viral genome encodes structural protein such as the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins are P7 polypeptide, the NS2-3 autoprotease and the NS3 serine protease, RNA helicase, NS4A polypeptide, NS4B, NS5A proteins, and NS5B RNA-dependent RNA polymerase (21).

The viral protein, known as polyprotein gets modified by the complex translation and post-translational machinery that include the cooperation of the host and viral proteases, which facilitate the generation of the viral proteins that mediate hepatocarcinogenesis (reviewed in (34)). Similar to HBV, host viral interactions are essential for the viral life cycle and hepatocarcinogenesis (33). HCV is one of the common causes of chronic liver disease and HCC in the United States.

Unlike HBV, HCV is commonly transmitted horizontally through unsafe body fluid exchange such as sharing needles and unprotected sexual intercourse (14). HCV is believed to mostly present as a chronic infection and cause chronic liver disease. Patients with HCV infection are more likely to develop HCC than patients with HBV and HCV (4). This is because HCV is susceptible to mutations in the genome due to inefficient viral polymerase enzyme, which will increase the susceptibility to replication errors in the viral replication system. As a result, mutations will generate variants of the virus that won't get recognized by the immune system because of variation in epitopes thereby helping the virus to escape the host immune system (16).

There is a 10-fold increase in the likelihood for developing liver cirrhosis in HCV compared to HBV. It is estimated that a range of 5% to 10% of HCV infected individuals develop cirrhosis 10 years post infection (35). Unlike HBV, HCV doesn't integrate into the host genome, and viral load and HCV genotype are thought to influence the incidence of HCV-related HCC (21, 36).

Moreover, the host genetic background, environment and genetic variations are also thought to play a role in facilitating the development of HCC in HCV infected individuals, which might explain why only a portion of HCV infected individuals develop HCC (36). The viral-host protein interactions regulate the initiation and development of HCC. The cascade of events that drive the transformation of hepatocytes to HCC are mostly similar between HBV and HCV. Both trigger chronic liver disease resulting in cirrhotic microenvironment which is thought to drive the transformation of hepatocyte to HCC (Figure 2) (21).

It is also worth noting that HCV infected patients who do not manifest liver fibrosis or manifest mild liver fibrosis are less likely to develop HCC (4). In regard to the difference in genetic signatures between HCV and HBV hepatocarcinogenesis, a recent infection related micro-RNA (miRNA) expression analysis showed that miRNAs associated with HCV initially altered lipid metabolism, cell cycle and the inflammatory pathways, while in HBV primarily altered pathways were cell death and DNA repair pathways (37).

The HCV driven hepatocarcinogenesis is thought to be through viral proteins (core, NS3, NS5A and NS4B) and host interaction, which regulate a multitude of events involved in cell signaling, transcriptional regulation, apoptosis membrane rearrangement and translation. (reviewed in (21)). While the carcinogenic interaction of HCV core protein with the host DNA is still not fully elucidated since HCV does not integrate into the host genome, it has been shown that HCV core protein enhances the activity of transcription factors such as, signal transducer

and activator of transcription (STAT) 3 and sterol regulatory element-binding protein (SREBP)-1c, which in turn promote hepatocarcinogenesis. (38). Moreover, HCV core protein has been recently shown to induce Vascular Endothelial Growth Factor (VEGF) through upregulation of Hypoxia-Inducible Factor-1α (HIF1α) in cirrhotic microenvironment (38).

Similar to HBV, HCV core protein has been shown to bind and nullify p53 function, activate MAPK, alter Wingless-int (Wnt) pathway and enhance the generation of Reactive Oxygen Species (ROS) and cancer stem cells in cell culture and animal models. Phosphorylated Retinoblastoma (pRB) protein is also shown to be inactivated by the core protein (21, 39, 40).

The envelope protein E2 has been shown to abrogate Protein Kinase R (PKR), which exerts resistance to Interferon inhibitory function. Moreover, E2 binds to CD81, a tetraspanin transmembrane protein, and leads to the inability to activate T lymphocytic cells and the Natural killer cells. The binding of E2 with CD81 has been also shown to modulate the MAPK-ERK pathway.

The inactivation of the p53 by HCV was also shown to be mediated by the NS3 protein trough binding to the C-terminal domain (oligomerization domain) of the wild type p53. NS5A viral protein has been proposed to dominate its oncogenic effects through inactivating p53, Forkhead transcription factor and the negative phosphorylation of Glycogen synthase kinase 3 (GSK-3), which lead to nuclear aggregation of β-catenin (21). With all these cytoplasmic and nuclear interactions, HCV is believed to initiate a chronic inflammatory state, which facilitates the development of HCC. Co-morbidity of Viral induced HCC with other factors such as Alfatoxin B1 or chronic alcohol consumption has been shown to reduce onset and promote the progression of HCC (14). Moreover, co-infection of HCV in HBV occult patients, which is

defined by the absence of HBV antigen but the persistence of HBV DNA in the host, has been reported to accelerate the development of HCC (19).

1.1.1.3 Alfatoxin B1:

Alfatoxins are a group of fungal toxins that are generally secreted by the fungus *Aspergillus flavus*. Natural common food and feedstuff contaminants aflatoxins are characterized as G1, G2, B1 and B2. Alfatoxin B1 (AFB1) is the most toxic and hepatocarcinogenic. When Alfatoxin B1 is ingested, the microsomal mono-oxygenases in liver oxidizes it. The oxidation of Alfatoxin B1 generates a carcinogenic metabolite AFB1- exo-8,9-epoxide which can bind to guanine residues of the DNA forming AFB1-N7-Gua adducts that are mutagenic with adverse effects (25).

Generation of mutagenic/carcinogenic AFB1 has been reported to cause mutations in the tumor suppressor p53 (Figure 1) (16), and activating mutations in Harvey Ras (H-Ras) oncogene (41). Farazi et al. speculated that the consequences of the mutagenic AFB1 are enough to initiate the development of HCC since there are no well understood links connecting the pre-requirement of cirrhotic microenvironment for the development of HCC in AFB1-related HCC cases (16). However, co-morbidity with HBV or HCV are possible and this will lead to an increased risk to develop HCC (4).

1.1.1.4 Chronic alcohol consumption:

Chronic alcohol consumption or heavy alcohol intake is defined by the daily intake of more than 60 grams of alcohol. Donato et al examined the association of risk of heavy alcohol intake and HCC. The association was seen to be linear with an increase of risk in heavy drinkers (>60grams/day) with no significant variations between males and females. The association also

seemed to be independent of the occurrence of viral hepatitis. However, the coexistence of alcohol and viral hepatitis would escalate the risk (42).

The toxicity of ethanol occurs when Alcohol Dehydrogenase type 3 (ADH3), a key enzyme, processes the metabolism of ethanol to acetaldehyde. Acetaldehyde is cytotoxic and causes hepatocyte damage. Interestingly, Single Nucleotide Polymorphisms (SNPs) near loci harboring genes that encode enzymes involved in metabolism of alcohol have been associated with an increased risk for some alcohol related carcinogenesis (43).

Chronic alcohol consumption has been shown to stimulate the inflammatory immune response through Interleukin 6 (IL6), IL1β, Tumor Necrosis Factor α (TNFα) and prostaglandin E2 secretion from monocytes. As a result, the inflammatory immune response facilitates damaging of hepatocytes. Similar to HBV, the degeneration/regeneration cycle leads to propagation of cells harboring mutation in key genes regulating normal homeostasis of hepatocytes, activation of stellate cells, fibrosis and eventually hepatocarcinogenesis. Moreover, alcohol ingestion was also shown to enhance the generation of oxidative stress, leading to fibrosis, genomic instability and mutations that promote HCC (Figure 1) (16).

1.1.1.5 Non-alcoholic fatty liver disease (NAFLD):

Obesity is a common morbidity affecting public health in the United States. The percentage of overweight and obese (BMI equal or more than 30kg/m^2) individuals is estimated to account for 66% and 32% of the population, respectively (44). The emergence of obesity to the list of risk factors for HCC is a relatively recent hypothesis that arose from a number of studies showing that approximately 30% to 40% of studied patients with HCC did not have underlying viral hepatitis. These studies speculated another shared factor that would increase the risk. In fact, studied patients showed a common risk factor that is type 2 diabetes, which is known to be influenced by obesity, and another group of patients showed non-alcoholic fatty liver disease (4).

The first documentation of HCC as a consequence of NAFLD was in 1990. However, available data show that the risk of developing HCC as a consequence of NAFLD is less than HCV. The cumulative risk of NAFLD-related HCC is 2.6% as opposed to 4% in HCV-related HCC (45). Interestingly, HCV core protein has been shown to promote hepatic steatosis in mice (46). In the United States NAFLD is prevalent. The increase in prevalence comes hand in hand with the increased rate of obesity in the country. Current estimates predict that obesity will become the common risk factor for HCC in the United States as HCV infection is predicted to go down in upcoming years (45).

NAFLD is a broad name that is associated with type 2 diabetes, insulin resistance and obesity. NAFLD is characterized by the excessive deposition of lipid in the liver as a complication of obesity. The excessive deposition of lipids due to unbalanced consumption of high calorie diet impairs liver function. As a result, the liver synthesizes triglycerides but cannot export them. The excessive accumulation of triglycerides in parenchyma of the liver leads to steatosis, which then results in low-grade inflammation that progress to Non-Alcoholic Steatohepatitis (NASH).

As a consequence, NASH leads to cirrhosis and HCC (Figure 4) (47). The increased risk to HCC in the majority of cases is believed to be due to NASH. It is worth noting that there are variations in the ramifications of NAFLD (i.e. hepatosteatosis) among U.S. population. For example, African Americans tend to have lower prevalence of Non-Alcoholic Steatohepatitis (NASH) compared to non-Hispanic whites and Hispanics due to genetic variations yet to be discovered (44).

The molecular mechanism behind obesity, and the associated metabolic diseases i.e. NAFLD and NASH is believed to be due to insulin resistance, an increase of plasma insulin and Insulin Growth Factor-1 (IGF-1), which enhance the secretion of pro-inflammatory cytokines (i.e. IL-6) and adipokines. Moreover, the increased availability of adipocytes facilitates the activation of inflammatory cells such as Kupffer cells (liver macrophages).

Collectively, all these events increase the oxidative stress and create a suitable environment that is mostly thought to be influenced by cirrhosis, which then facilitates the development of HCC (reviewed in (47)). However, there are reports implicating non-cirrhotic NAFLD in HCC development (45). However, it has been claimed that although a number of studies implicated obesity in HCC development, there are no clear associations between obesity and HCC due to lack of data that demonstrates a true link, and therefore, the calculation of risk of obesity to the development of HCC is still controversial (4).

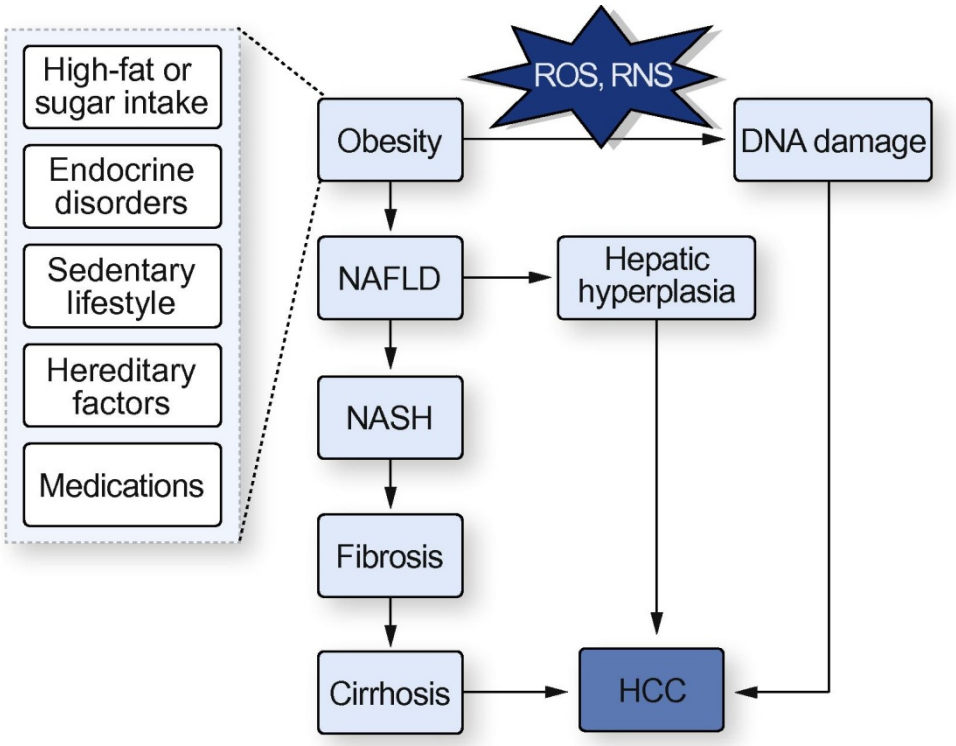

Figure 4:

A diagram showing the current proposed mechanisms behind obesity related Hepatocellular Carcinoma (HCC). A multitude of factors, such as genetic variants, unbalanced diet, medication, endocrine disorders and sedentary life style, are shown to be associated with obesity. The impact of obesity to the development of HCC is thought to be through the initiation of a low-grade inflammation leading to generation of oxidative stress such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) and progression of NAFLD to NASH. Figure adopted from (47).

1.1.2 Molecular mechanisms of hepatocarcinogenesis:

The genomics driving the transformation of a single or group of hepatocyes to proliferate, undifferentiate and migrate from a primary tumor and settle into a secondary organ is complicated and much of it is still unknown. (48). Hanahan and Weinberg defined cancer as "acquired functional capabilities that allow cancer cells to survive, proliferate, and disseminate; these functions are acquired in different tumor types via distinct mechanisms and at various times during the course of multistep tumorigenesis" (49).

The development of HCC requires a long process of cellular alterations starting from a stem cell or differentiated hepatocyte leading to changes that create hallmarks of cancer (50). In most cases HCC is believed to arise from a cirrhotic microenvironment, which will allow the initiation of hyperplastic nodules that will progress to dysplastic nodules and eventually HCC. For these histological changes to occur, a multistep deregulation of epigenetic and genetic processes that are involved in regulating cellular behavior and interaction with neighboring normal cells and microenvironment need to occur (Figure 1) (4, 49, 50). As mentioned earlier in the chapter there are reported cases where HCC arise from a non-cirrhotic microenvironment. The reason is still unclear, however there are suggestions implicating the exposure of additional risks that might accelerate the process to surpass the need of cirrhosis (14, 45). The inherent complexity and heterogeneity of HCC might be due to the variability in the impact of risk factors (19). Although gene expression studies of HCC demonstrated that genetic signature of every patients is different than the other, the majority showed a common deregulation that include: EGFR-Ras-MAPKK Pathway, c-MET Signaling, IGF Signaling, PI3K/Akt/mTOR Pathway, Wnt β-Catenin Pathway, hedgehog pathway, the inflammatory pathway, such as IL-6, and p53 (reviewed in (50, 51)).

1.1.2.1 EGFR-Ras-MAPKK Pathway:

Epidermal Growth Factor Receptor (EGFR, also known as Her1) is a tyrosine kinase receptor from a family of ErbB receptors that include four receptors (ErbB2 (Her2), ErbB3 (Her3), ErbB4 (Her4). Following ligand binding, EGFR form homo and heterodimers that result in autophosphorylation of the receptor to transmit downstream signal. (reviewed in (50)).

The association between activated EGFR and HCC was seen in overexpression of EGFR in most of HCC cases. Overexpression of EGFR correlated with poor prognosis. According to Cervello et al., recent studies demonstrated that high levels of tissue and serum EGF in cirrhotic patients increased the adjusted risk for HCC compared to unaffected individuals (51, 52).

It is worth noting that, activating mutations of Ras are relatively low in HCC. Interestingly, studies on HCC showed that Ras is activated in most of HCC patients (16). Activation of Ras-MAPKK pathway was seen to be through downregulation of Ras inhibitors such as Spred or Raf inhibitors such as RKIP (16) (Figure 5). Overexpression EGFR leads to the activation of Ras-MAPK. Moreover, HBV and HCV were reported to activate MAPKK pathway (16).

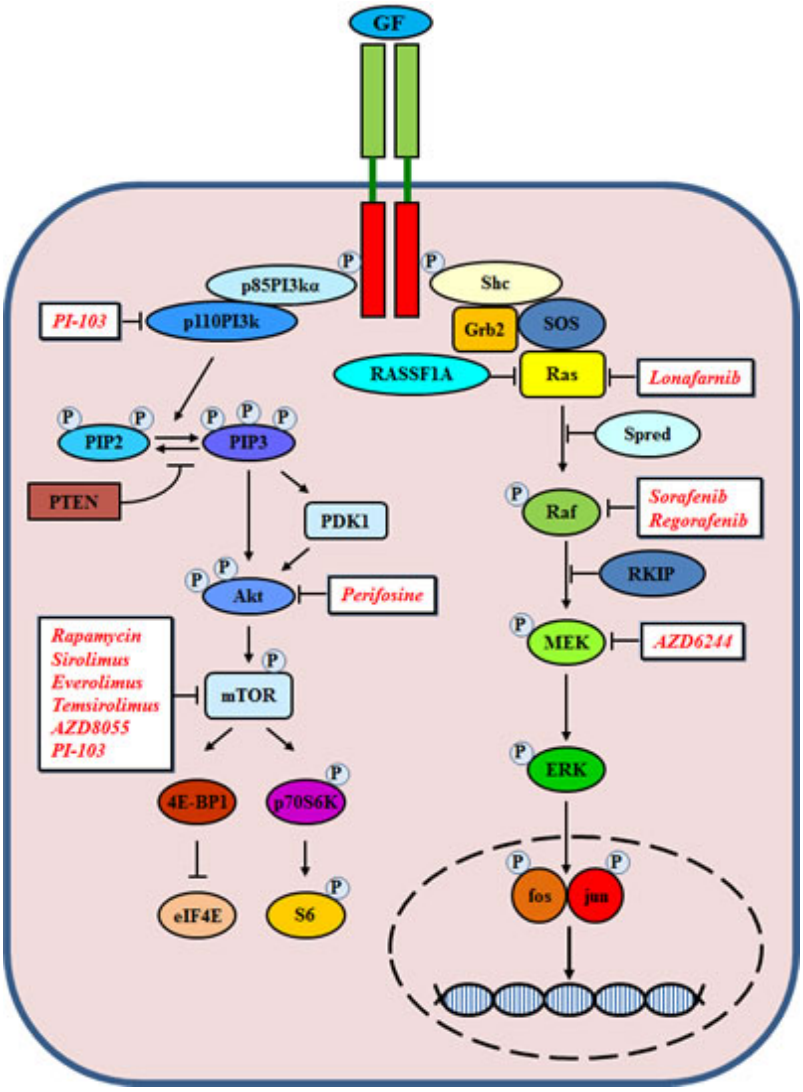

Figure 5:

The figure is a representation of a receptor tyrosine kinase receptor signaling upon ligand binding (GF: Growth Factor) such as EGF binding to EGFR. White squares represent current drugs used to block signaling pathway in HCC. "P" denotes the event of phosphorylation. Figure was adopted from (51).

1.1.2.2 c-MET (mesenchymal-epithelial transition factor) Signaling:

Binding of Hepatocyte Growth Factor (HGF) to its receptor c-Met, induces dimerizationof the receptor and downstream signaling. HGF is an essential growth factor that enhances proliferation of hepatocyte. Upon liver damage stellate cells get activated and secrete HGF, which then binds c-Met, and enhance downstream signaling such as MEK/ERK, and PI3K/Akt pathways. In HCC, overexpression of c-Met has been reported in a large number of cases. HGF/c-Met signaling is commonly altered in HCC and is correlated with poor prognosis and metastasis (50, 53) and recently, HGF independent activation of c-Met via upregulation of osteopontin has been reported in HCC (53).

1.1.2.3 IGF Signaling:

Insulin like growth factor is from a family of growth factors that regulate a multitude of cellular events such as fetal development, proliferation, differentiation, cell growth and apoptosis. The family includes IGF-1 and IGF-2 that binds to IGF- receptors (IGF-R) and induce downstream signaling (reviewed in (51)). IGF2 is mostly overexpressed in HCC in around 40% of the cases . The overexpression of IGF-2 is thought to be through dysregulated methylation of the promoter region of IGF-2, the interaction of HBx protein of HBV or HCV core protein (54-56). According to Llovet et al, there are no reports implicating IGF-1 in HCC and surprisingly IGF-1 was shown to improve cirrhosis (50). The deregulation of the IGF signaling in HCC is also coupled with the downregulation of tumor suppressor proteins IGF bindin protein-1 (IGFBP-1), IGFBP-3, IGFBP-4 and more recently IGFBP-7 (50, 57).

1.1.2.4 PI3K/Akt/mTOR Pathway:

The deregulation of PI3K/Akt/mTOR pathway is a common phenomenon in carcinogenesis. Alteration of the pathway has been reported in a number of cancers including HCC (49, 50). Following binding of extracellular signals such as EGF, PI3K gets activated and induce downstream signaling (Figure 5). PI3K/Akt/mTOR pathway regulates many cellular mechanisms such as proliferation, growth, survival, motility and protein synthesis (reviewed in (51). In HCC the activation of PI3K/Akt/mTOR pathway takes place at multiple levels. Constitutive activation of PI3K might result from mutation in PIK3CA (catalytic domain of PI3K), which has been reported in HCC patients (58, 59).

Another possible mechanism of constitutive activation of the pathway was shown to be through epigenetic aberrations leading to downregulation of PTEN (tumor suppressor; negative regulator of PI3K) or Ras inhibitors. However, it is worth noting that PTEN mutations are low in HCC and the most possible mechanism for PTEN silencing is through loss of heterozygosity (50, 51, 60). Downregulation of PTEN and activation of the PI3K/Akt/mTOR pathway was reprted to be mediated by HBx protein (61). The activation of the PI3K/Akt/mTOR pathway is associated with poor prognosis in HCC (51).

1.1.2.5 Wnt β-Catenin Pathway:

In the canonical Wnt β-Catenin signaling, the binding of the extracellular Wnt to the Frizzled receptor rearranges the membrane anchored β-Catenin complex so that it inhibits the negative phsphorylation of β-Catenin, which then leads to its nuclear translocation to activate target genes such as c-myc (reviewd in (62)). Wnt β-Catenin pathway is an essential pathway regulating, embryogenesis, neurogenesis, apoptosis, proliferation and carcinogenesis. The activation of the

Wnt β-Catenin pathway is a key mechanism regulating invasive capability of cancer cells(50). In viral hepatitis related HCC, the HBx protein was shown to interact with and activate β-Catenin pathway. Similarly, HCV core protein was shown to act as a Wnt ligand and induce downstream signaling (51). Constitutive signaling of Wnt pathway has been reported in one third of HCC cases (50). Deregulation of Wnt pathway and translocation of β-Catenin into the nucleus was shown recently to be mediated by Astrocyte elevated Gene 1 (AEG-1), an oncogene overexpressed in HCC (63). Activating mutation of β-Catenin, Axin1 or APC are relatively low in HCC, the most common alteration is through the overexpression of the Frizzled receptor (51).

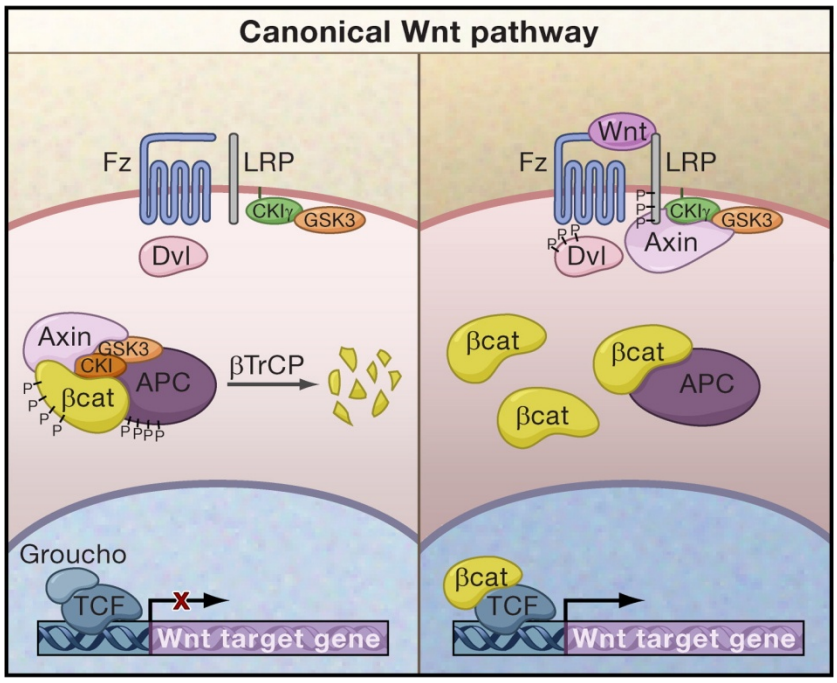

Figure 6:

Left: In the absence of Wnt, β-Catenin gets phosphorylated by CK1 and GSK3α/β and targeted for βTrCP (member of E3 ubiquitin ligase complex) mediated degradation by the proteasome. Right: Following binding of Wnt, Frizzled receptor induces rearmament signaling cascade that prevents the negative phosphorylation of β Catenin leading to its translocation into the nucleus to activate target genes. Adopted from (62).

1.1.2.6 Inflammatory Pathway:

Increasing evidence linking inflammation to cancer lead Hanahan and Weinberg to add inflammation to the hallmarks of cancer to their recent review (49). The inflammatory pathway plays a critical role in the development of HCC (4, 6, 51). Nearly, all risk factors for HCC activates the inflammatory pathway and the development of HCC in most cases is preceded by cirrhotic/chronic inflammatory microenvironment (4, 45, 47, 50).

The mechanism behind the modulation of chronic inflammation in HCC is yet far from getting elucidated. The available data in the literature implicate the involvement of cytokines such as IL-6 and other inflammatory mediators such as tumor necrosis factor α (TNFα), prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX-2) (reviewed in (64)). Moreover, STAT3, downstream of IL-6, was also implicated in HCC. Activation of STAT3 (pTyr705) induced transformation of NIH3T3 and 3Y1immortalized fibroblasts and tumor formation in mice (65). Another player in the inflammatory cascade of cancer is the NF-κB. Several reports suggested the association of NF-κB cascade with cancer development (reviewed in (66)). In HCC, NF-κB is overexpressed and aberrantly activated in many HCC cases and HCC cell lines (51).

The General theme behind inflammation induced HCC is believed to be through the activation and enhanced secretion of pro-inflammatory factors such as cytokine that lead to the activation of STAT3 and activation and recruitment of inflammatory cells such as macrophages. Infiltration of macrophages increases the oxidative stress and genomic instability. Collectively, all these events are thought to create a favorable environment for a tumor to arise (16, 47, 51, 67).

1.1.2.7 Hedgehog Pathway:

Hedgehog signaling pathway is an important pathway that is involved in development, cell polarity and differentiation (reviewed in (68). The pathway is silenced in adult hepatocytes, however, it gets reactivated when the liver is in regenerative state i.e. liver damage (51). In general, alterations of the hedgehog pathway have been reported in cancers including HCC (51, 68). The implication of the aberrant activation of the pathway is not well understood, however, recent reports showed that Hedgehog facilitate invasion and metastasis of human HCC via increasing MMP-9 expression by modulating ERK pathway (69). Moreover, a cross talk between Hedgehog pathway and Wnt/β-Catenin pathway has been reported in literature (51).

1.1.2.8 p53 pathway:

The tumor suppressor p53 is an important regulator of genomic stability of the cell, often called "Guardian of the genome". It is a transcription factor that can have nuclear and cytoplasmic functions regulating a plethora of cellular events such as apoptosis and DNA repair. The p53 gene is mutated and implicated in carcinogenesis in most reported cancers including HCC (49, 50). The inactivation of p53 in HCC can be by either loss of heterozygosity, genomic mutation of the p53 gene such as in Alfatoxin B1 or by nullifying its function through protein-protein interaction such as the case with viral hepatitis related HCC (4, 16, 50).

1.1.3. Staging of Hepatocellular Carcinoma (HCC)

A number of staging systems are being used around the world to evaluate the development, progression and treatment options for HCC patients. All systems characterize patients based on cytopathological changes, patient symptoms or liver function tests and none of them have incorporated the molecular and genetic alterations yet (reviewed in (70), (4)). The available staging systems are Barcelona Clinic Liver Cancer (BCLC), Cancer of the Liver Italian Program (CLIP), TNM (tumor, node and metastasis), Okuda, and Japanese Integrated Staging Score (JIS) systems.

In the United States, American Association for the Study of Liver Diseases (AASLD) uses the BCLC system as standard for staging HCC. The preference to use the BCLC system over the others is because it has an implemented treatment algorithm that can predict treatment based on assessing tumor stage, patient physical status, symptoms and liver function (Figure 7) (14, 70). Although, BCLC is probably the best system for predicting treatment, it has an inherit limitation in that the molecular alteration are not implemented in the system. This might be due to the lack of diagnostic molecular markers and the complexity of HCC due to the heterogeneity seen in patients (4, 14, 70). Therefore, identification of new diagnostic markers is immensely required to diagnose and treat patients at very early stage when prognosis is highly favorable (4, 48)

1.1.4. Treatment:

Cancer in general and Hepatocellular Carcinoma (HCC) in particular are not easy to treat. The idea of one drug works for all is no longer acceptable in cancer therapy. This is due to the fact of heterogeneity and resistance to conventional treatments (4, 48, 50). The BCLC treatment algorithm treats patients based on the stage. For example, surgical resection and liver

transplantation are offered for patients diagnosed at an early stage with equal or more than 75% survival rate (Figure 7) (4). However, unfortunately due to lack of early diagnostic markers, most patients are diagnosed at an advanced stage where prognosis is dismal (4, 71).

Molecular targeted therapy has ushered in a new era in the treatment of advanced stage HCC. For instance, clinical trials lead to the validation of sorafenib, BRAF/VEGFR/PDGFR multi-kinase inhibitor, as a standard drug for treating advanced stage HCC. Trials have shown that sorafenib by itself improved overall survival by ~3 months (4, 48, 50).

The need for personalized medicine is increasing as each HCC patient has a cancer genetic signature that is different than the other. Anti cancer drugs should be tailored based on the signature of molecular alterations of patients. This can only be approached through the identification of new markers and potential therapeutic targets for combinatorial therapy (36, 71).

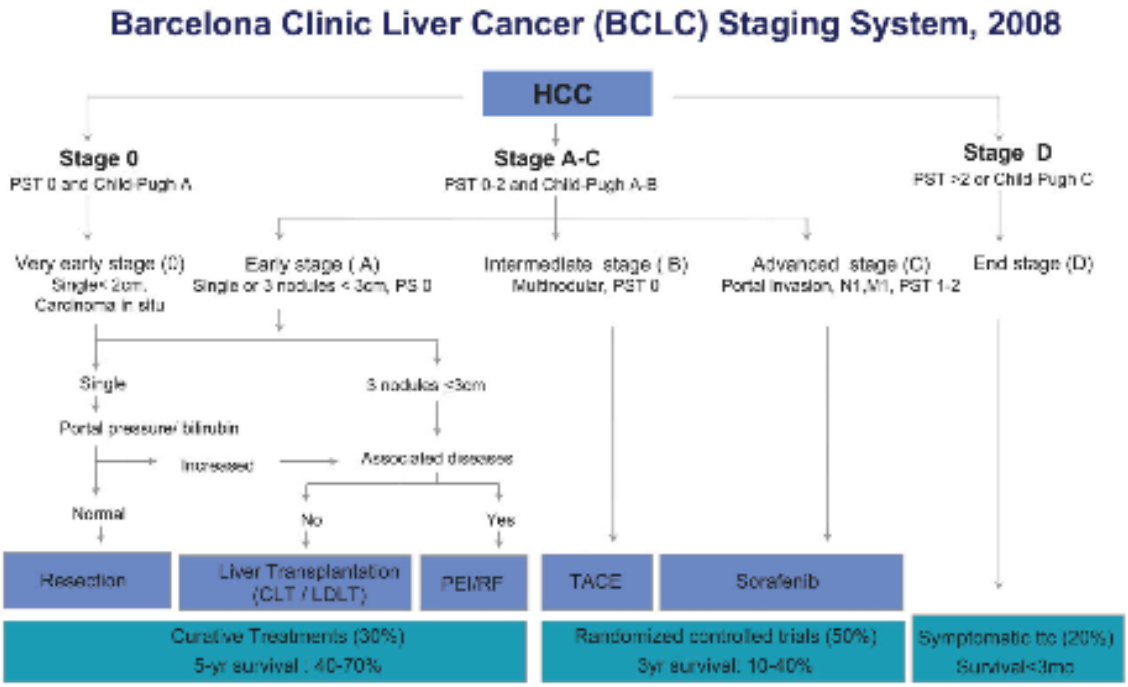

Figure 7:

The BCLC algorithm that is endorsed by AASLD. Stage 0: patients at a very early stage. Stage A: patients with early HCC. Stage B: patients with intermediate HCC; no portal vein invasion. Stage C: patients with advanced HCC, portal vein invasion and extrahepatic metastasis. Stage D: terminal HCC, at that stage patient receive treatment to ameliorate symptoms. CLT: Cadaveric Liver Transplantation. LDLT: Living Donor Liver Transplantation. PEI: Percutaneous Ethanol Injection. RF: Radio Frequency ablation. TACE: Transarterial chemoembolization. Figure adopted from (50).

1.2 Angiogenesis in Hepatocellular Carcinoma (HCC):

Angiogenesis is a phenomenon that describes the generation of new blood vessels from an extant vasculature. It is a highly controlled physiological phenomenon that gets activated in the case of menstruation, wound healing, and tissue repair. The link of angiogenesis with carcinogenesis was first suggested by the pioneering work of Judah Folkman. Folkman et al. showed that cancer cells are capable of altering the "net-balance" between pro-angiogenic and anti-angiogenic molecules to the advantage of angiogenesis leading to "angiogenic-switch" (72-75). As a result, the work of Judah Folkman lead to the recognition of angiogenesis as a hallmark of cancer (49).

Briefly, the mechanism of angiogenesis initiates vasodilatation and destabilization of the vessel (mediated by VEGF, ANG2) resulting in increase in the vessel permeability. Exuded plasma proteins act to guide the migrating endothelia cells to the target (mediated by integrins). Then, upregulation of Matrix Metalloproteinases (MMPs) facilitate the breakdown of the extracellular matrix, migration and proliferation of endothelial cells. Next, biding of endothelial cells (mediated by VE-cadherins and integrins) and tube formation is initiated (mediated by TNF-α, FGF and PDGF). After that, the primitive endothelial layer is formed by the differentiating endothelial cell and recruited pericytes (mediated by VEGF, PDGF and TGFβ). Finally, the mature vessel is re-stabilized (mediated by ANG-1), and new extracellular matrix is formed (reviewd in (76)).

HCC is a highly vascular cancer, preceded in most cases by chronic liver disease. As mentioned earlier in the chapter, irrespective of the etiological agent the chronic inflammation leads to the activation of resident macrophages (i.e. Kupffer cells) and stellate cells. As a result, Kupffer cells secrete pro-inflamatory cytokines such as TNFα, and increase levels of ROS and

Platelet Activating Factor (PAF). Moreover, the secretion of proinflamatory cytokines has been shown to increase levels of HIF1α. Similarly, activated stellate cells secrete a number of pro-inflammatory cytokines and pro-inflammatory mediators such as, VEGF, PlGF, PDGF and nitric oxide (NO). Together, stellate cells and kuppfer cells recruit inflammatory cells that also secrete cytokines and promote angiogenesis. For example, it has been shown that leucocytes secrete a number of pro-angiogenic factors such as Ang-2, VEGF, and many others (Figure 8) (77).

Collectively, all these events are implicated to promote angiogenesis (76). The progression of CLD to cirrhosis increases the portal vein pressure and induces portal hypertension that creates a hypoxic condition in the surrounding tissue, which then promotes induction of HIF1α and angiogenesis. In HCC, transformed hepatocytes showed increased production in pro-angiogenic factors such as VEGF, FGF and many others and downregulation of anti-angiogenic factors such as thrombospondin-1. Angiogenesis is a major mediator in HCC progression, and currently potential inhibitors of angiogenesis are being evaluated in clinical trials (76).

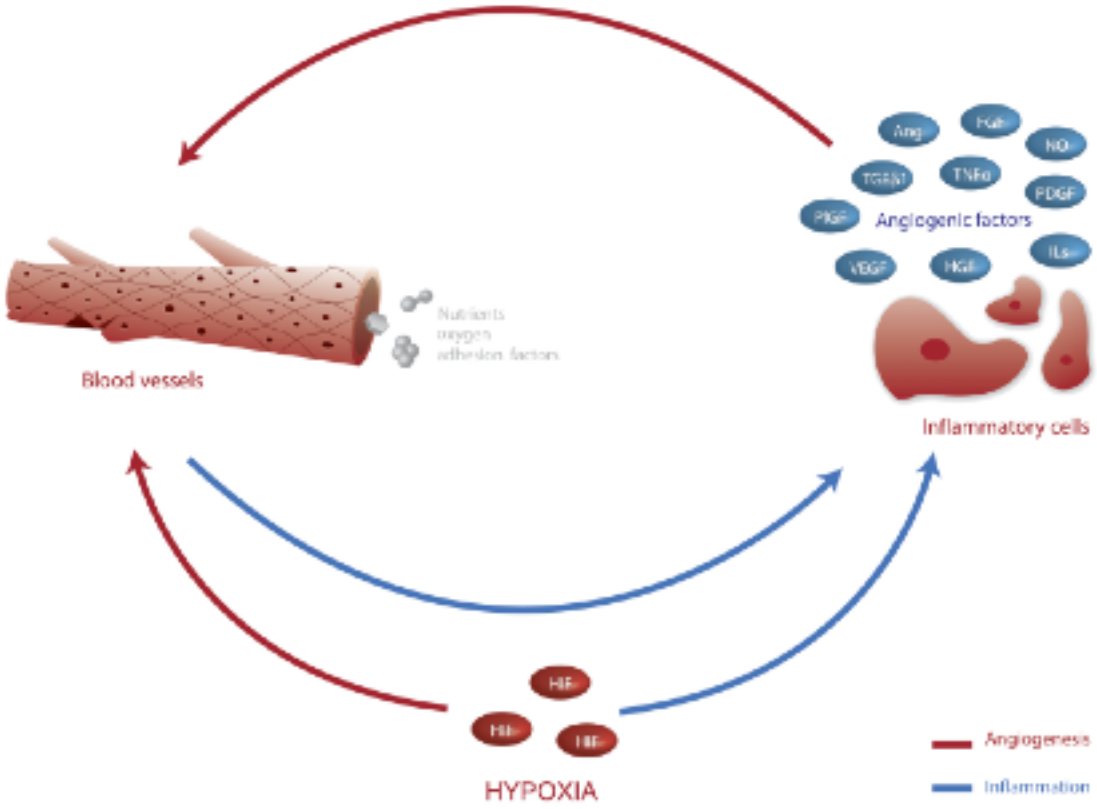

Figure 8:

Although angiogenesis and inflammation come hand in hand, they are two different events. The figure illustrates the interplay between angiogenesis and inflammation. Hypoxia induces both inflammation and angiogenesis. Upon arrival to the target site, activated inflammatory cells secrete pro-angiogenic factors that promote angiogenesis to supply them with oxygen and nutrients to meet their metabolic activity. Figure adopted from (76).

1.3 Metastasis:

Metastasis is the death sentence for cancer patients. It is a name given to a wide spectrum of complex events ranging from migration from a primary organ to colonization of a secondary organ. Hanahan and Weinberg defined metastasis as a process that encompasses four key events: First, the cancer cell has to migrate from the primary site (known as "local invasion"). Second, cancer cell has to pass the endothelial cell membrane and invade lymphoid and blood vessels (known as "intravasation"). Third, cancer cell has to migrate, move out from the circulation and invade the cell membrane of the blood vessels and settle in a secondary site (known as "extravasation". Fourth, cancer cell has to acquire the capability to expand from micrometastatic to macrometastatic nodule and colonize the new environment (known as "colonization") (49).

Given this complexity, it is possible to think that for a cancer cell to metastasize it has to acquire a large number of alterations in genes regulating cellular behavior and interaction with the microenvironment. However, Hanahan and Weinberg by interpreting available data on tumor-microenvironment interaction stated an untested hypothesis that tumor- microenvironment interaction might be enough in some cancers to facilitate metastasis. Cancer cells might not need additional mutation other than the ones responsible for tumor initiation (49).

Molecular alterations of cells undergoing metastasis are at the level of cell adhesion, proteolysis of extracellular matrix, invasion and tumor tissue microenvironment interaction (reviewed in (78)). The interepithelial adhesion of primary tumor cells is disrupted by down-regulation or disruption of the interaction of E-cadherin. Several reports linked alterations in E-cadherin to the aberrant methylation of its promoter region or to mutations in the β-catenin/E-cadherin complex leading to endocytotosis and degradation of the complex. Moreover, transcription silencers of E-cadherin such as Snail, Slug, and Twist and many others have been

shown to be overexpressed and downregulate E-cadherin. In fact, the above-mentioned transcription factors are key mediators of Epithelial-to-Mesenchymal Transition (EMT) suggesting that the cadherin switch that is seen by the downregulation of E-cadherin and upregulation of N-cadherin in cells undergoing metastasis is a characteristic of EMT (78). Alteration in E-cadherin by the proteolytic cleavage of the extracellular domain by MMPs such as MMP3 and MMP7 is reported in literature (78, 79).

Interaction with integrins is an important mechanism that orchestrate the migratory phenotype. Integrins have diverse functions in regulating migration, proliferation and survival. The regulatory role of integrins vary depending upon the cell context. For example, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ mediate epithelial cell adhesion and decrease metastasis in some experimental models but promote invasion and metastasis in other models (78).

Alterations in cell surface proteoglycan such as CD44 have been reported in cancers. CD44 is constitutively activated in mesynchymal cells and fibroblasts. CD44 can bind with ErbB, c-Met and co-localize with MPPs and by doing so it increase invasion through promoting migration and survival (78). Cancer cells can upregulate the expression of MMPs themselves or enhance the secretion of MMPs in the stroma and surrounding microenvironment to clear up the way by degrading the ECM. However, this is not applicable to all cancer metastasis (49, 78).

Increasing evidence suggests that cancer metastasis depends on the interaction with microenvironment, as in some cancers we see that cell undergoing metastasis lack key features of metastasis and depends on the supply of the stroma for these factors (49). The concept of whether metastasis is a late or an early event is being debated. Microarray analysis data of some very early primary tumors showed a group of cells that resemble a metastatic genetic signature

(80). To better understand metastasis, we need to take cancer cells-microenvironment into account (49).

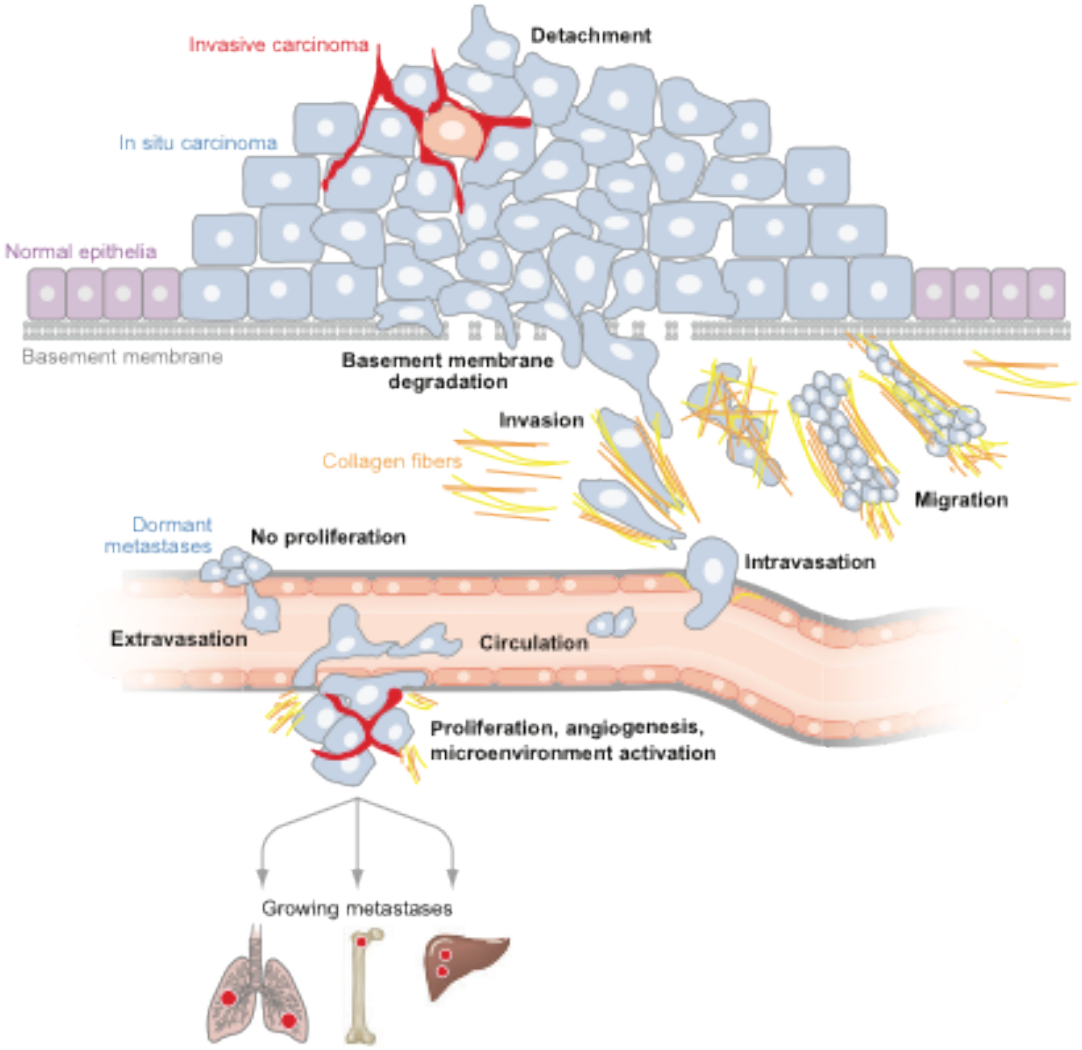

Figure 9:

A schematic representation of four key events in metastasis. Hyperplastic tissue progress to carcinoma in-situ and subsequent alteration and microenvironment interactions facilitate local invasion, intravasation, extravasation and colonization of a secondary organ. Figure adopted from (78).

1.4 Astrocyte elevated gene-1 (AEG-1):

1.4.1 Identification:

The laboratory of Dr. Paul B. Fisher first reported the identification and cloning of Astrocyte elevated gene-1 in 2002. The identification of AEG-1 came from studies of deregulated genes in Primary Human Fetal Astocytes (PHFA) upon HIV-1 infection that are responsible for neuronal degeneration seen in late stage Acquired Immune Deficiency Syndrome (AIDS). AEG-1 was identified by rapid subtraction hybridization (RaSH) approach comparing gene expression between HIV-1 infected and uninfected PHFA and was shown to be an HIV-1 and TNFα inducible gene (Figure 10) (81, 82).

Two years later, three different laboratories in 2004 identified AEG-1 in different cellular contexts and locations. In an effort to study breast cancer metastasis to the lung using phage screening approach Brown and Ruoslahti identified AEG-1 as a protein that is located on the cell membrane and mediates breast cancer metastasis to the lung, thereby naming it metadherin (MTDH) (83). Britt et al indentified mouse/rat AEG-1 as a tight junction protein and named it LYsine-RIch CEACAM1 co-isolated (LYRIC), while Sutherland et al. identified it as a protein present in ER and nuclear membrane as well as in the nucleolus by gene trapping techniques and named it 3D3/lyric.(84, 85).

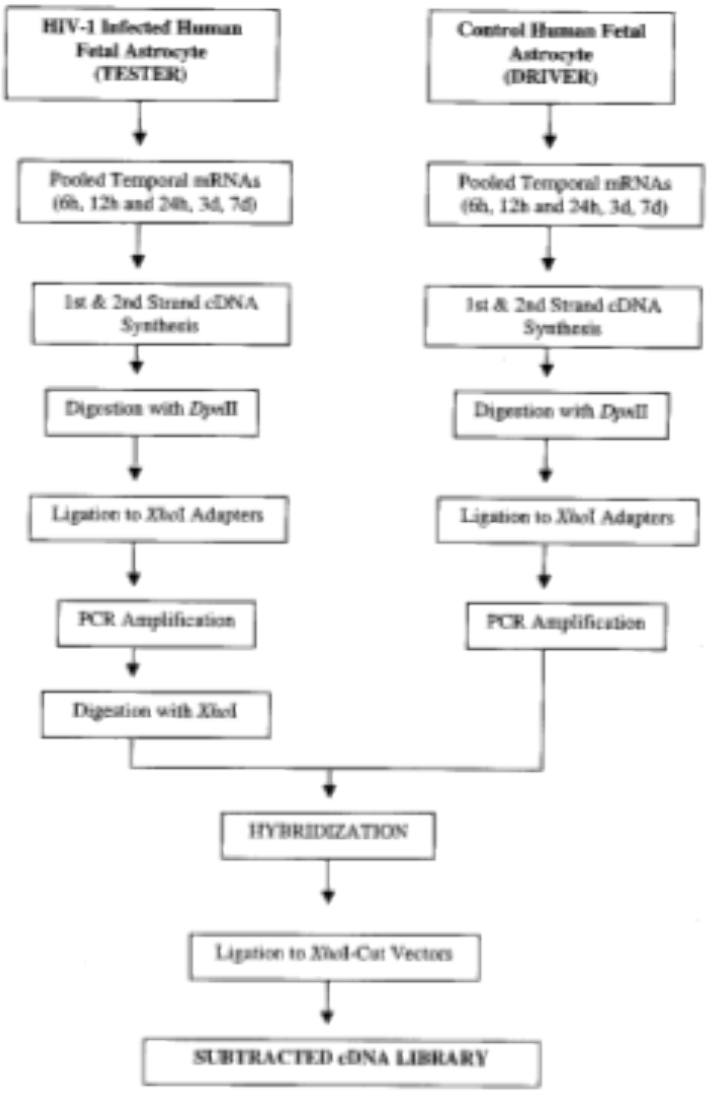

Figure 10:
A diagram representing the rapid subtraction hybridization (RaSH) on the infected and uninfected Primary Human Fetal Astrocyte (PHFA). Figure adopted from (81).

1.4.2 AEG-1 locus and protein structure:

1.4.2.1 AEG-1 locus:

Human AEG-1 gene is located at chromosome 8q22. This location is specifically significant as it is a hot spot for genomic amplifications in a number of malignancies, including HCC (86). Studies using microarray and SNP array in HCC demonstrated significant gains in copy number of AEG-1 that is associated with the increased expression of AEG-1 (63). Studies in breast cancer confirmed the genomic amplification in 8q22 using Fluoresence in situ hybridization (FISH) and genomic DNA quantitative PCR (qPCR), which correlated with the overexpression of AEG-1 (87). The gene has 12 exons and 11 introns with high conservation among vertebrates with 90% identity. The mRNA encodes a single transmembrane protein with a predicted molecular weight of 64 Kilo-Dalton (KD).(88).

1.4.2.2 AEG-1 protein structure:

AEG-1 is a lysine rich, highly basic protein with a single transmembrane domain. The protein is predicted to contain a transmembrane domain (TMD) at amino acid residues 51 to 72 and three putative nuclear localization signals (NLS) between residues 79 to 91, 432 to 451, and 561–580 (Figure 11). AEG-1 is located in the nucleus, nucleolus and ER/nuclear envelop. The C-terminal extended NLS-3 (residues 546–582) is responsible for the nuclear localization. The NLS-1 (residues 78–130) controls nucleolar localization. The NLS2 (residues 415–486) is believed to be subjected to monoubiquitination, which results in sequestration of AEG-1 in the cytoplasm (88).

The protein is predicted to be subjected to post-translational modifications since it contains a variety of putative amino acid residues for post-translational modifications. For example, the C-terminal 435-GALPTGKS-442 is putative binding site for ATP/GTP. Moreover there are a

number of phosphorylation sites such as tyrosine, serine, and threonine amino acids that might be subjected to phosphorylation by Protein Kinases A and C (PKA and PKC) (88).

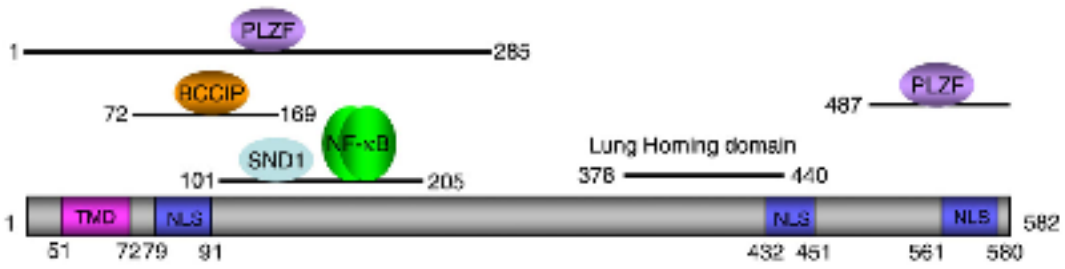

Figure 11:
Representative figure of the predicted protein structure of AEG-1, and site of interacting proteins. Numbers are amino acid residues. TMD: transmembrane domain; and NLS: nuclear localization signal. Figure adapted from (88)

1.4.3 Physiological function of AEG-1:

The physiological function of AEG-1 is yet to be elucidated. The expression of AEG-1 is detected in a number of normal tissues with highest level in skeletal muscle, heart, thyroid and the adrenal gland (89). Moreover, recent studies implicated AEG-1 in embryonic development in mice. Whole-mount immunohistochemistry showed expression of AEG-1 at E9.5 (embryonic day 9.5) in dorsal midbrain and fronto-nasal process. The expression between E9.5 to E10.5 was detected in the forelimb, the hindlimb and the pharyngeal arches. Moreover, at E12.5 to E18.5 the expression was seen in the brain, olfactory and skeletal systems. Expression of AEG-1 at these specific timings and sites implicates AEG-1 in embryonic development (90).

1.4.4 AEG-1 in carcinogenesis:

AEG-1 is aberrantly overexpressed in a large number of cancers. The overexpression of AEG-1 was reported in breast, prostate, gastric, renal, colorectal cancers, non-small cell lung cancinoma, esophageal squamous cell carcinoma, melanoma, glioblastoma multiforme, neuroblastoma, oligodendroglioma and hepatocellular carcinoma. Microarray expression analysis of HCC patients showed overexpression of AEG-1 in tumor compared to non-tumor tissue. The expression was also correlated with the disease stage and level of differentiation. AEG-1 was seen to be significantly upregulated in HCV-related HCC as opposed to cirrhotic and normal tissue (63, 88).

AEG-1 play a critical role in carcinogenesis as it interplays with a number of biological phenomena of cancer such as proliferation, anchorage-independent growth, migration, invasion, chemoresistance, angiogenesis and in vivo tumorogenesis and metastasis, suggesting that AEG-1 mediate the hallmarks of cancer (reviewed in (88)). Ectopic expression of AEG-1 promotes proliferation in large spectrum cancer cells including HCC (63, 91, 92). Experimental

upregulation of AEG-1 in normal human cells, such as the immortal primary human fetal astrocytes (IM-PHFA) and normal immortal melanocytes (FM516-SV) induce resistance to serum starvation- induced apoptosis, implicating AEG-1 in onogenesis by increasing survival of cells (93). Moreover, ectopic expression of AEG-1 was shown to mediate H-Ras-induced anchorage independence in normal cloned rat embryonic fibroblasts (CREF), IM-PHFA, and FM-516-SV, suggesting that AEG-1 promote transformation of normal cells via H-Ras (89, 94, 95). AEG-1 was shown to be a downstream gene of H-Ras. H-Ras activates PI3K/Akt, which in return promote expression of AEG-1 that enhances PI3K/Akt activity suggesting that AEG-1 induces a positive feedback loop (Figure 12) (reviewed in (88)). Microarray expression analysis showed increased expression of genes related to chemoresistance in human HCC cells overexpressing AEG-1 when compared to control cells. Overexpression of AEG-1 upregulated dihydropryimidine dehydrogenase (DPYD), a drug-metabolizing enzyme, and the transcription factor LSF/TFCP2 (reviewed in (88)). Overexpression of LSF, increases the expression of thymidylate synthase (TS), a target of 5-Fluorouracil (5-FU). As a result, cells gain resistance to 5-FU (96). AEG-1 modulation of the chemoresistance phenotype was also seen through increasing the stability of proteins implicated in resistance to chemotherapeutic drugs. For instance AEG-1, enhances the association of multidrug resistance gene 1 (MDR1/ ABCB1) mRNA thereby increasing its translation via activation of PI3K pathway (97). Angiogenesis was also seen to be induced by AEG-1. HCC cells that overexpress AEG-1 showed upregulation of a number of pro-angiogenic factors and matrix metalloproteinases such as vascular endothelial growth factor (VEGF), placental growth factor (PIGF), fibroblast growth factor α (FGFα), HIF1α and MMP-2 (63, 88, 94). Moreover, AEG-1 overexpressing cells showed upregulation of genes involved in Wnt signaling such as LEF1, metastasis such as TSPAN8, and down

regulation of genes involved in senescence such as IGFBP-7(57, 63). Experimental studies of AEG-1 tumorigenic properties showed that it cross talks with PI3K/AKT, NF-κB , MAPK and WNT/β-catenin pathways and by doing so AEG-1 augments proliferation, migration, invasion, anchorage-independence, chemoresistance, angiogenesis, and metastasis and inhibits senescence (Figure 12) (reviewed in (88),(98).

1.4.5 AEG-1 in neurodegenerative disorders:

AEG-1 was originally identified as an overexpressed transcript in HIV-1 infected astrocytes. Overexpression of AEG-1 is implicated in neuronal death associated with HIV-1 activated dementia (HAD) (89). In support of this finding, Astroglial glutamate transporter (excitatory amino acid transporter-2/EAAT2) that mediates the clearance of glutamate from neuronal synapses in the Central Nervous System (CNS) is downregulated by AEG-1 (53). As a consequence, toxic accumulation of glutamate induce neuronal degeneration, a phenomenon that is seen in most neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, a number of forms of epilepsy, ischemia/stroke, HAD, traumatic brain injury and hepatic encephalopathy (88, 89). Moreover, the colocalization of AEG-1 with ribosome synthesis 1 (Rrs1) in the endoplasmic reticulum (ER) implicates AEG-1 in the pathogenesis of Huntington Disease (HD) since it was shown that in HD transgenic mouse models Rrs1 is upregulated and might regulate the pathogenesis of HD (88, 99, 100).

1.4.6 AEG-1-interacting proteins:

Current evidences indicate that AEG-1 most likely functions as a scaffold protein interacting with other proteins and thereby mediating oncogenesis by regulating a variety of events. AEG-1

was shown to interact with NF-κB and the transcription co-activator cyclic AMP-responsive element binding protein (CREB)-binding protein (CBP). The interaction with CBP was shown to modulate AEG-1-NF-κB interaction, which leads to activation of target genes such as IL-8 (93). AEG-1 was shown to interact with BCCIPα, a tumor suppressor that binds to p21 (mda-6/CIP-1) and enhance the inhibition Cycline dependent kinase 2 (Cdk2 kinase). Inhibition of BCCIPα abrogates G1/S checkpoint activation following DNA damage. Cooperation of BRCA2 and BCCIPα is essential in homologous recombination repair of DNA damage and maintains chromosome stability. AEG-1 was shown to interact with Staphylococcal nuclease domain containing 1 (SND1) and mediates the cytoplasmic function of SND1 in facilitating RNA interference (RNAi) and angiogenesis (reviwed in (88), (101, 102)).

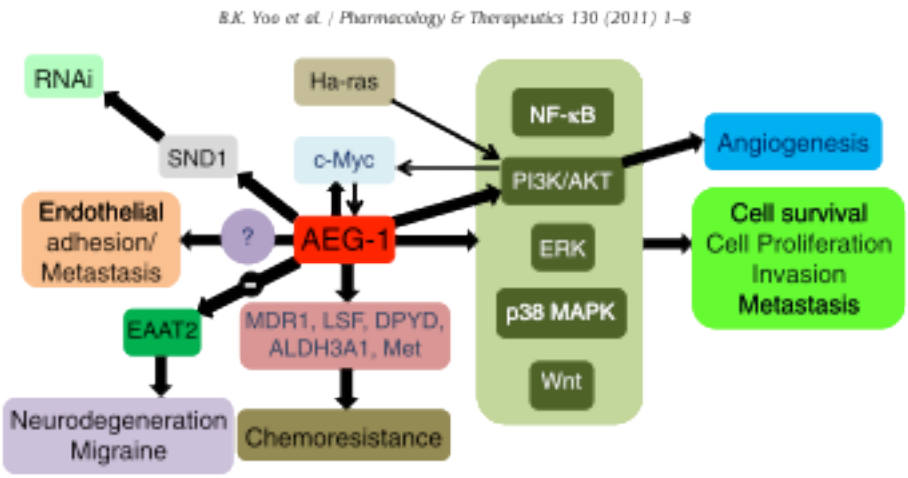

Figure 12:

A schematic representation of the current understanding of the network of molecular interactions of AEG-1 promoting tumorogenesis. Figure adopted from (88).

1.5 Tetraspanins:

Oren et al first reported the identification of tetraspanin proteins in 1990 (103). The family of tetraspanins participates in a wide range of physiological phenomena within the cell membrane that include fertilization, synaptic contacts at neuromuscular junctions, platelet aggregation, and maintenance of skin integrity and immune response induction. In addition, tertraspanins are implicated in pathological phenomena such as parasite and viral infection and carcinogenesis (104). Having these critical functions across a spectrum of cellular activities, it is not surprising to find that tetraspanins are highly conserved in evolution across species (105).

1.5.1 The protein structure of tetraspanins:

Tertaspanins are a group of small transmembrane proteins with short N (amino) and C (carboxyl) tails. Tetraspanins cross the membrane four times, and have a small extracellular loop that is located between transmembrane 1 and 2 (TM1, TM2) and a large extracellular loop in-between TM3 and TM4. The protein also has a small intracellular loop between TM2 and TM3 (Figure 13) (104). The large extracellular loop is subdivided into a constant and a variable region. The constant region is responsible for dimerization with tetraspanin members. The variable region is responsible for interaction of tetraspanins with non-tetraspanin members. In addition, tetraspanins harbor conserved cysteines in the large extracellular loop that are believed to act as a signature for tetraspanins. Transmembrane regions contain polar amino acids that are believed to fix the tertiary structure of the protein. Just like any protein, tertraspanins are subjected to post-translational modifications. Tertraspanins have palmitoylation sites. Palmitoylation of intracellular juxtamembrane cysteines is essential for tetraspanin web formation and prevents the protein from lysosomal breakdown, acts as an association site to cholesterol and gangliosides, and enhances cell–cell contact. It is worth noting that

palmitoylation of intergrins has also been shown to promote tetraspanin web formation. The C-tail of the protein of some tetraspanins such as TSPAN8 contains a tyrosine-based sorting motif that is believed to mediate internalization of tetraspanins (reviwed in (104)).

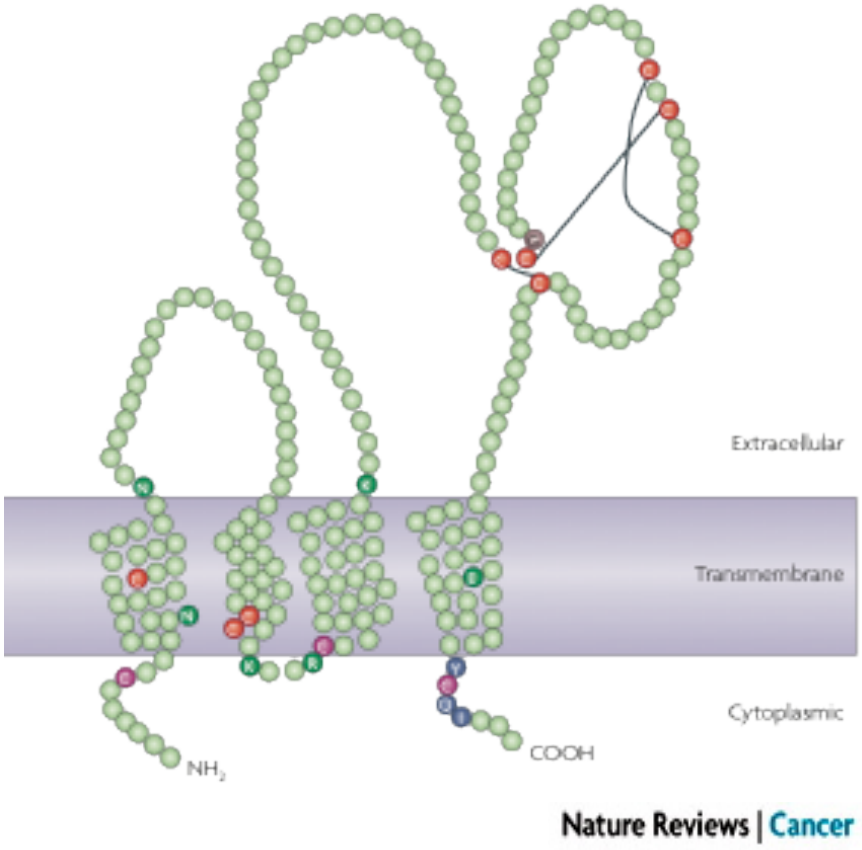

Figure 13:
Since tetraspanins have similar structure, TSPAN8 is used a representative of tetraspanins in this cartoon. Large extracellular loop has 6 conserved cysteines (Red) and CCG motif; disulfide bonds are shown. Palmitoylation of cysteine residues (pink), and the carboxy tail with a sorting motif (blue) are shown in the cytoplasmic region. Polar amino acids (green) are present in transmembrane 1 and 4. Figure adopted from (104)

1.5.2 Tetraspanin Enriched Microdomain (TEM):

Tetraspanins associate and interact with other tertraspanin members and non-tetraspanin partners of cytosolic proteins. Given this wide range of interactions, the function of tetraspanins depends of the nature of associated proteins, some tetraspanins might have diverse functions depending on the cellular context (104). The major transmembrane non-tetraspanin interating proteins are integrins, specifically α3β1, α4β1 and α6β1, G-protein-coupled receptors (GPCRs) and associated intracellular heterotrimeric G-proteins, peptidases, CD44, epithelial cell adhesion molecule (EPCAM), immunoglobulin (Ig) superfamily members such as EWI-F and EWI-2. Cytosolic signaling proteins such as protein kinas C (PKC), phospholipase Cγ (PLCγ) and type II phosphatidylinositol 4-kinase (PI4KII) are shown to interact with tetraspanins (reviwed in (104)).

The aforementioned associations of tetraspanins with other proteins were delineated using different concentrations of detergents. The interactions were classified based on the required concentration of a detergent to break the association. The associations are classified into Class I, II and III. Class I interactions are strong and mainly homotrimers and homotetramers, and some heterointeractions between TSPAN8, CD81, CD9 and EWI proteins, and CD151 and integrins. These types of interactions may proceed through the large extracellular loop and or TM2 or TM3 or TM4; Class I interaction are direct and infrequent.

Most of tertraspanin-tetraspanin interactions and tetraspanin-integrin interactions are class II interactions (medium strength; maintained in mild detergents).

Class II interactions require palmitoylation of the tetraspanin partners or tetraspanins themselves.

Lastly, Class III (weak; disrupted by milder detergents) interactions are mainly the cytosolic interacting proteins such as kinases. Palmitoylation of the cytosolic protein or tetraspanins enhances the interaction complex. In addition to all these associations, tetraspanins associate with lipid molecules and cholesterol and gangliosides. Such intereraction enhance the formation and increase the complexity of high micromolecular structures called Tetraspanin Enriched Microdomain (TEM) (104).

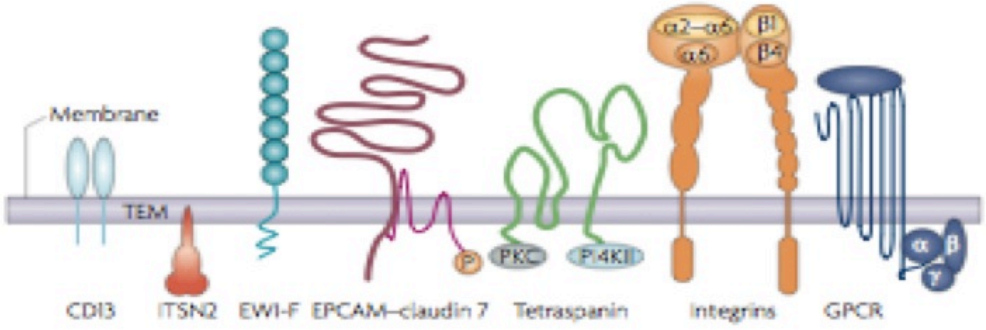

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Figure 14:
A representation of the Tetraspanin Enriched Microdomain (TEM), and the interacting partners. TSPAN8 is shown as a representative. Figure adopted from (104).

1.5.3 Main roles of tetraspanins in carcinogenesis:

The functions of tetrapsanins are dependent on the context of the tetraspanin-enriched microdmain (TEM) and the cell type. Tetraspanins can exert their effects through direct association with tetraspanin partners or infrequently through ligand binding. The function of the associated proteins might be enhanced or inhibited depending on the TEM (106). Moreover, tetraspanins are enriched in secreted exosomes, which suggests that they might play a role in cell communication with neighboring cell (104).

Tetraspanins are implicated in a wide range of cellular operations. These include: A) In cancer tetraspanins are mainly implicated in augmenting migration and tube formation that is mainly due intergrin internalization, compartmentalization and signaling. B) Tetraspanins are also implicated in cell adhesion via their ability to regulate the biosynthesis and trafficking of integrins. C) Tetraspanins are involved in inhibiting cell migration via association of EWI that inhibit the phosphorylation of Ezrin-Radixin-Moesin proteins (ERM). D) Tetraspanins modulate the invasive phenotype of cancer cells probably through association with peptidases, such as A disintegrin and metalloprotease (ADAMS; such as ADAM10), and MMPs. CD151, a tetraspanin member, has been shown to regulate transcription of MMPs such as MMP7 (reviwed in (104), (107)).

1.5.4 Functional classification of teraspanins in carcinogenesis:

Tetraspanins are classified based on their correlation with the metastasic profile in malignancies. Based on reported cases thus far, tetraspanins are found to have both antagonistic and agonistic effects on metastasis. Such contradictory effects of tetraspanins on metastasis are related to the diversified nature of associating partners i.e. the context of the TEM. Tetraspanins

that were reported to suppress metastasis are CD9 and CD82. On the other hand, metastasis-promoting teraspanins are TSPAN8 and CD151 (reviewed in (104)).

1.5.4.1 Metastasis suppressing tetraspanins:

- CD82:

  CD82 contains 6 cysteines in the large extracellular loop and internalization motif and is detected on the endosomal-lysosomal compartment of exosomes. CD82 is downregulated and loss of CD82 correlates with poor prognosis in a number of malignancies such as cervical, ovarian and breast cancer and melanoma (104, 108). In-vitro and in-vivo studies demonstrate that the forceful expression of CD82 abrogates metastasis in experimental models (104). The molecular mechanism behind the metastasis suppression capability of CD82 is believed to be through its ability to associate with a number of interacting proteins. For example, CD82 interacts with α6 integrin chain and EGFR resulting in inhibition of laminin adhesion and migration.

  Another example is the association with EWI protein that has been shown to facilitate the tumor suppressor function of CD82. In regards to the cellular context of TEM, it is worth noting that the ability of CD82 to inhibit or promote EGFR mediated proliferation is contingent on the presence of gangliosides, GD1a. GD1a is proposed to induce re-localization of the CD82-EGFR complex that might abrogate the ability of CD82 to recruit proteins that inhibit the phosporylation of EGFR and thus CD82 loses the ability to inhibit proliferation. These examples from studies on CD82 indicate that the diversity in function seen in tetraspanins is because of the capability to form higher complexity of the micromolecular structure, the tetraspanin enriched microdomain, and the ability of tetraspanins to associate wide spectrum of proteins. (reviewed in (104)). The possibility

of reintroducing the expression of CD82 as a therapeutic strategy requires further experimentations, especially after the finding of Tsai et al. that CD82 could be ubiquitinated through its ubiquitin ligase gp78. This finding suggests potential use of proteasome inhibitors for therapy (104, 109)

- CD9:

The classification of CD9 on whether it is considered a metastasis suppressor tetraspanin or metastasis-promoting tetraspanin is questionable. This is due to the fact that the available reports on CD9 in metastasis are paradoxical. In some malignancies, CD9 promote metastasis but in others it inhibits metastasis. For example, in ovarian carcinoma CD9 has been shown to associate with integrin chains such as β1, α2, α3, α5 and α6. Consequently CD9 inhibit metastasis through inhibiting the integrin-mediated motility that is associated with low matrix adhesion, and downregulation of CD9 is correlated with poor prognosis (110). Moreover, studies of microarray analysis demonstrated that in CD9 overexpressing fibrosarcoma cells genes involved in EMT, such as the WNT pathway, are downregulated, and thus CD9 act as a metastasis suppressor (111).

One the other hand, it has been shown that forceful overexpression of CD9 in melanoma cell lines, upregulates the expression of MMP2, which implicates CD9 in promoting metastasis by participating in the overexpression of an ECM degrading enzyme and transendothelial migration (112). The diverse effects of CD9 are consistent with the properties of tetraspanins to associate with multiple proteins, CD9 effects on metastasis are dependent on the cellular context and TEM (reviewed in (104)).

- CD63 and CD81:

Several reports on literature implicated CD63 and CD81 in suppressing tumor metastasis. In-vitro studies showed that CD63 inhibits migration through inducing integrin endocytosis and MMP14 breakdown and recruitment of tissue inhibitor of metalloproteinase TIMP1 (reviewed in (104, 113). CD 81 has been associated with metastasis suppression in hepatomoa (104).

1.5.4.2 Metastasis promoting tetraspanins:

- CD151:

The characterization of CD151 as metastasis promoter tetraspanin was first reported in in-vivo studies of human epidermoid carcinoma line (114). Margot Zöller in her recent review suggests that CD151 should be used as a marker to predict prognosis in prostate cancer since its overexpression was seen in a wide variety of cancers such as breast, pancreatic, colorectal and non-small-cell lung cancer, and was associated with dismal prognosis in prostate cancer (104).

The mechanism by which CD151 promotes metastasis is believed to be through its ability to induce pericellular activation of MMPs such as MM9, MMP7and MMP2 that then results in degradation of the ECM and recycling of integrins. CD151 mediates the expression of MMP9. In-vitro studies on human melanoma cells showed that CD151 associate with, $\alpha 3\beta 1$ or $\alpha 6\beta 1$ via CD151 homodimerization and mediates the integrin dependent c-Jun activation and subsequent MMP9 expression that results in enhanced motility. CD151 dependent internalization of integrins i.e. integrin recycling, also plays a role in promoting migration of tumor cells (104, 115).

Zijlstra et al. (116) demonstrated in an in-vivo study that blocking CD151 with an antibody abrogated the invasive ability of cancer cells via inhibiting intravasations but not extravasations, primary tumor growth, secondary tumor growth or tumor cell arrest. Zijlstra et al showed that the detachment of tumor cells is affected and that resulted in inhibition of metastasis in CD151 blocked cells. Margot Zöller speculated that the findings in Zijlstra et al is because the inability of CD151 antibody treated cells to recruit MMPs (on the primary site) that are required to degrade the ECM to invade the local stroma (reviewed in (104). On the other hand, Sadej et al showed that the CD151 knockout breast cancer cells co-cultured with endothelial cells affected proliferation and angiogenesis in xenograft models but not in three-dimensional ECMs under standard culture conditions. This means that not only CD151 association with integrins is important in CD151 induced tumorigenesis, the interaction with the surrounding stroma is also important (117). Colocalization of CD151 with other tertraspanins is also variable in malignancies. For expamle CD151 was shown to colocalise with TSPAN8 and α6β4 integrin resulting in augmentation of cell motility in human pancreatic adenocarcinoma (118). In contrast, in HCC CD151/integrin β1 complex drives the enhanced migration and invasion. Overexpression of CD151/integrin β1 complex in patients correlates with poor prognosis. Integrin β1 is expressed at high level in low invasive cells such as HepG2 cell lines, however, overxpresion of CD151 increasead invasion of HepG2 cells, which suggests that the formation of CD151/integrin β1 complex is the critical regulator in invasion and migration of HCC (119). These findings further strengthen the complexity seen in tetraspanins that is mediated by the tetraspanin enriched microdomain (104).

- TSPAN8 (also known as CO-029/ TM4SF3):

The first report implicating TSPAN8 in tumor metastasis was in colorectal cancer in 1989 by Sela et al. (120). TSPAN8has been shown to complex with CD9, CD81, CD151 and a number of integrins such as α3β1 and α6β4. The association of TSPAN8 with integrins can be distrupted with mild detergents suggesting a weak association. On the other hand, the association between CD151 and TSPAN8 is stronger.

The TSPAN8 induced migratory phenotype of cancer cells is mediated mainly through the association with α6β4. The increased motility is mediated by temporary internalization of CD151 and α6β4, by the tyrosine based internalization motif and PKC activation, that results in morphological changes toward the migratory phenotype (104, 121, 122). Moreover, TSPAN8 has been shown to complex with EPCAM, EWI-F, PKC, CD13 and PI4KII (122). The association with EPACM has been shown to increase cell survival (104).

In HCC, TSPAN8 has been shown to correlate with poor differentiation and intrahepatic metastasis of hepatoma (123, 124). Similiary, in colona cancer TSPAN8 was upregulated in metastasized tumor as opposed to primary tumor (104). In esophageal cancer, TSPAN8 increases migration mainly via modulation and upregulation of ADAM12 expression (104). D6.1A (TSPAN8 homologue) knockout mice display disseminated intravascular coagulation (DIC). Angiogenesis was also shown to be mediated by D6.1A and is dependent on the association with α6β4 (104). The implication of TSPAN8 extends to embryonic development as it has been shown to be critical for dorsal-ventral pancreatic bud fusion in *Xenopus laevis* (125). Surprisingly, TSPAN8 knockout mice showed reduced body weight, which implicates TSPAN8 in obesity (126).

Similar to other members of tetraspanins the ability of TSPAN8 to associate with various partners dictates its function and therefore TEM should be taken into account when analyzing tetraspanins (reviewed in (127)).

1.6 Rational of the study:

Hepatocellular Carcinoma is a major worldwide illness and is resistant to conventional chemotheraputic strategies, which might be due to its inherent complexity and to the heterogeneity seen in HCC patients (4, 48, 50). Although surgical resection and liver transplantation provided promising results and increased survival in patients with early HCC, the majority of patients are diagnosed at late stage when prognosis is poor and conventional treatment options are limited and no longer effective (4). The introduction of molecular targeted therapy provided a new era for treating advanced HCC. Sorafenib, a multikinase inhibitor, increased the survival of advanced HCC patients by ~3 months (4, 51).

Thus far, the availability of systemic therapy is limited, which means that the need for characterization of additional molecular mechanisms driving the progression of HCC and that could be targeted for therapy is immense. Our lab had previously identified AEG-1 as an oncogene that is overexpressed with the stages and grades of HCC. Microarray studies of AEG-1 overexpressing cells defined the set of genes deregulated by AEG-1. TSPAN8 that is known to be implicated in metastasis was upregulated significantly by AEG-1 (63, 104). The role of TSPAN8 in HCC is not fully understood. To our knowledge only two reports in the literature implicated TSPAN8 in HCC. The authors showed that TSPAN8 is overexpressed in metastatic tumor cell compared to non-metastatic tumor cells and when overexpressed in HCC cells it

enhances metastasis (123, 124). However, the molecular mechanisms behind this phenomenon are lacking. The aim of this study is to characterize TSPAN8, a downstream target of AEG-1, in the context of AEG-1 mediated metastasis and tumor progression. As a scaffold protein with no conventional domains or motifs, it is difficult to block AEG-1 with small molecule inhibitors. Identification and characterizations of downstream target of AEG-1 are necessary to develop insights for potential therapeutic targets.

1.6.1 Aim 1: Analyze the role of TSPAN8 in mediating the oncogenic functions of AEG-1.

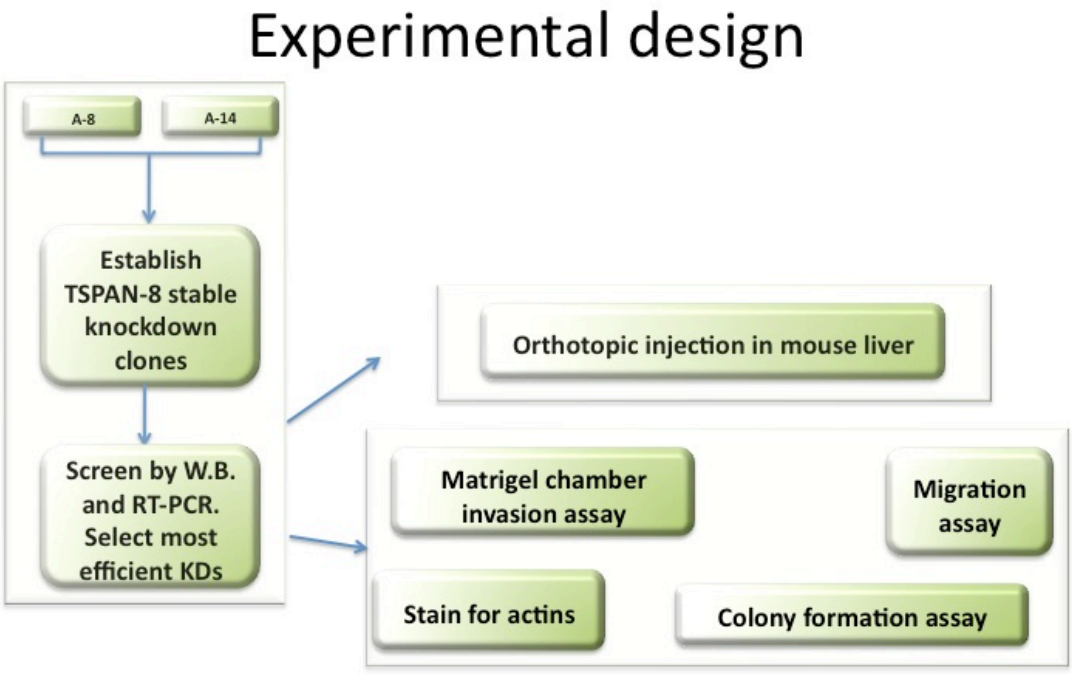

Figure 15:

- AEG-1-8 (A-8) and AEG-1-14 (A14) cell lines: Two clones of HepG3 cells stably overexpressing AEG-1 (63).
- AEG-1-14AEG-1-8Knockdown of TSPAN8 will be performed using plasmids expressing shRNA for TSPAN8.

Aim 2: Investigate the mechanism by which AEG-1 regulates TSPAN8 expression.

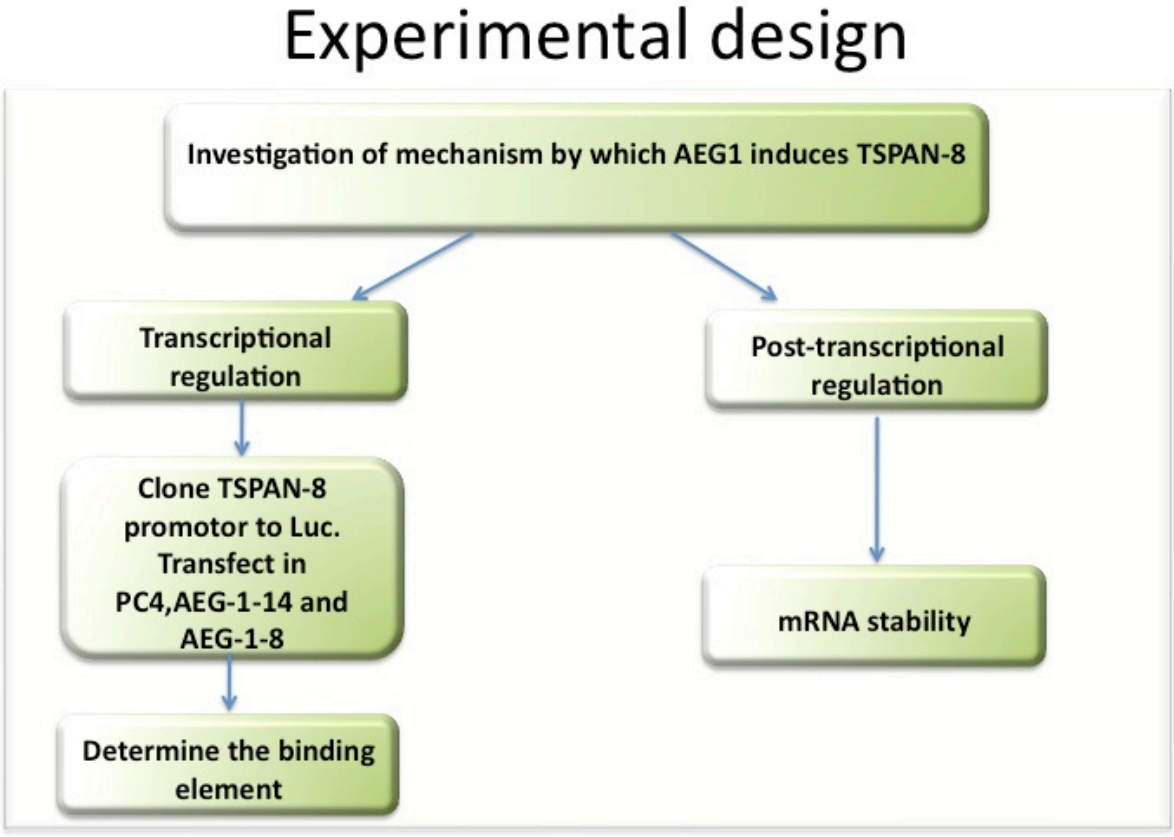

Figure 16: A schematic diagram for Aim 2

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell lines and culture condition:

Cells lines used in this study are AEG-1-8 and AEG-1-14, which are stable clones of HepG3 cells overexpressing AEG-1 (63). The cell lines were cultured at 37°C, in a 5% CO_2/95% atmosphere and cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing:

Table 1: Contents of the DMEM media used in culture

Contents	Percentage/500 ml of DMEM
Fetal Bovine Serum (FBS) from Sigma aldrich	10%
Penicillin and streptomycin from Hyclone	1%
Hygromycin	0.02%
Ciprofloxacin	0.05%

2.2 Construction of TSPAN8 knockdown stable cell lines:

The plasmid expressing short hairpin RNA (shRNA) for TSPAN8 was obtained from Santa Cruz Biotechnology, Inc. The TSPAN8 shRNA plasmid contains three target-specific lentiviral vector plasmids each encoding 19-25 nucleotides (plus hairpin) and a puromycin resistant gene. The trasnfection of AEG-1-8 cell lines with TSPAN8-shRNA plasmid and the control shRNA plasmid was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturers protocol. Cells were selected in 10μg/ml of puromycin in same media conditions used in (63) for two weeks. To ensure the establishment of stable knockdown clones (KDs), single colonies were isolated and maintained for 4 weeks in a DMEM media containing:

Table 2: Content of DMEM media used for selecting stable TSPAN8 knockdown clones.

Contents	Percentage/500 ml of DMEM
Fetal Bovine Serum (FBS) from Sigma aldrich	10%
Penicillin and streptomycin from Hyclone	1%
Hygromycin	0.02%
Ciprofloxacin	0.05%
10μg/ml of puromycin	0.0002%

Western blotting and Real Time PCR (RT-PCR) were used to screen clones with good knockdown of TSPAN8.

2.3 Whole-cell lysates preparation and western blot analysis:

Whole cell lysates were prepared as in (63); 5x10e5 cells were lysed using a lysis buffer from cell signaling (CATLOG# 9803) containing phosphatase inhibitors from Roche (PhosSTOP CATLOG# 04-906-837-001) and protease inhibitors from Roche (complete CATLOG# 11-836-145-001) . Protein estimation was performed using Bio-Rad protein assay kit using the Bradford method (128). For detecting TSPAN8 and AEG-1 100µg and 30µg of protein was loaded, respectively, and ran on an SDS-PAGE. 12% gel was used for TSPAN8 and 8% Gel was used for AEG-1.

Table 3: preparation of polyacrylamide gel

Resolving gel	8%	12%
Protogel	2.67ml	4ml
Resolving buffer	2.6ml	2.6ml
Water	4.62ml	3.29ml
10% APS	100µl	100µl
TEMED	10µl	10µl

Stacking gel	
Protogel	1.3
Stacking buffer	2.5
Water	6.1
10%APS	50µl
TEMED	10µl

The primary antibodies used were anti-AEG-1 (1:500; chicken polyclonal) and anti-TSPAN8 (1:1000 rabit;Sigma-Aldrich). After transferring to a nitrocellulose membrane and blocking for1 hour, the blots were incubated in the primary antibodies overnight in blocking buffer (5% dry non fat milk in TBST) as recommended by the manufacturer.

Secondary antibodies used for anti-AEG-1 and anti-TSPAN8 were (1:1000; anti-chicken) and (1:1000; anti-rabbit), respectively. Blots were stripped and equal loading was checked using anti-EF1-α antibody.

2.4 RNA extraction and Real time PCR:

Total RNA was extracted from 5x10e5 cells using QIAGEN miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. After measuring the RNA concentration using a spectrophotometer, 2µg/µl of RNA was used for cDNA preparation. cDNA synthesis was performed according to the manufacturer's protocol using high capacity cDNA reverse transcription kit from Applied Biosystems. Real-time PCR was performed using Applied Biosystems ViiA7 Fast Real-Time PCR System and TaqMan Gene Expression Assays for TSPAN8 according to the manufacturer's protocol.

Table 4: Amounts required for one reaction for synthesis of cDNA Lot# 1204164.

cDNA synthesis (kit)	
25X dNTP (100mM)	0.8µl
Reverse transcription buffer	2.0µl
10X Random primers	2.0µl
Reverse Transcriptase (50Units/µl)	1.0µl
RNA	2µg/10µl
Nuclease –free water	4.2µl

Table 5: Amounts required for one reaction of RT-PCR

RT-PCR reaction	
Taqman master mix	10µl
Nuclease free water	7.5µl
cDNA	1.5µl
Probe	1µl

2.6 Colony formation assay:

Colony formation was performed as in (63). One thousand cells were plated on 6 cm dishes (4 dishes per group). Plates were incubated for two weeks till colonies were visible. After that, cells were fixed by directly adding 500µl of 37% formaldehyde (from Sigma Aldrich) to the media for 30 minutes. Cells were carefully rinsed with distilled water and 3ml of 10% giemsa (in PBS) was added and plates were incubated for 4 hours. Finally, cells were carefully rinsedand allowed to dry overnight. Colonies >50 cells were scored.

2.7 Matrigel Invasion assay:

Invasion assay was performed using 24-well BioCoat cell culture inserts (BD) with an 8-µ–porosity polyethylene terephthalate membrane coated with Matrigel Basement Membrane Matrix (100 µg/cm2; BD). The procedure was followed as in (63).

2.8 Migration assay:

Migrations assay was performed as in (93) with few modifications. 5x10e4 cells were plated on 6 well plate. Culturing conditions were same as in (63). Cells were allowed to grow till reaching a confluency of 80% to 90%. Then cells were scratched with a pipet tip and observed at 0 hours and 48 hours using a bright-field microscope.

2.9 Actin staining:

Rhodamine phalloidin from Invitrogen, which was developed by (130), was used. The staining procedure was according to the manufactures with one modification, 4% paraformaldehyde solution in PBS was used instead of 3.7% formaldehyde. Briefly, 1x10e4 cells were plated on chamber slide and allowed to grow till reaching a confluency of 80% to 90%. Cells were scratched and after 24 hours cells were stained with Rhodamine phalloidin. The images were analyzed using a Zeiss confocal laser-scanning microscope at the VCU Department of Neurobiology and Anatomy Microscopy facility supported, in part, with funding from NIH-NINDS Center core grant 5P30NS047463.

2.10 Co-culturing with Human Umbilical Vein endothelial cells (HUVEC):

Tube formation by HUVECs was performed using a Cultrex basement membrane extract (R&D Systems) as recommended by the manufacturer. HUVECs were obtained from Lonza and maintained according to the manufacturer's protocol in endothelial cell growth medium-2 (EGM-2). The experiment done as described in (102) with few modifications, 4x10e4 of

HUVECs were co-cultured with 10,000 , 5,000 and 1,000 of AEG-1-8 and TSPAN8 knockdown clones in EGM-2 for 4 hours then tube formation was analyzed using a bright-field microscope.

2.11 Orthotopic xenograft in nude mice:

Cells were orthotopically implanted by intrahepatic injection in athymic nude mice (6-8 weeks of age). The mouse was placed into a plexiglass chamber for induction of anesthesia with 2% isoflurane and 2-liters/min oxygen flow for a mouse of 25 g body weight. After anesthetization, the animal was transferred onto a Styrofoam pad and anesthesia was maintained by isoflurane inhalation through a suitable mouthpiece. The mouse was placed in the left lateral decubitus position and the skin was disinfected with betadine/ethanol scrub. A small skin and muscle incision (about 0.5-1 cm long) was made longitudinally (parallel to the spine) in the right flank to expose the liver. The liver was retracted and using a 30G needle 1 X 10^6 cells in 0.1 ml PBS was injected into the parenchyma of the liver. A visible pale wheal indicated a successful injection. The needle was retracted and a Q-tip was placed over the injection site for 30 seconds to prevend bleeding and spillage of material. The liver was returned to the peritoneal cavity. The peritoneum was closed with a 5-0 suture and the skin was closed by using wound clips. After closing the abdomen the skin was wiped surrounding the suture with betadine and the animal was placed on a warming pad for recovery. The animals were monitored by measuring body weight and observing posture, feeding and grooming behavior. The animals were sacrificed eight weeks after the implantation, and burden was calculated by the sum of all metastatic lesions multiplied 1x (micro), 2x (small) and 3x (large).

2.12 TSPAN8 promoter cloning:

20 μg of human male genomic DNA from Promega (CATLOG# G1471) was used, and digestion of genomic DNA was performed using XhoI.. Appropriate primers were used to isolate the promoter region (Table 6) and Taq PCRx kit from Invitrogen.Inc was used for performing the PCR, 1Kb plus ladder from invitrogen was used. BLAST search confirmed that the primers are unique. Cloning of the promoter was performed as in (95). To assess the efficiency of cloning the promoter, miniprep kit from QIAGEN was used after the transformation ligation reaction of the pGEM®-TEasy vector into DH5α competent cells from Promega; transformation was performed according to the manufacturer protocol.

TABLE 6: Designed primer used for the isolation of TSPAN8 promoter region. Bold nucleotides are digestion sites for restriction enzymes used.

Primer	Primer Sequence (5'-3')
TSPAN8 promoter Forward with **Nhe1 site**	**GCTAGC**GCTAAGGCAGAGAGGAAC
TSPAN8 promoter Reverse with **Xho1 site.** Located in non-coding exon**.**	**CTCGAG**GCTTGTCATAGCTCCTGG
TSPAN8 promoter Forward without **Nhe1 site**	GGAGAAACTGCCAGGGAAAT
TSPAN8 promoter Reverse without **Xho1 site.** Located in the first intron	CGCAAAGGCTATTAACCCAC

Bioinformatics analysis showed that TSPAN8 has a promoter of 2KB in length and does not contain Xho1, Nhe1 and EcoRI sites. The following sequence is the sequence of promoter:

TCAATAAGTATTTCTCAAAAAGAATTTTAAAGTGCTAAGGCAGAGAGGAACTGGG

AGAAACTGCCAGGGAAATTAATTCTCATTCTATTGACACAGCTCAAGTAAGAAATG

ATAAAGGACTGAATAATGAAATGCAAAAGGATGAGATGGAAGAAATAGTTTTGACA

GTAATTTAGGAGCTACACAGACCAGTAAGAGTGATTGGATGGAAAGAAAGAGAAAT

ACAAAATATCTGTACAAATTCAGCCTGGGAGAGTGTTGTGTGATTATAACATTAATA

GGAATAGGAATTTCCAGGAGTGAACTGTTTCATATAGGTTTTGAATTAGACTAGGGG

TTCTATTACACAGTCATTCTCCTGTACAAACACATCCATTGATTTTTGTGTTACAGAG

TTCAAGAGTGATTCAACTAAAGACTACACAGTATTCTTTTTAAGGATGTTCAGCATC

CACCAAAATATCTATTTCAAACTAAAATTTGGTAGTATTCAATACATTCTTTTCAACA

AAGCAGCATTTAAATCTGTAATTTTTTTTTAATTTTAGCCTCTGATAAATAATCATTT

CTGGGACTATGAATCAATTTTGGTTTTCATTCAATCTGAAATTATTCTTTCAGCCTTG

GATTATTCTATATACAAAATATCTCAATAGAAGGTTGTGAAACTATCCATTTTCTAGT

AGGAAAGAGAACACAAACAATTTTAGTGATGATTTATATATTAAGCCATTATTTTTG

GAATTAATCCTAAATTTGGCCTTTAACGCTATATATTATTGTTGATAATGCATTAAAA

TATATTGTATTTGTAGTCAGGGAGTAGAATTGTTTCAAAAACATTGTATGCCAGTGA

AAATTAGAAATAATATTTTTCTTTTGTAAGGAGTTCATGCTTGCTTATCGTGTAAAAC

TTAGCAAAGATTCTAAAGAATAATTTTCTCAACAACCTTTTAAAGTAGAGGGTTCTT

TTATTTAGGGGGAGAAGAACTCCCTTTGAGAGGCAGGATGTGGAAACTAACATGAC

CCAGGCTGTTTACACAAAGCAGTCCACACCTCCCTGATGATGTCGGAGCATTTTGCC

TTTACCTGAGACAGCTGCCTTCTATTAGCACTAGAAACAAATAGTAAAATCTATGGT

CACTGATCCATGACCACCTTCCCATTTTGAATTTTTTTGTCCGGGAGGACCTGGAATC

CAGAAACTGAGTTGGTGGAGAACAGCTGTTGCTAGATACAATTTATCAGAGGAAGG

AAATTACAGTGTCCACCTTAAAAACAAACCAAGCTATCAGAATGACTACGCATTATA

AAATGAAAACAATGTTGCAGTCAGTGCCACTTTATGATTTCTTTCCCTTTGTATCATA

AAGAAATAAAGATAAAAAGCAAATGGTCTTATCATGATAGGCAAGGCTGCCTCTCC

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AATGTCCATTTAACTCAGAACAAAACAACCTTATTTCCCCATAGTTGAAATGGAGGG

CGGGGAAGTCAGGCAGTGGTTTCTGAAAGCCAAGAACTTAGTAGCACTGTGCCATT

CTCTTGCCTGATCCAGTGCCATTCCCTTCACTTGATATCTGTTTACTTTAGAGGAGGC

AGTTTTTGAGAAAGGATCATAAATATCCTGGCCCAGTGCC**CCAGGAGCTATGACAA**

GCAAAGGAACATACTTGCCTGGAGATAGCCTTTGCGATATTTAAATGTGTGAGTTAA

TAGCCTTTGAGATATTTAAAT**GTGTGGGTTAATAGCCTTTGCG**ATATTTAAATGTG

TGGG

- Colored bold nucleotides are positions of the primers used in this study.

CHAPTER 3: RESULTS AND DISSCUSSION

3.1 Establishment of stable TSPAN8 knockdown clones:

We have previously shown in a microarray study that AEG-1-8 and AEG-1-14 cells compared to pc-4 cells (control clone transfected with an empty plasmid in HepG3 background) display more than 25 fold change in upregulation of TSPAN8 (Table 7) (63). To confirm the hypothesis that AEG-1 might upregulate TSPAN8, we first checked the basal expression of TSPAN8 in a number of cell lines, namely, pc-4, AEG-1-8, AEG-1-14 and si-AEG-1 (HepG3 cells trasfected with short-interfering-RNA against AEG-1) by Western blot analysis (Figure 17; a). Both AEG-1-8 and AEG-1-14 clones showed significantly higher levels of AEG-1 and TSPAN8 compared to pc-4 and siAEG-1 clones indicating that TSPAN8 expression might be regulated by or correlate with AEG-1.

To analyze the role of TSPAN8 in mediating AEG-1 function, we established TSPAN8 stable knockdown clones in AEG-1-8 background by transfection with a plasmid expressing TSPAN8 shRNA and selecting in the presence of puromycin for 2 weeks. Individual clones were amplified and screened by Western blotting and Taqman RT-PCR. Clones 9 and 10 (T9 and T10) and control clone 17 (C17 expressing control scrambled shRNA) were chosen for subsequent experiments based on TSPAN8 expression level (Figure 17;b and c).

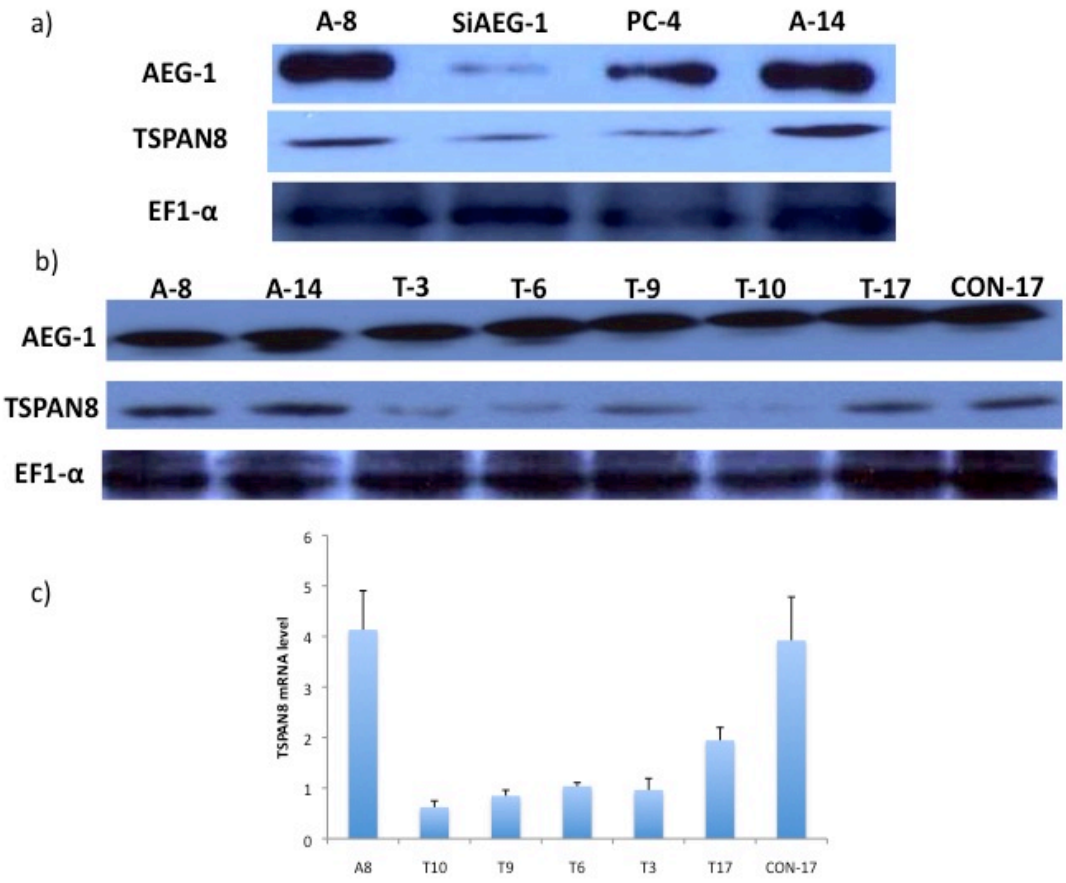


Figure 17:
Establishment and screening of stable TSPAN8 knockdown clones. a) Basal expression of TSPAN8 in AEG-1-8 (A-8), SiAEG-1 (knockdown clone of AEG-1), PC-4 and AEG1-14 (A-14). b) Western blot of AEG-1-8 (A-8) of stable knockdown clones. c) RT-PCR for TSPAN8 expression level in AEG1-8 stable knockdown clones. Results were normalized against GAPDH as an endogenous control.

Differentially regulated genes by AEG1 identified by microarray

Gene	Direction	Function	Fold change in Hep-AEG1-14 (microarray)	Ratio: Hep-AEG1-14 vs. Hep-pc-4 (TaqMan QPCR)	Ratio: Hep-AEG1-8 vs. Hep-pc-4 (TaqMan QPCR)
LEF1	Up	Wnt signaling	12.35	25.28	8.84
CTBP2	Down	Wnt signaling	33.76	0.001	0.008
APC	Down	Wnt signaling	2.32		
DPYD	Up	5-FU metabolism	24.7	6.02	5.02
CYP2B6	Up	Drug metabolism	37.66	15.16	2.08
AKR1C2	Up	Drug metabolism	18.48		
ABCC11	Up	Drug transport	12.51		
TFCP2	Up	Transcription factor	22.73	15.72	15.43
TSPAN8	Up	Invasion and metastasis	25.9	12.64	10.44
CLDN4	Up	Invasion and metastasis	25.32	3.85	2.08
TAGLN	Down	Invasion and metastasis	24.78	0.054	0.177
IGFBP7	Down	Senescence	26.38	0.12	0.001
PK	Up	Glycolysis	26.96		

CYP2B6, cytochrome P4502B6; *AKR1C2*, dihydrodiol dehydrogenase; *ABCC11*, ATP-binding cassette transporter (also known as MRP8); *TSPAN8*, tetraspanin 8; *CLDN4*, claudin 4; *TAGLN*, transgelin; QPCR, quantitative PCR.

Table 7:
The set of deregulated genes by overexpression of AEG-1. Table is from (63).

3.2 TSPAN8 knockdown clones have reduced proliferation:

Given the observation that tetraspanins in general are capable of associating with a variety of membrane and cytosolic proteins allowing them to regulate tumor cell growth, invasion, migration and metastasis (104), we tested if proliferation is affected in TSPAN8 knockdown clones compared to the parental clone (AEG-1-8). TSPAN8 has been previously implicated in promoting metastasis but not proliferation in a number of malignancies including HCC. However, the role of TSPAN8 is not well understood in HCC (104, 124).

To check for proliferation, we performed colony formation assay. Proliferation is significantly reduced in the knockdown clones (T9 and T10) as they have more than 50% less colonies compared to the parental (AEG-1-8) and control (C-17) clones.

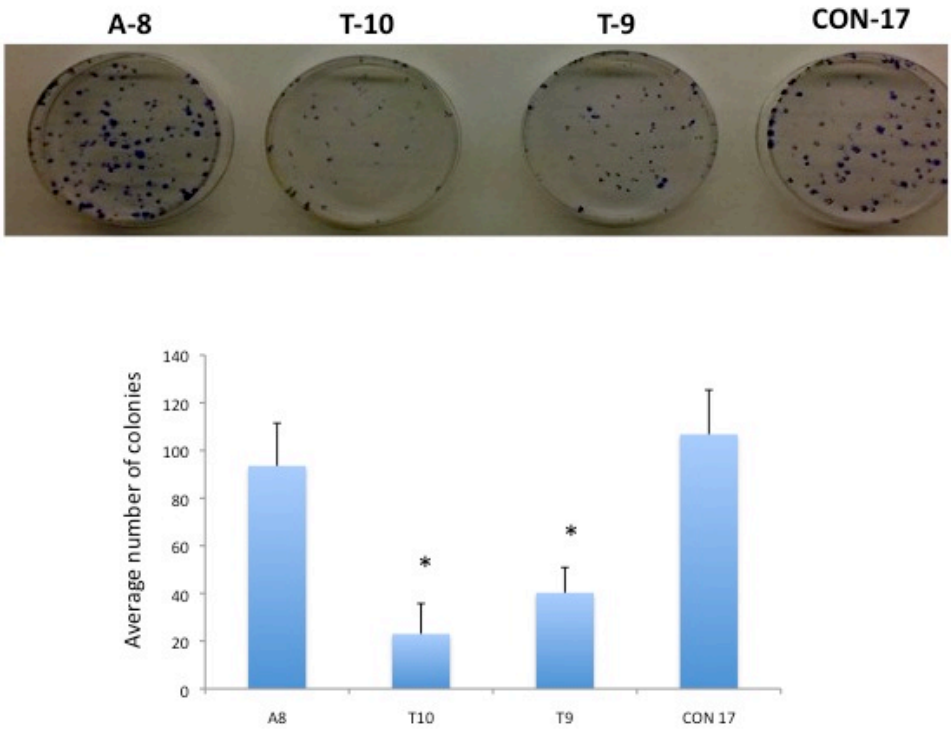


Figure 18:
Knockdown of TSPAN8 in AEG-1-8 cells reduces proliferation. Colony formation assay each group is a representative of 4 samples. *: Indicates a p-value of less than 0.05 between AEG-1-8 (A8; parental cell) and knockdown clones, and between CON-17 (control clone) and knockdown clones. The difference between A8 and CON-17 is not statistically significant.

3.3 Knockdown of TSPAN8 reduces migration and invasion:

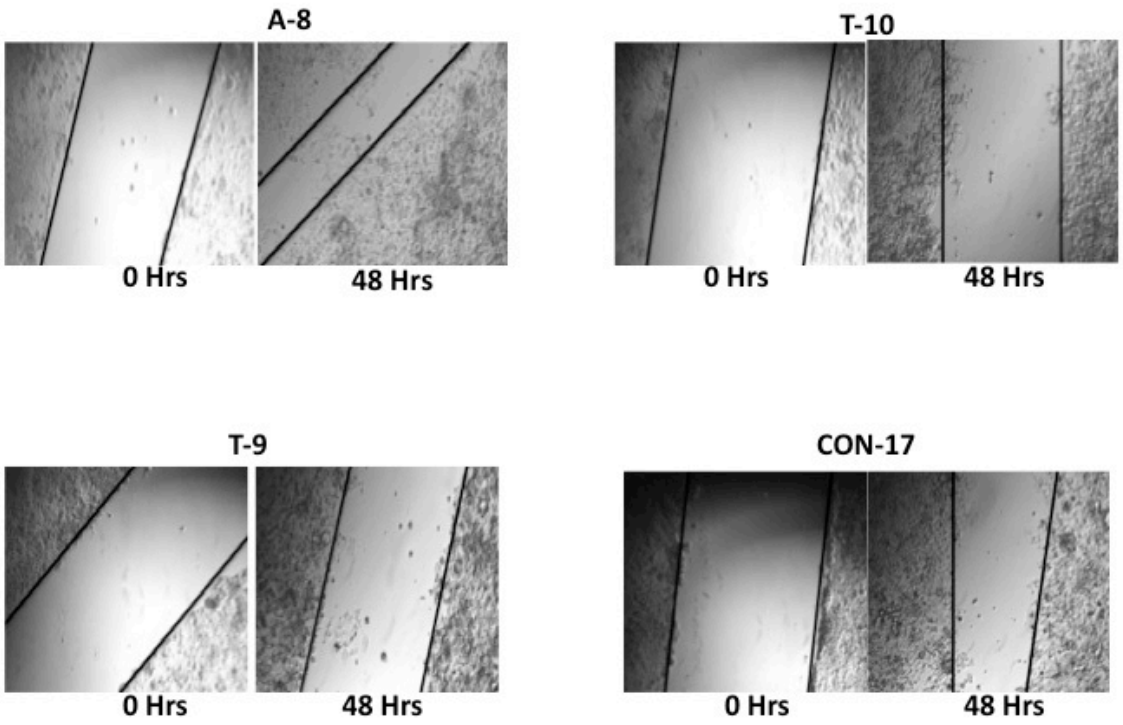
Increasing evidence suggests the involvement of TSPAN8 in mediating tumor invasion and metastasis (104). In melanoma for example, overexpression of TSPAN8 correlated with an invasive phenotype in a number of melanoma cell lines. Experimental knockdown of TSPAN8 in metastatic melanoma cells decreased their invasive ability when plated on Matrigel (131). In HCC, the involvement of TSPAN8 in metastasis is not well understood. To our knowledge only two reports in the literature implicated TSPAN8 (formerly known as CO-0029) in metastasis demonstrating that increased expression of TSPAN8 was associated with intrahepatic metastasis. However, functional analysis on the role of TSPAN8 in HCC is lacking (123, 124). Moreover, the contribution of TSPAN8 in mediating the oncogenic function of AEG-1 is also not known.

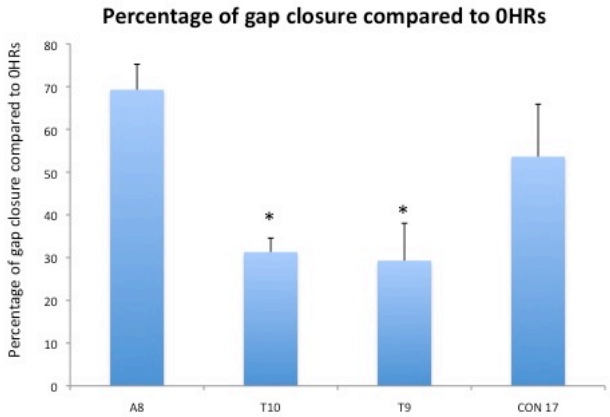
To test whether TSPAN8 mediates AEG-1-induced enhancement in migration and invasion, we performed scratch assay and Matrigel invasion on AEG-1-8 (parental cell line), T9, T10 (knockdown clones) and control clone C17. Results show that knockdown of TSPAN8 abrogated cell migration. T10 and T9 clones migrated ~31% and ~13% of the distance compared to 0 hours, respectively. On the other hand, AEG-1-8 and C17 migrated 69% and 50% of the distance compared to 0 hours, respectively (Figure 19; a). Matrigel invasion assay showed that knockdown of TSPAN8 reduced invasive ability of cells 24hrs after plating on Matrigel. Results show that the number of T10 and T9 cells that crossed the matrigel are significantly reduced compared to AEG-1-8 and CON-17 cells.

We next asked whether the reduction in migration and invasion in knockdown clones is because of reduction in the formation of lamellipodia since that TSPAN8 is a transmembrane protein that was previously shown to localize at the lamellipodia (104, 121, 132). We stained the cells for actin cytoskeleton to detect lamellipodia While AEG-1-8 and C17 cells showed ruffled

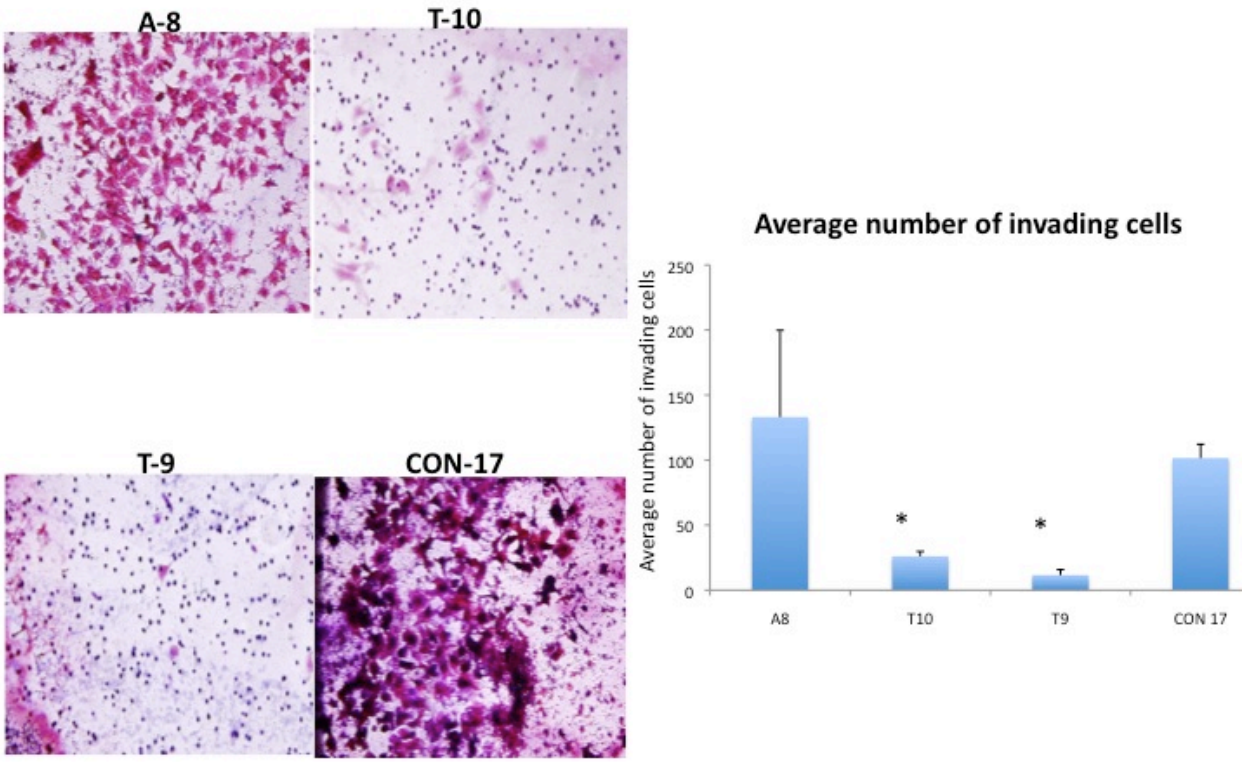
membrane with lamellipodia, the membrane of T9 and T10 cells were blunted in appearance (Figure 19 c; arrow).

a)





b)


A-8
T-10
T-9
CON-17
Average number of invading cells
Average number of invading cells
250
200
150
100
50
0
*
*
A8
T10
T9
CON 17

c)

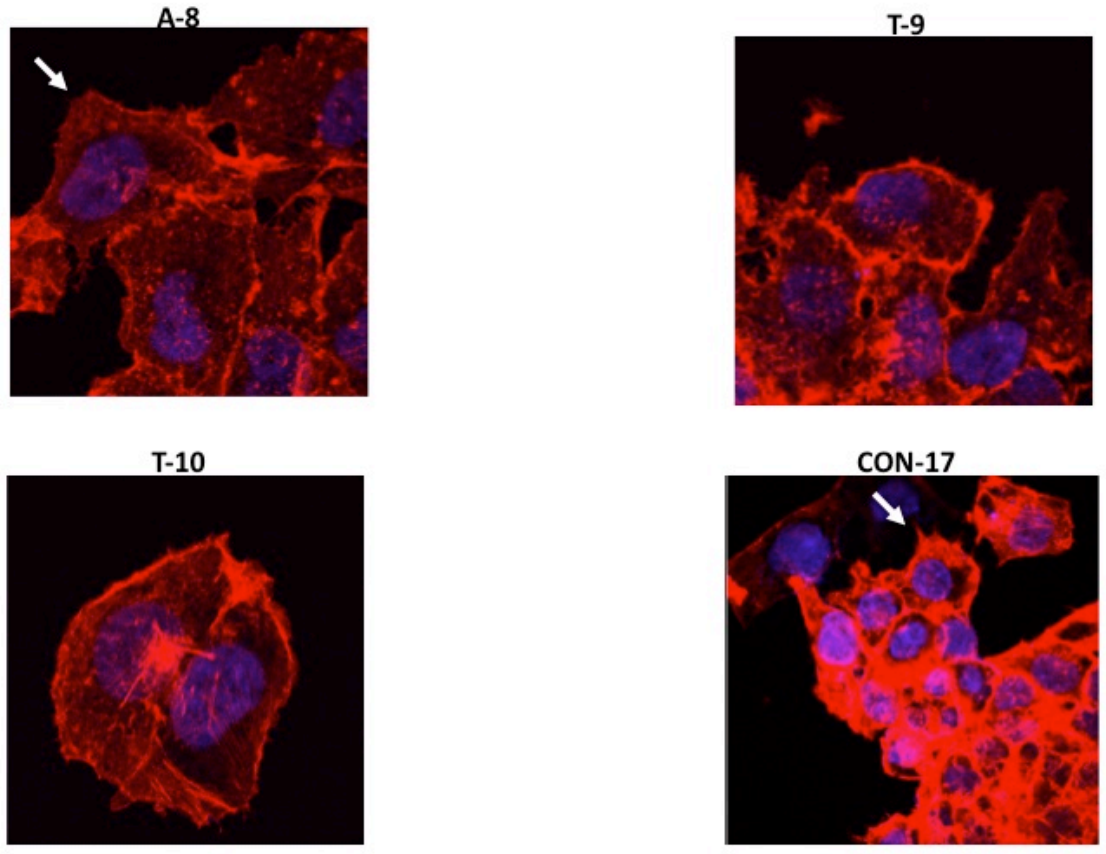


Figure 19:

Effect of TSPAN8 knockdown on migration and invasion of cells. a) Scratch assay at 0 Hrs and 48 Hrs. Each group is a representative of 2samples; 3 readings at different sites. *: Indicates a p-value of less than 0.05 between AEG-1-8 (A8; parental cell) and knockdown clones, and between CON-17 and knockdown clones. The difference between AEG-1-8 and CON-17 is not statistically significant. b) Matrigel invasion chamber assay. Each group is a representative of 2 samples; 4 readings at different sites. . *: Indicates a p-value of less than 0.05 between AEG-1-8 and (A8; parental clone) and knockdown clones, and between CON-17 and knockdown clones. The difference between AEG-1-8 and CON-17 is not statistically significant. c) Actin staining using confocal microscopy at magnification of 100X.

3.4 Knockdown of TSPAN8 reduces endothelial cell activation (HUVECs):

One major funciton of TSPAN8 is regulation of angiogenesis. Overexpresion of D6.1A (mouse homologue of TSPAN8) in low metastatisizing pancreatic adenocarcinoma cells induced disseminated intravascular coagulation (DIC), and (AS) increased metastasis as well as increased hemorrhage around tumors upon establishment of xenografts in syngeneic mice (133). Recently, TSPAN8 secreted exosomes were shown to induce endothelial cell activation through induction of several pro-angiogenic proteins such as VEGF and von Willebrand factor in rat pancreatic carcinoma model. In general, the involvement of TSPAN8 in angiogenesis in cancers and in HCC in particular is still not well understood. To our knowledge there are no reports that implicated TSPAN8 in angiogenesis of HCC.

AEG-1 profoundly augments tumor angiogenesis and we hypothesized that overexpressed TSPAN8 in the membrane of AEG-1 overexpressing cells might facilitate interaction with endothelial cells and their differentiation. To test this hypothesis, we co-cultured 10,000, 5,000 and 1000 AEG-18, T10 or T9 cells along with with 4x10e4 of HUVECs and analyzed differentiation by measuring tube formation. Results show that knockdown clones have significantly reduced number of tubes compared to AEG-1-8 clone(Figure 20). We also collected conditioned media from AEG-18 and TSPAN8 knockdown clones and cultured HUVECs in the presence of the conditione media. However, no significant difference was observed in tube formation (data not shown). These findings indicate that physical interaction between TSPAN8 in tumor cells with endothelial cells might be required for activation of endothelial cells by AEG-1.

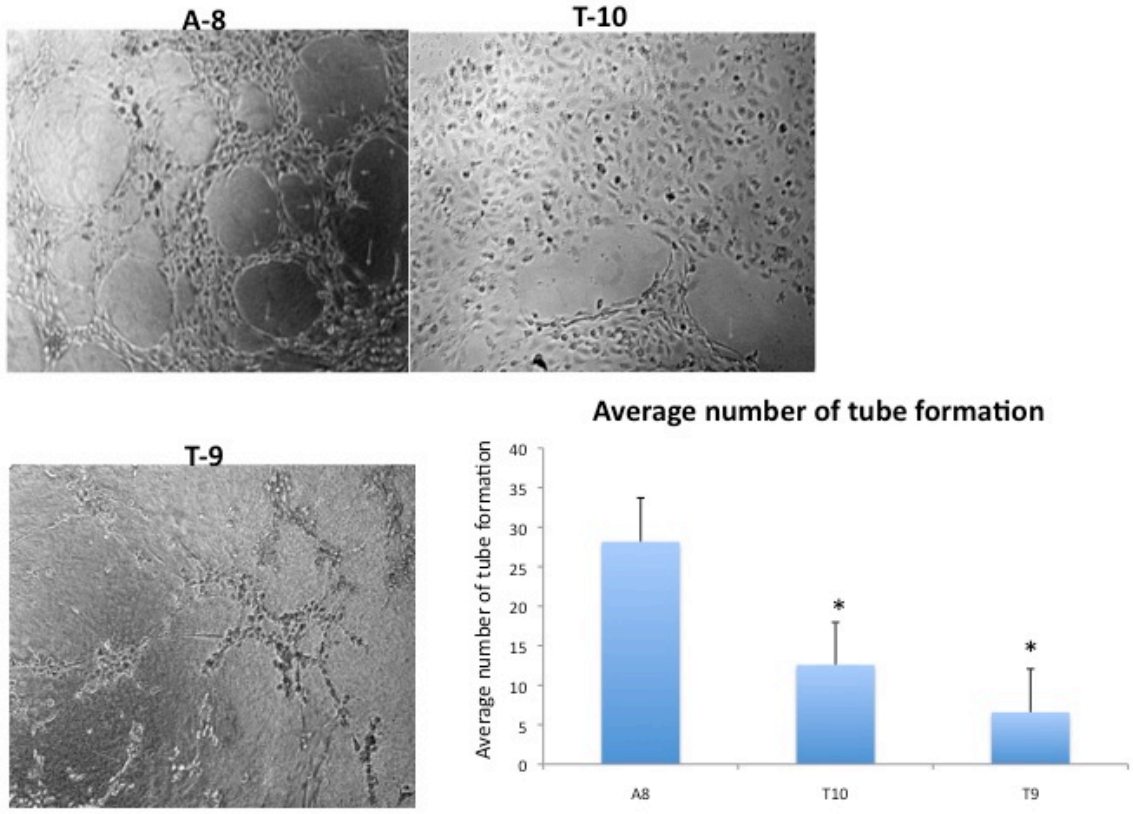


Figure 20:
Co-culture of HUVECs with A8 and knockdown clones (T10 and T9). Each group represents co-culture of (10,000, 5,000, 1000) cells of parental and knockdown clone; each number of cell has two samples and reading from 2 different sites. *: indicates a p-value of less than 0.05 between AEG-1-8 (A8; parental cell) and knock down clones.

3.5 TSPAN8 knockdown clones have reduced intrahepatic metastasis in-vivo:

To check the role of TSPAN8 in AEG-1-mediated metastasis in-vivo, we established orthotopic xenografts in the livers of athymic nude mice using AEG-1-8, T10, T9 and C17 clones. Mice were followed for 8 week. Results show that knockdown clones have reduced intrahepatic metastasis and reduced tumor size compared to AEG-1-8 and C17 clones (Figure 21).

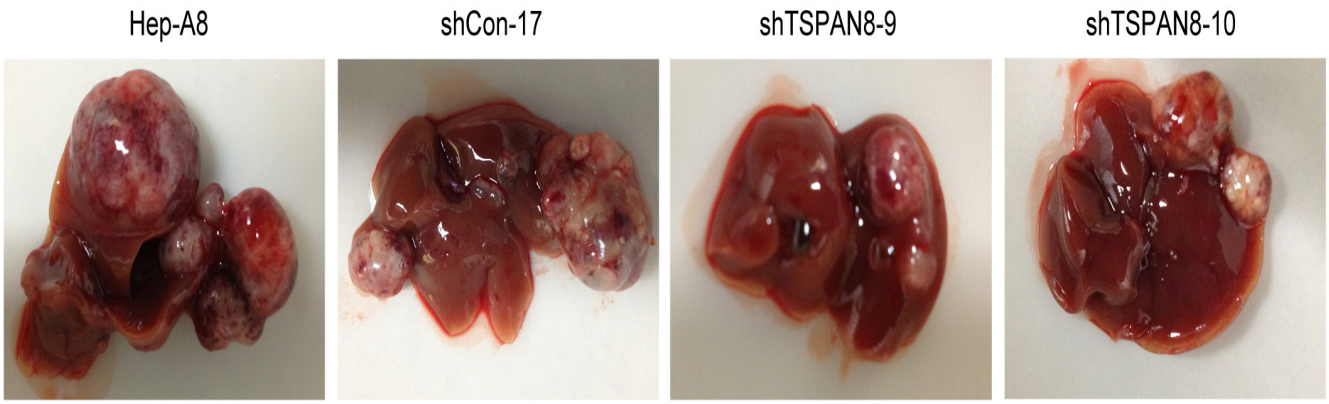


Figure 21:
Orthotopic injection of AEG-1-8 and TSPAN8 knockdown clones. Each group is representative of 4 mice.

3.6 Cloning of TSPAN8 promoter region:

Overexpresion of AEG-1 causes upregulation of TSPAN8 at the mRNA level (63, 98). To test whether AEG-1 upregulates TSPAN8 at the transcriptional level, we attempted to clone the promoter region of human TSPAN8 gene for further experimentation to identify the essential elements in the promoter region of TSPAN8 gene for regulation by AEG-1.

Human genomic DNA was used as template to clone ~2kb region of TSPAN8 promoter by PCR. A single band corresponding to the expected size was generated by PCR (Figure 22; a). The PCR product was ligated into pGEM®-TEasy vector. Upon transformation into DH5α competent cells random colonies were picked, the plasmid DNA was extracted and the presence of the insert was screened by digestion with *Xho*I and *Nhe*I.

Even though a 2kb band was detected upon digestion, sequencing of the plasmid DNA did not reveal the presence of the expected TSPAN8 promoter sequence in the constructed clones. (Figure 22;b). In another attempt we used different set of primers without adding the *Xho*I and *Nhe*I sites. Even though we obtained a PCR product of the expected size (data not shown), cloning the PCR product into pGEM®-TEasy vector and subsequent sequencing still did not reveal TSPAN8 promoter sequence. It is anticipated that there might be problems with ligation that might interfere with the cloning of the promoter. Using new ligation kit still did not resolve the problem. Attempts are being made with new primers and new ligation kit to clone the TSPAN8 promoter.

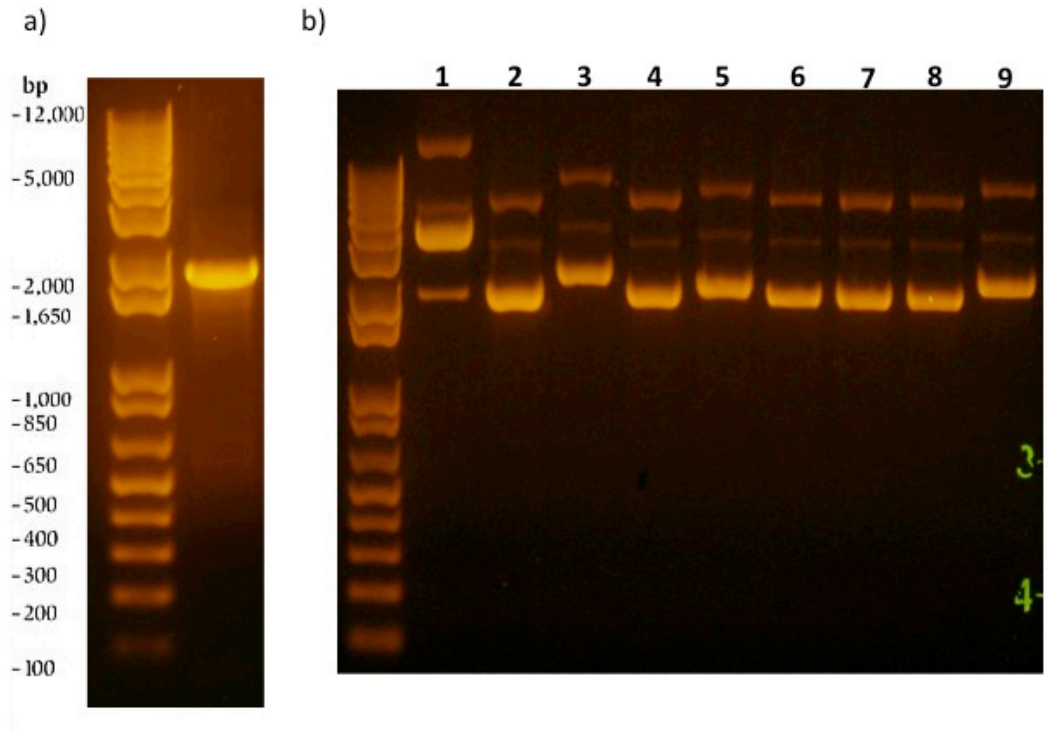


Figure 22:
Gel electrophoresis for extracted DNA from a cloning attempt of TSPAN8 promoter. a) 2Kb isolated band after digestion of human genomic DNA and use of appropriate primers. b) Screening of transformed colonies; Bands extracted from 9 randomly selected transformed colonies.

3.7 Discussion:

Hepatocellular carcinoma (HCC) is a devastating form of liver cancer with dismal prognosis and a five-year survival of less than 12% in the United States (2, 4). This is because of metastasis and lack of systemic effective therapeutic strategies. The heterogeneity seen in HCC patients might explain the inherent complexity of the illness. Available data demonstrates the difficulty of designing a drug that works for all patients as every patient has a genetic signature that is different than the other. This suggests that the pathogenesis driving the progression of HCC is still puzzling. To solve the puzzle, we need to further characterize the molecular mechanisms behind the development of HCC to obtain insights into potential therapeutic targets that in turn will help to develop diagnostic markers and therapeutic profiles for the use of personalized medicine (4, 36).

We, in previous studies, have identified AEG-1 as a major protein regulating the development and progression of HCC (63). Overexpression of AEG-1 was seen in late stage HCC and was seen to cross talk with a number of pathways modulating major hallmarks of cancer that drive tumorigenesis and complicates the use of available treatment strategies such as surgical resection in late stage HCC with metastasis. In a microarray study, TSPAN8 was shown to be upregulated in AEG-1 overexpressing cells (AEG-1-14 and AEG-1-8), (Table 6), and AEG-1 transgenic mice (98). TSPAN8 is from the family of transmembrane proteins that cross the membrane 4 times. TSPAN8 is classified as a metastasis promoting tetraspanin as it was shown in recent reports to be overexpressed in metastasizing tumor but not primary tumor (104). Moreover, experimental overexpression of TSPAN8 in non-metastatic pancreatic cell lines augmented metastasis (126).

In this study we analyzed the role of TSPAN8 in AEG-1 mediated tumor progression. Since TSPAN8 is associated with metastasis, we were interested in cellular events that mediate

metastasis (104). Experimental knockdown studies provided clues to investigate the function of proteins of interests and the function of tetraspanins (104, 134). Therefore, to understand the role of TSPAN8 in AEG-1 mediated oncogenesis, we looked at migration, invasion and Human Umbilical Vein Endothelial cells (HUVECs) activation in AEG-1-8 cells with stable knockdown of TSPAN8. Our results show that the knockdown of TSPAN8 reduced migration, invasion, endothelial cell activation and in-vivo intrahepatic metastasis. Knockdown of TSPAN8 abrogated cell motility (migration). Both T9 and T10 clones displayed ~50% reduction in motility. This result is consistent with reports of TSPAN8 mediating cell migration (104, 126, 135). The potential mechanism underlying this observation is that the knockdown of TSPAN8 abrogates the ability of TSPAN8 to recruit key interacting protein to the tetraspanin enriched microdomain by which tetraspanin can modulate various biological processes such as migration, cell contact and cell fusion and many other processes (98). Integrins, for example, are non-tetraspanin interacting protein that are found to complex with the TEM and to implicate motility and migration (119). The associaton of tetraspanins with integrin partners is diverse and leads to changes in adhesive and migratory phenotype. In some tetraspanins, such as CD9, the pro or anti migratory effects is dependent on the type of integrins that are associated. TSPAN8 has been shown to mainly associate with $\alpha 6\beta 4$ integrin (104, 136). Recnlty, in colon cancer the ability of TSPAN8 to switch between $\alpha 1\beta 1$ to $\alpha 2\beta 1$ was shown to enhace motility of highly invasive colon cancer cells (ovexpression of TSPAN8) compared to less invasive colon cancer cells.; the switch was thought to be mediated by silencing of E-cadherin/p120ctn (137). The mechanism by which TSPAN8 in our model associates with integrins is still need to be elucidated.We do not know what type of integrins are associated with TSPAN8 in our model and further experiments are required to idntify the associating integrins. Similarly, the ability of the TSPAN8 knockdown

cells to invade into matrigel was significantly reduced. T10 and T9 clones displayed more than 70% reduction in invading cells compared to parental cells and control clone. Tetraspanins in general were shown to mediate invasion through their ability to associate MMPs in the TEM. This association facilitates matrix degradation (104). Association of TSPAN8 with ADAM12 promotes metastasis through degradation of ECM in eosophigal cancer (135). The abrogation of invasion in TSPAN8 knockdown clones was consistant with the role of TSPAN8 in invasion of melanoma (131). The most striking feature of AEG-1 is that it robustly promotes invasion (138). The observation that TSPAN8 knockdown profoundly abrogates AEG-1-induced invasion indicates that TSPAN8 might be a key molecule mediating AEG-1-induced invasion.

Interestingly, in contrast to other studies, our results also showed that proliferation was also affected in TSPAN8 knockdown clones indicating that the consituents of the TEM in the contexts of HCC might be different from that of melanoma. This finding further extends the importance of analyzing the TEM as a whole. The mechanism by which knowckdown of TSPAN8 affected invasion and proliferation is still not fully understood and further expermients are required to identify the possible interacting partners. The alteration in the balance of MMPs has also been shown to promote angiogenesis. TSPAN8 dervived exosomes induced upregulation of pro-angiogenic factores in rat models and activation of endothelia cells (139, 140). Although the endothelia cell activation was not seen to be affected when we used conditioned media from knockdown and parental cell (data not shown), the co-culturing of the knockdown cells with HUVECs affected the activation suggesting that the interaction between tumor cells and the endothelial cells was affected. The cell-cell interaction hypothesis is consistent with the reported functions of TSPAN8 in development as the knockdown of TSPAN8 (Tm4sf3) affected the fusion of the dorsal and ventral bud in *Xenopus laevis* (125). We further

show that the effect of knockdown of TSPAN8 on metastasis stands true in-vivo. Knockdown clones have impaired intrahepatic spreading compared to control and parental cells.

Current microaray data from our laboratory suggests the regulation of TSPAN8 by AEG1 at mRNA level (63, 98). We speculated that AEG-1 reguates TSPAN8 through interplaying with the transcriptional machinery or through the post transcriptional machinery. AEG-1 either might activate specific transcription factors regulating TSPAN8 transcription. Alternatively, AEG-1 might directly regulate transcription of TSPAN8. AEG-1 has 3 nuclear localization signals and translocates to the nucleus acting as a coactivator of NF-κB by interacting with CBP to induce expression of IL-8 (93). Additionally, AEG-1 also interacts with CBP and the transcriptional repressor YY1 to inhibit transcription of the astrocyte specific glutamate transporter, EAAT2 (53). AEG-1 interacts with the transcription repressor PLZF and squelches it away from the c-myc promoter thereby augmenting c-myc transcription (88). Thus a direct regulation of TSPAN8 transcription is plausible.

However, AEG-1 has been shown to localize predominantly in the cytoplasm in cancer cells, including HCC, as well as in a transgenic mouse with liver-specific expression of AEG-1, regulating translation and miRNA function (88, 98). Thus, posttranscriptional regulation by increasing mRNA stability and/or by altering levels of miRNAs that regulate TSPAN8 might be possible mechanisms. More in-depth analysis is required to dissect the molecular mechanism by which AEG-1 regulates TSPAN8 expression.

Our results in this study further strengthen the rationale of using TSPAN8 as a diagnotsic marker and potential theraputic target. Animal studies have shown that antibodies against TSPAN8 were efficient in abrogating the internalization of TSPAN8 complex and hence

inhibiting the migratory phenotype (104). Whether this strategy could be used for therapy remains to be elucidated.

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VITA

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