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IZMIR INTERNATIONAL BIOMEDICINE
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POTENTIAL REGULATORY EFFECTS OF FLUIDIC SHEAR STRESS THROUGH c-MET SIGNALING ON BIOLOGICAL BEHAVIORS IN HEPATOCELLULAR CARCINOMA CELLS

DEHAN ÇÖMEZ

MOLECULAR BIOLOGY AND GENETICS

MASTER DISSERTATION

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Assoc.Prof. Dr. Güneş Özhan

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DOKUZ EYLÜL ÜNİVERSİTESİ

İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ

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ABBREVIATIONS

EMT: Epithelial-to-Mesenchymal Transition

FSS: Fluidic Shear Stress

HCC: Hepatocellular Carcinoma

HGF: Hepatocyte Growth Factor

HGFA: Hepatocyte Growth Factor Activator

HUVEC: Human Umbilical Vein Endothelial Cell

RTK: Receptor Tyrosine Kinase

RT-qPCR: Reverse-transcriptase quantitative polymerase chain reaction

SF: Scattering Factor

WB: Western Blot

LOC: Lab-on-a-Chip

MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)

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ABSTRACT

Potential Regulatory Effects of Fluidic Shear Stress Through c-Met Signalling on Biological Behaviours of Hepatocellular Carcinoma Cells

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Tumour metastasis is the most common reason of the cancer related death. Metastasis process requires cancer cells to detach primer tumour and intravasation to blood circulation and survive the shear stress in blood stream, homing and proliferation in the secondary organ. Fluidic shear stress (FSS) in blood circulation effects tumour cell's survival, apoptosis, invasion, metastasis and proliferation. Several clinic-pathological studies underline that in HCC intravascular invasion is poor prognosis factor and it is significantly important in metastasis. When tumour cells intravasate to sinusoidal capillaries, shear stress stimulate apoptosis. Frequency of intrahepatic metastasis is high in HCC and it occurs as invasion dependent manner indicating survival of tumour cells under FSS is crucial step for intrahepatic and extrahepatic metastasis. Nevertheless mechanisms behind process hasn't been illuminated adequately yet. Our previous studies showed that HGF/c-Met pathway plays crucial role in cell survival, invasion, metastasis during hepatocarcinogenesis. In this study we aimed to examine the role of HGF/c-Met pathway on survival and proliferation of HCC cells under FSS. In order to do this, we used peristaltic pump to apply FSS to HCC cells which have low (HuH-7 and HepG2) and high (SNU-449) c-Met levels. c-Met expression and activation levels were determined by RT-qPCR and Western blotting, cell survival and proliferation analysis by trypan blue and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)(MTT) in HCC cells. The effect of FSS on colony formation, epithelial-mesenchymal transition (EMT) and invasion were determined by 3D microfluidic system. Our data showed that, FSS treatment increased the expression and activation of c-Met stimulating EMT phenotype, cell survival, proliferation and invasion in HCC cells and c-Met inhibitor (SU11274) reversed these effects suggesting HGF/c-Met pathway has critical role on the survival of HCC cells under FSS and induces aggressive phenotype and c-Met inhibitors could be used to inhibit survival of circulating tumour cells' metastasis in HCC.

Keywords: HCC metastasis, fluidic shear stress, circulating tumour cell, cancer

ÖZET

Sıvı Teğetsel Gerilme Stresinin Hepatoselüler Karsinoma Hücrelerinin Biyolojik Davranışlarına c-Met Sinyal Yolağı Aracılıklı Etkilerinin Araştırılması

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Tümör metastazı kanser ile ilişkili ölümlerin en yaygın nedenidir. Bir tümörün metastaz yapabilmesi için primer tümörden ayrılarak dolaşıma geçmesi, dolaşımda sağ kalması, hedef organda yerleşerek çoğalması gerekmektedir. Solid tümörlerde kan akımı, doku ve lenf sıvılarına bağlı ortaya çıkan mekanik stres-sıvı teğetsel gerilme stresi (fluidic shear stress: FSS) tümör hücrelerinin proliferasyonunda, apoptoz, otafaji, invazyon ve metastaz gelişiminde önemlidir. Birçok klinikopatolojik çalışma, hepatoselüler karsinomada (HCC) intravasküler yayılımın kötü prognostik faktör olduğuna ve metastaz oluşumundaki önemine dikkati çekmektedir. Tümör hücreleri sinüzoidlere veya kan dolaşımına geçerek FSS ile karşılaştığında genelde anoikis veya mekanik travma nedeniyle ölmektedir. HCC'de intrahepatik metastazın yaygın olduğu, metastazın genelde invazyon bağımsız olarak gerçekleştiği, gerek intrahepatik gerekse ekstrahepatik metastaz için FSS'de sağkalımın kritik bir basamak olduğu bildirilmiştir. Ancak bu sürecin mekanizmaları ile açıklandığı yeterince çalışma bulunmamaktadır

Daha önceki çalışmalarımız Hepatosit Büyüme Faktörü (HGF) /c-Met yolağının HCC'de hücre sağkalımı, invazyon ve metastazı düzenlediğini göstermiştir. Bu çalışmada, HGF/c-Met yolağının, FSS varlığında HCC hücrelerinin sağkalımındaki rolünü araştırdık. Bu amaçla mikroakışkan bir sistem kullanarak c-Met ekspresyonu yüksek (SNU-449) ve düşük olan (HuH-7 ve HepG2) HCC hücre hatlarında, c-Met'in aktive ve inhibe edildiği koşullarda, FSS uyguladık. c-Met ekspresyon ve aktivasyonu qRT-PCR ve WB, sağkalım ve proliferasyonu tripan mavisi, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (MTT) ile zaman bağımlı olarak inceledik. Ayrıca FSS'nin koloni oluşumu ve epitelyal mezenşimal dönüşüme (EMT), motilite ve invazyonuna etkisini belirledik.

Verilerimiz FSS koşullarının HCC hücrelerinde EMT fenotipini uyardığını; c-Met ekspresyon ve aktivasyon düzeyinin yüksek olduğu FSS varlığında sağkalımın, adhezyon ve proliferasyon kapasitesinin daha yüksek olduğunu; c-Met aktivasyonunun bu etkiyi artırdığını,

buna karşın spesifik olarak c-Met yolağını inhibe eden küçük molekül inhibitör (SU11274) uygulamasının ise bu etkiyi tersine çevirdiği gösterilmiştir.

Çalışmamız, HGF-Met sinyal yolağının insan HCC hücrelerinin FSS varlığında sağkalımında ve daha agresif karakter kazanmasında kritik bir role sahip olduğunu, c-Met'i bloklayan inhibitörlerinin, dolaşımdaki tümör hücrelerinin sağkalımının veya HCC de metastazın engellenmesinde kullanılabileceğini düşündürmektedir.

Anahtar kelimeler: HCC, metastaz, sıvı teğetsel gerilme stresi, sirküle tümör hücresi, kanser

1 INTRODUCTION

1.1 Hepatocellular Carcinoma

In 2005 American Cancer Society declared that there is more than new 667.000 liver cancer patients around the world and only %8.9 of those patients survive after aggressive conventional therapy. Hepatocellular carcinoma(HCC) is the most common type of liver cancer which is %83 of all liver cancer patients (Farazi and Depinho., 2006.). Globally, liver cancer is the sixth cancer type nearly 850.000 new cases per annum and second leading cause of cancer-associated deaths nearly 800.000 per year. Hepatocellular carcinoma comprises approximately 85-90% of total primary liver cancers. The etiology and risk factors of HCC is well known amongst other cancer types. Hepatocellular carcinoma is frequent in patients with advanced hepatic fibrosis or cirrhosis as a result of chronic liver disease. Chronic liver damaged promoted by HBV or HCV infection and alcohol consumption. HCC appears on the background of cirrhosis that caused by chronic HBV infection (85% of patients) which is a DNA virus that can induce mutations in host genome (which seen in highest prevalence in Asia). On the other hand HCV infection by an RNA virus, hepatocellular carcinoma appears infrequently due to lack of advanced hepatic fibrosis or cirrhosis (Llovet, 2016). The other factor for HCC development is aflatoxin B1 which is a fungal toxin. It functions as a mutagen and causes specific p53 mutations and provide activation of oncogenes such as HRAS56. Although the mechanistic understanding of the synergy is not known yet aflatoxin B1 and HBV virus appears to be synergistic for HCC development especially in African countries. It is known that aflatoxin B1 exposure does not lead to cirrhosis which differs from HCV infection might show that aflatoxin B1 exposure might be a primary driver for HCC development (Farazi & DePinho, 2006) (Llovet, 2016). There are other reasons for cirrhosis related HCC such as unhealthy alcohol use, non-alcoholic steatohepatisis (NASH), haemochromatosis and α1-antitrypsin deficiency. There are also other factors for HCC development such as gender predilection, three times more prevalent in males than females. Majority of HCC patients are over 45 years old. Studies on tobacco usage can not show consistent relevance between smoking and HCC. Moreover a rising consideration is the metabolic syndrome following diabetes and obesity is highly related with liver diseases like as non-alcoholic fatty liver disease (NAFLD) and NASH relevant to hepatocellular carcinoma development. These diseases might be the exception of the postulation that hepatocellular carcinoma is always relevant with advanced hepatic fibrosis

or cirrhosis. Studies show 40% of HCC patients who have NAFLD or NASH caused HCC may not have cirrhosis at all (Manley, 2013).

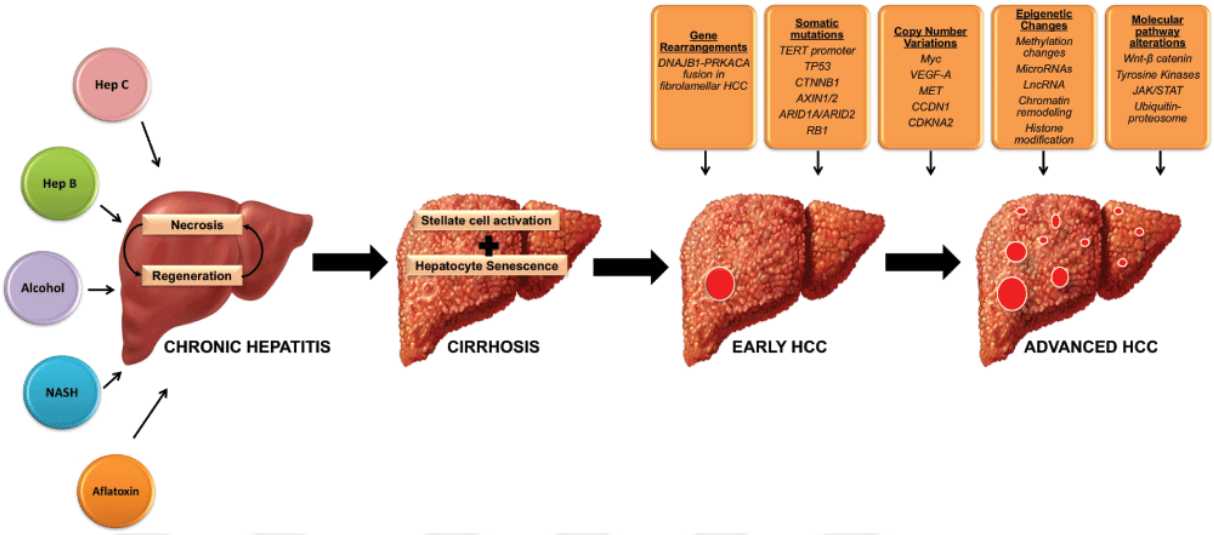

Figure 1.1. The pathogenesis of Hepatocellular carcinoma(Desai A, Sandhu S, Lai JP, 2019).

The molecular alterations in HCC consist of several genetic and epigenetic changes. These alterations cause reorganization of oncogenes and tumour-supressor genes such as TP53, β-catenin, ErbB receptor family members, MET and its ligand hepatocyte growth factor (HGF), p16(INK4a), E-cadherin and cyclooxygenase 2 (COX2). As guardian of genome P53 mutations can lead to tumorigenesis since it plays vital roles in DNA damage repair as a tumoru-supressor gene. In HCC p53 mutations widely seen is late stage HCC. β-catenin is a pivotal downstream signalling molecule of Wnt signalling pathway. When Wnt signalling is activated APC and Axin proteins do not bind β-catenin which causes translocation to the nucleus to link with Tcf transcription factors family. Tcf family regulates the cancer-related genes such as MYC, cyclin D1, COX2 and MMP7. The ErbB family of receptor tyrosine kinases have four members. ERBB1 (also known as epidermal growth factor receptors (EGFR)), ERBB2 (HER2) show different overexpression levels in HCC and plays pivotal role like in many other cancer types. Telomere shortening demonstrated pivotal factor in chronic hyperproliferative liver disease. Genomic instability caused by telomere shortening leads to HCC. On the other hand elevated telomerase activation which can be determined as increased levels of TERT mRNA has been shown in 90% of HCC patients. Besides HBV translocate and alter the TERT locus of human hepatocytes and increases the TERT gene expression leading to HCC. One of the key molecular

alterations in HCC is overexpression of MET receptor tyrosine kinase and its ligand human hepatocyte growth factor (HGF) (Farazi & DePinho, 2006).

HCC is one of the rare cancer types that can be prevented by vaccination. HBV vaccination research demonstrated that in the populations with highly frequent HBV infection HCC cases are reduced with vaccination. On the other hand due to an increase of metabolic syndrome, diabetes and obesity prevalence HCC cases are increased in recent years (Chang et al., 2009).Lifestyle changes such as reducing alcohol consumption for cirrhosis related HCC and diet for NAFLD and NASH caused HCC may decrease HCC prevalence. Although avoidance of HCC depends on avoidance or treatment of fundamental diseases. The therapy options and surveillance of the patients depend on the stage of the disease. When symptoms are seen in the patients related with HCC, the disease mostly at advanced stage and the mortality can be seen within a few months. Early-stage HCC tumours are generally tiny and mostly remediable with minimal invasive procedures. There are protocols for HCC patients depending on the development of the disease such as stage and location. The Barcelona Clinic Liver Cancer (BCLC) classification which arise from 5 stages of HCC provides an algorithm which supports clinicians for treatment options due to tumour stages. Patients with early stages (stage 0 or A) of HCC are convenient for radical therapies such as resection, local ablation and transplantation, patients with multinodular HCC (stage B) are treated with transcatheter arterial chemoembolization (TACE), advanced HCC patients with invasive tumors (stage C) may take chemotherapy as sorafenib a multikinase receptor tyrosine kinase inhibitor and end-stage patients (stage D) are the palliative patients (Chianchiano et al., 2019) (Takamura et al., 2001). After resection, post-operation for patients with HCC major problems are recurrence, intrahepatic and extrahepatic metastasis. Studies on HCC research needs to focus on metastasis mechanisms.

1.2 Metastasis and Circulating Tumour Cells

1.2.1 Metastasis

Metastasis constitutes 90% of the cancer related deaths. Although oncogenic transformation and cancer formation has been most focused and illuminated in cancer research metastasis process has many question marks and needs to be understood. One reason for that metastasis come forth from the evolution of genetically diverse tumour cell population under the selective constraint of an environment enforcing strict orders on cell behaviour (Gupta & Massagué,

2006). These rate-limiting steps are the explanation why millions of tumour cells intravasate to the circulation and only a trifle minority of these cells are able colonizing other organs. To accomplish metastasis, tumour cells must evade the several orders and obstacles which were developed over millions years of evolution. On the other hand cancer cells evolves through a selection process via genetically heterogeneous lines within the organism to accomplish tumorigenesis and metastasis. Tumour cells must overcome these steps of cascade of metastasis in order to accomplish metastasis; 1-)loss of cellular adhesion, 2-)increased motility and invasiveness, 3-)intravasation and survival in the circulation, 4-)extravasation into new tissue and 5-)colonization of a distant site.

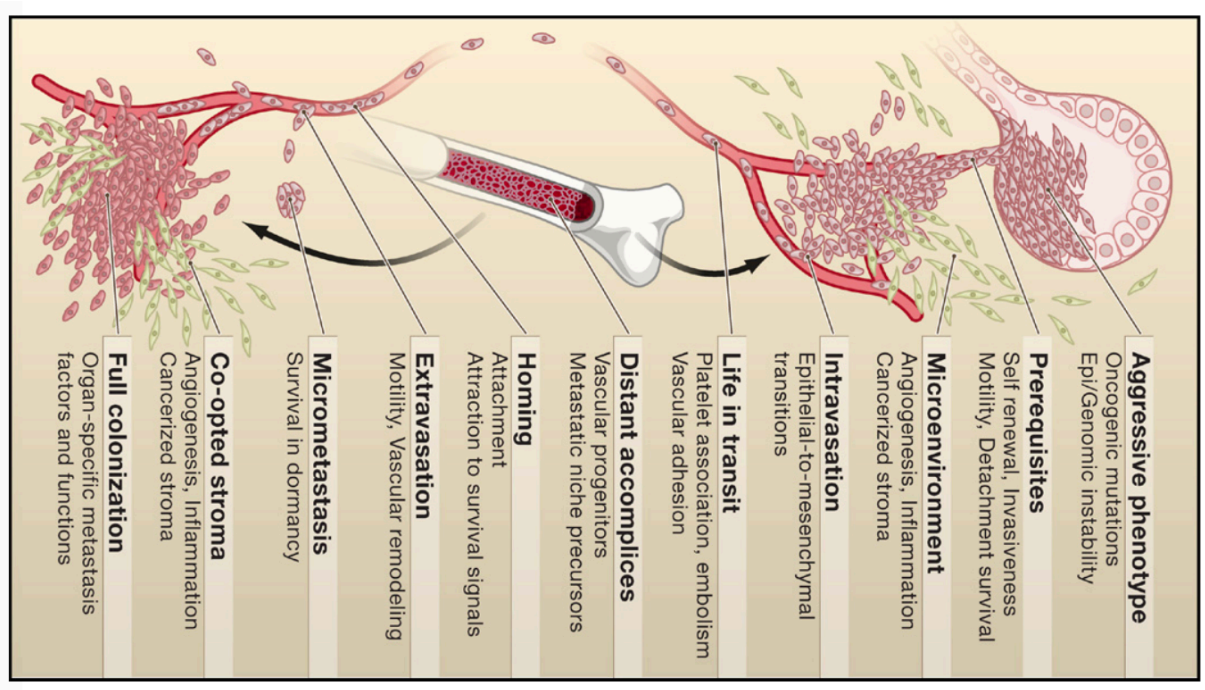

Figure 1.2. Metastasis limiting-rate steps of tumour cells (Gupta & Massagué, 2006).

1.2.2 Circulating Tumour Cells

Solid tumours are foremost reason of the morbidity and mortality globally. Circulating tumour cells (CTCs) are the cells which derived from primary tumours circulate through the bloodstream to diverse tissues and organs nevertheless only modest amount of the cells finally advance into metastasis (Massagué & Obenauf, 2016). Multiple clinical studies have been suggesting that circulating tumour cells are culpable for most post-surgical recurrences and distant metastasis in patients with malignant tumours. Not only that, metastatic colonisation of circulating tumour cells is not proficient as entering into vessels. Although millions of tumour cells transform into CTCs per day, only 0.02% can survive to successfully form metastasis

(Wirtz, 2012). The cancer science has known the appearance of circulating tumour cells since detected by Thomas Ashworth during an autopsy of a patient in 1869. After the first observation almost 100 years later the first isolation techniques were reported in early 1960s but for many years it was challenging to isolate CTCs still. With the immunomagnetic separation techniques progression CTC research field advanced quickly thanks to relatively high specificity of CTC separation in 1990s. Despite the advances in the field the recognition that CTCs are tremendously heterogeneous necessity for more specific marker dependent separation and determination of subpopulations of CTCs arose. The improvement of microfluidic systems for CTC separation strengthen the isolation yield significantly in recent years. In 2016 first circulating tumour cell-based test were clinically approved and used for therapeutic separation of prostate cancer showing that CTC research are has high clinical impact (Poudineh, Sargent, Pantel, & Kelley, 2018). The history line of CTCs can be seen on Figure 1.3.

There is positive correlation between tumour size and CTC numbers. To comprehend CTC biology and mechanisms can play important part to develop targeted therapy for cancer. In addition CTCs can be useful for non-invasive diagnostic applications. On the other hand CTC isolation and identification have some challenges. To study and use clinical applications for CTC require to solve these challenges. One of the challenges is CTCs might be found as single cells or as clump through blood flow and are capable to alter their phenotype due to microenvironment in body fluids such as immune cells, glucose and other substance levels of blood, endothelial cell diversity or different shear stress levels in vascularity or lymph systems. Furthermore the detection of CTC can be tough as a result of low number of CTCs which can be a few to hundreds per millilitre amongst one billion blood cells per millilitre. Due to the requirement for the solutions of those challenges development of advanced techniques are the hotspot of CTC research field. Regardless these challenges, enormous capacity for clinical implementation remain to be promiser to understand function of CTCs in metastasis (Das et al., 2015).

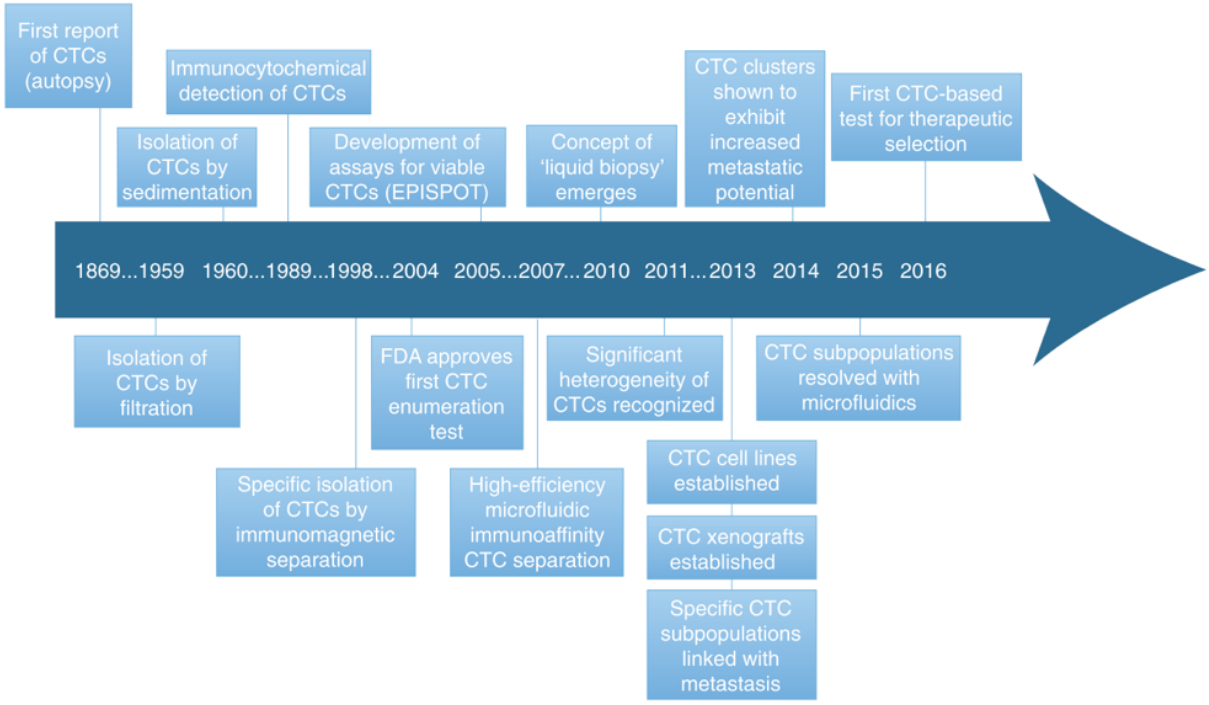

Figure 1.3. Timeline of outstanding milestones of CTC research and applications (Poudineh et al., 2018)

CellSearch system which has been clinically applied tool for circulating tumour cell analysis approved by US Food and Drug Administration (FDA) that can enrich and enumerate circulating tumour cells from blood. It relies on counting epithelial tumour cells from blood using magnetic immune-targeting of the epithelial cell adhesion molecule (EpCAM) identifying circulating tumour cells relying on fluorescent tagged antibodies against cyto-keratins. In recent years the presence and the number of CTCs in various groups of cancer like breast and colorectal cancer have been shown with the CellSearch system demonstrated high number of CTCs are significantly relevant with mitigated survival in patients (Bidard et al., 2014), (Romiti et al., 2014). Another research demonstrated that the CTC number after chemotherapy is relevant with good prognosis and improved overall surveillance in prostate cancer patients (Benz, 2017). Although it seems that CTC number detection is the criteria for prognosis and predictor for survival and efficacy of therapy only enumeration is not sufficient by itself. Deeper understanding to illuminate the physiology of metastasis and the metastatic process involving trans-endothelial migration and intravasation of the tumour cells in blood circulation, survival of tumour cells through blood circulation, mechanical forces, transmit of the cells through vessels as extravasation and colonisation and formation of metastatic lesions.

1.3 Fluidic Shear Stress and Tumour Metastasis

Every cells subsist in a molecular and physiological environment which is designated by chemical and physical components; and those determinants lead to tissue growth, arrangement and different cellular behaviours. Moreover they also may lead to diseases like cancer. Although many research is going on in cancer microenvironment area the physical stimuli of the cancer microenvironment still need more attention and research in order to be understood impactfully (Hyler et al., 2018). Recently due to generation of microfluidic technology and mechanical measurement techniques on cancer research the tumour fluid mechanics have been one of the hot topics in biological research. Recent findings shows that fluidic shear stress (FSS) is a critical factor that effects fluid mechanics and interest for its function on cancer metastasis has been escalated lately. Fluidic shear stress is defined as the internal frictional force between moving layers in laminar flow (Huang et al., 2018). Moreover fluidic shear stress as a result of fluid viscosity and shear rate is significant parameter of cellular stress in flowing liquids, measured in Newtons per square meter (N/m^2) or dynes per square centimetre (dyn/cm^2)(Wirtz, 2012). FSS is an important organizer of vascular endothelial organization and it can induct the cell polarity in endothelial cells as well as cytoskeletal reorganization and post-translational modification such as phosphorylation and gene expression of various genes (Haddad et al., 2010). Fluidic laminar flow established in organisms are categorised as blood, lymphoid and interstitial flow. Recent findings show that FSS has binary impacts. It may foster tumour invasion, adhesion, metastasis, extravasation in contrast it may mechanically eliminate CTCs and at the same time they may cause the arrest on cell cycle in tumour cells (Huang et al., 2018).

The moderate flow of fluids in tumour tissues has been known as interstitial flow and it provides the material exchange between capillaries and adjacent tissue. In tumours it has been known that the high vascular permeability occurs which causes interstitial shear stress increased to nearly 0.1 dyn/cm^2 while the general fluidic shear stress in veins, capillaries and arteries are 1- 4 dyn/cm^2, 10-20 dyn/cm^2 respectively. The mean of FSS in lymphatic vessels is 0.64 ± 0.14 dyn/cm2 and the top is 4-12 dyn/cm2 that is less than blood FSS. It has been noted that FSS may upregulate ICAM-1 in lymphoid endothelial cells and promote lymph gland metastasis. Clinically brain, lung and liver are highly metastatic organs and they have unrivaled blood microvasculature organizations. Their fluidic shear stress rates are shown at Figure 1.4. For example hepatic sinusoids and alveolar walls own a double blood supply system and organ-

specific metastasis by CTCs occur by divergent vasculature. Especially intrahepatic metastasis through hepatic sinusoids and central veins in liver vasculature in HCC is clinically prevalent between HCC patients (Chianchiano et al., 2019).

Mechano-transduction is postulated as five stages; sensing, signalling, gene expression, cellular response and function (Iskratsch, Wolfenson, & Sheetz, 2014). Recently molecular mechano-sensing is the well-known and most studied step. Mechanical sensitivity relies on the physical structure and conformation of mechano-sensor molecules. For instance FSS can foster a structural alteration in p130-Cas adhesion molecule and revealing phosphorylation site of Src protein consequently activation of downstream p38/MAPK signalling pathway. Besides integrins, adherent proteins and calcium channels are amongst the mechano-sensor cell surface molecules. They can sense the alterations in mechanical forces such as fluidic shear stress and activate extracellular and intracellular signalling cascades which sequentially regulate gene transcription and translation effecting cellular behaviours and responses. Nevertheless how those mechanosensitive molecules sense the alterations in amplitude and direction of mechanical forces is uncertain. Studies on fluidic shear stress is mostly focused on endothelial cells and recent developments on cancer metastasis studies and improvement of microfluidic systems allow the cancer and circulating tumour cell research on biomechanical area.

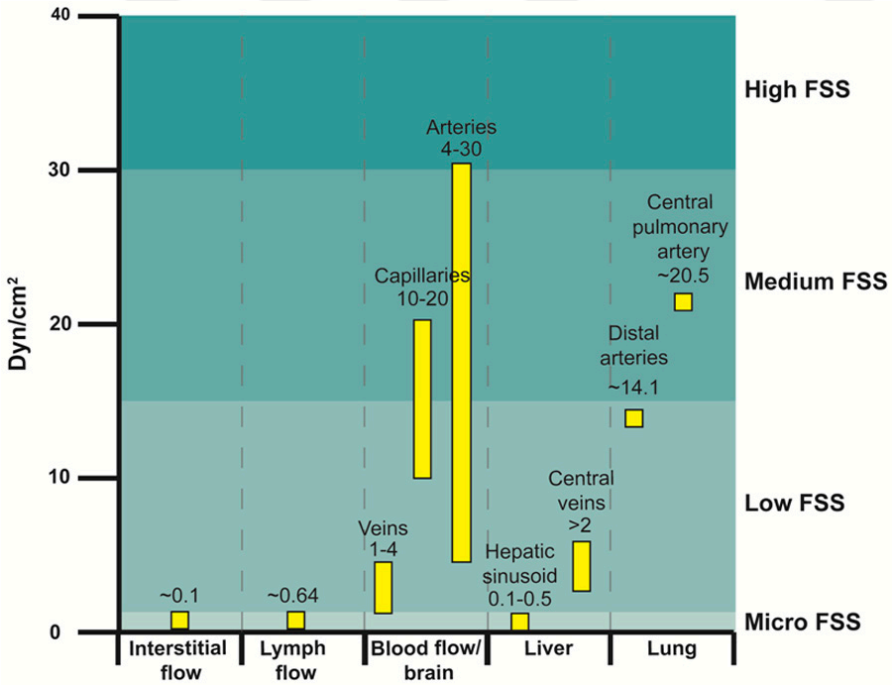

Figure 1.4. Fluidic shear stress rates of different organs and vasculature systems (Huang et al., 2018).

CTCs in vasculature system are exposed to FSS in different size , exposure time and acting form during metastasis. FSS range that CTCs exposed can fluctuate from 0.1 dyn/cm^2 to 1-40 dyn/cm^2 although sometimes it reaches to 3000 dyn/cm^2. These FSS sizes are classified in the literature as micro (0-0.5 dyn/cm^2), low (0.5-15 dyn/cm^2), medium (15-30 dyn/cm^2) and high (>30 dyn/cm^2) FSS as indicated in Figure 1.4. (Huang et al., 2018). Laminar shear stress (LSS) 0.5-12 dyn/cm^2 stimulate apoptosis in Hep3B HCC cell line while oscillation shear stress (OSS) cause the reverse effect on the cells. This data suggest that different forms of FSS results different cellular behaviour in cancer cells. Under liver fibrosis condition blood flow might be disturbed and that can cause oscillatory FSS which will cause decreased FSS-induced apoptosis in cancer cells. Due to this information high metastatic zones might depend on variant FSS forms. FSS leads to cancer cell apoptosis or autophagy in a time dependent manner. CRC cell line HCT116 under FSS has demonstrated no cell death under 2 minutes of 8-60.5 dyn/cm^2 FSS on the other after 20 hours of FSS caused cell death 60% of cancer cells (Rana, Liesveld, & King, 2009). Relatedly tumour cells exposed to high FSS (60 dyn/cm^2) survived 90% less than cells exposed to low FSS (15 dyn/cm^2) under 4 hours of FSS. Moreover apoptosis resumes for 16-24 hours after FSS. FSS induced apoptosis has molecular signalling mechanisms such as BMPRIB upregulation causing Smad1/5/p38 MAPK activation that results increased cleavage caspase-3 and LC3B-I endorse autophagy and apoptosis. Likewise it has been reported high fluidic shear stress escalate the reactive oxygen species (ROS) levels in cancer cells direct to oxidative stress and cell death (Huang et al., 2018). Other mechanism of cell death is cytoskeleton disruption through FSS induction that causes cell adhesion inhibition and anoikis (Guadamillas, Cerezo, & del Pozo, 2011).

1.4 HGF/c-Met Signalling Pathway

In addition to clinical, histo-pathological traits of HCC, latest data demonstrated HGF and its binding protein, c-Met receptor tyrosine kinase concerned in development, advancement of HCC . Besides, it was shown hepatocellular carcinoma patients with activated HGF/c-Met signalling pathway have critically poorer prognosis and related with aggressive metastasis than which does not have activated pathway . Various strategies were developed to inhibit HGF and c-Met gene and protein expression or activation at various steps of HCC treatment. Even though accelerated c-Met levels were demonstrated to lead in metastatic liver tumours and related with poor prognosis in HCC , a contradictory inadequacy of c-Met in hepatocytes were shown leading to tumorigenesis in liver (Gherardi, Birchmeier, Birchmeier, & Woude, 2012).

Hepatocyte Growth Factor/Scatter Factor (HGF/SF) receptor c-Met provides the growth, migration, surveillance and differentiation of epithelial cells. In 1984 it was defined as oncogene product and then it was occured that this form is the active form of receptor which is Tpr-Met and it was estimated that c-Met protooncogene express tyrosine kinase receptor. It has been shown that HGF/c-Met signalling pathway plays a crucial role in biological mechanisms like wound healing, postnatal organ regeneration, embryonic development, epithelial branching morphogenesis, as demonstrated in Figure 1.5. HGF is a paracrine signalling molecule released from mesenchymal cells to impact adjacent epithelial cells which express c-Met receptor tyrosine kinase. Protective roles of HGF demonstrated in endothelial injury, fibrosis and cirrhosis (Ma et al., 2007).

Met tyrosine kinase, as a result of proteolitic cleavage by one chained precursor molecule, it takes its heterodimer mature structure bound by disulfide bond. Heterodimer structure consists of a beta chain which pass through cell membrane one time and an extracellular alfa chain. Extracellular domain contains of 500 amino acids long, low affinity ligand binding provider Sema domain. This extracellular domain also contains sistein rich Met related sequence domain (MRS), immunoglobulin–plexin–transcription domain (IPT) and a protein-protein interaction domain. Intracellular part contains a catalytic domain following juxtamembrane region and at the c-terminal there is a regulator region. Juxtamembrane segment contains two tyrosine (TYR 1234, 1235) and it is responsible for enzyme activity control. Finally two tyrosines at C-terminal tail (Tyr 1349, 1356) are responsible for intracellular adapter molecule binding to the receptor and signal transduction through downstream signalling molecules. When Ser985 serine residues are phosphorylated receptor kinase activity is inhibited. At tyrosine 1003 CBL proto-oncogene binds and it results as Met degradation. (Organ and Tsao, 2011)

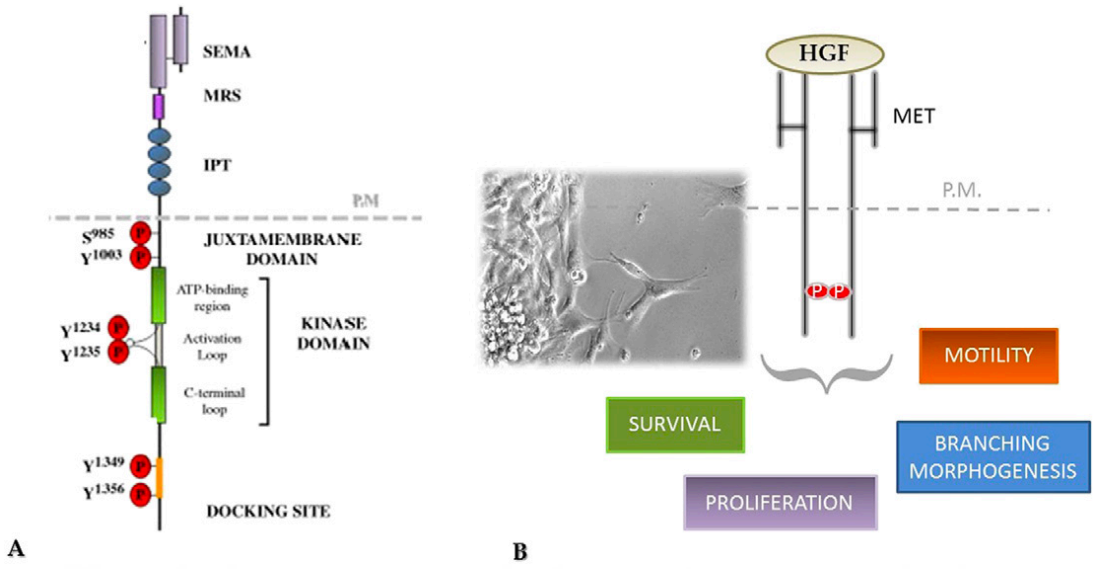

Figure 1.5. Structure and regulation: Structure and regulation of HGF/c-Met receptor tyrosine kinase pathway and its downstream cellular behaviour responses. (Stella, Benvenuti, Gentile, & Comoglio, 2017)

Activation of c-Met molecule receptor tyrosine kinase by its receptor ligand hepatocyte growth factor binding protein provides diverse signalling cascades including survival, mitogenic and motogenic responses through downstream signalling pathways and other signalling molecules that can crosstalk with MET. Hepatocyte growth factor is a multidomain protein which is associated to blood proteinase precursor plasminogen. Besides the transcriptional regulation of HGF gene, the post-translational regulation of HGF protein is proteolytic activation of pro-HGF to functional ligand of c-Met. The soluble HGF activator (HGFA), type II transmembrane enzymes matriptase (ST14) and hepsin are the three serine proteinases that activates HGF through structural reorganizations. HGFA is a soluble proteinase which activated by thrombin providing a vital connection between the coagulation cascade and HGF/MET organized regeneration process. On the other hand hepsin and matriptase are present on the membrane of c-Met expressed cells. Increased expression of these genes stimulate the cancer cell invasion and metastasis. Pro-HGF is excreted as a single-chain precursor and these enzymes provide protease activity to processing of HGF. HGF binds to MET on NK1 (N-terminal and first kringle domains) with high affinity while ß-chain binds with lower affinity through the SEMA-PSI domains on MET which is shown on Figure 1.6. by crystallographic structural analysis. This ligand binding through HGF provides stable dimerization of c-Met receptor results appropriate length to structural basis for MET activation. (Michael et al., 2019)

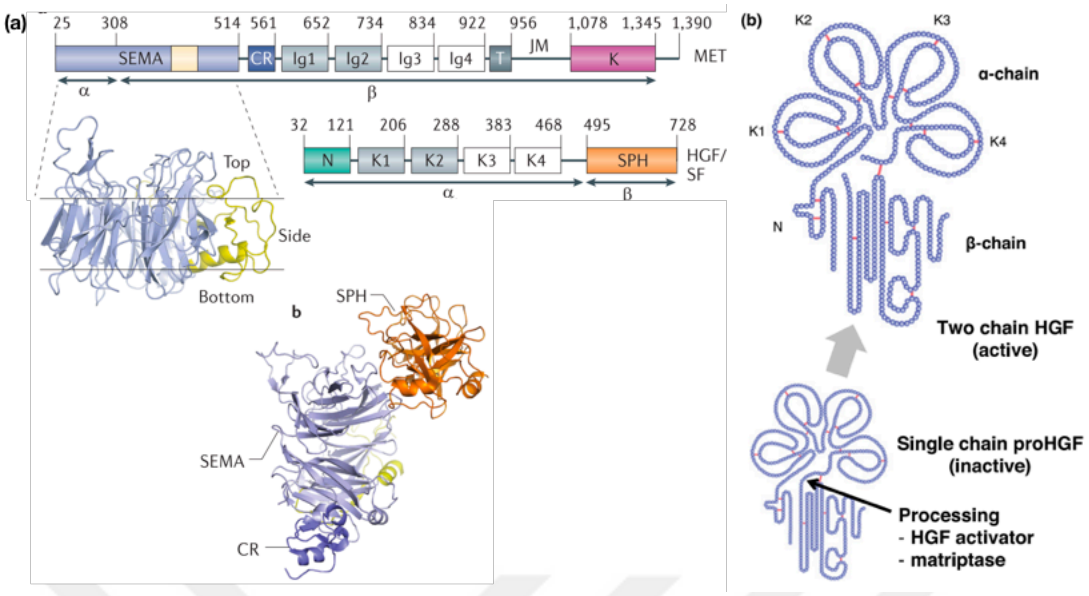

Figure 1.6. HGF activation of MET receptor tyrosine kinase. (a) The structure of c-Met and HGF protein domains and their crystal structure demonstrations (Gherardi et al., 2012). (b) The proteolytic activation of HGF through structural reorganization(Matsumoto, Umitsu, De Silva, Roy, & Bottaro, 2017).

c-Met dimerization and autophosphorylation of the tyrosine residues Y1003 in the juxtamembrane domain and Y1235 and Y1235 in the kinase activation ensuing the phosphorylation of the docking site residues Y1349 and Y1356 on the COOH terminus site is provided by HGF ligand binding. Y1349/1356 phosphorylation conducts to docking of intracellular adaptor and signalling molecules via src homology-2 domain (SH2)- mediated interactions on the c-Met downstream signal transduction. The p85 subunit of phosphatidylinositol 3- kinase (PI3K), growth factor receptor bound protein 2 (GRB2) , signal transducer and activator of transcription 3 (STAT3), GRB2-associated binding protein 1 (GAB1), , SRC, phospholipase C-γ (PLCγ) and Shc are the adaptor proteins that contains SH2 domain which can directly interact with c-Met protein. GAB1 is a multi-adaptor protein provides persistent phosphorylation via either directly binding on 13-amino-acid MET binding site (MBS) or indirectly via GRB2 link. Phosphorylated GAB1 also play role as a helper signal transduction via recruitment of diverse effector proteins such as (SHP2), PI3K, SHC and PLCγ. Several different downstream signalling cascades are activated consequently of those interactions and phosphorylations which are the PI3K-Akt pathway, SRC/FAK, Jun amino-terminal kinases (JNKs), STAT3, nuclear factor-κB (NF-κB), the extracellular signal- regulated kinase 1/2 (ERK1/2), Rho-like GTPases such as RAC1 and p38 MAPK cascades,. These

signalling pathways regulate various cellular behaviours and responses such as cell survival, proliferation, invasion, branching morphogenesis and motility through EMT process. HGF activated c-Met receptor tyrosine kinase co-organized with CD44v6 that binds to c-Met cytoplasmic site to the F-actin cytoskeleton through the ERM (Ezrin, Radixin and Moesin) protein complex and offer a docking site for SOS (son of sevenless) protein complex to activate RAS/MAPK pathway through carcinogenesis. (Bradley et al., 2017) All those activation of c-Met receptor tyrosine kinase by HGF secreted by adjacent stromal cells and its downstream signal transduction pathways and their cellular behaviour responses are shown in Figure 1.7.

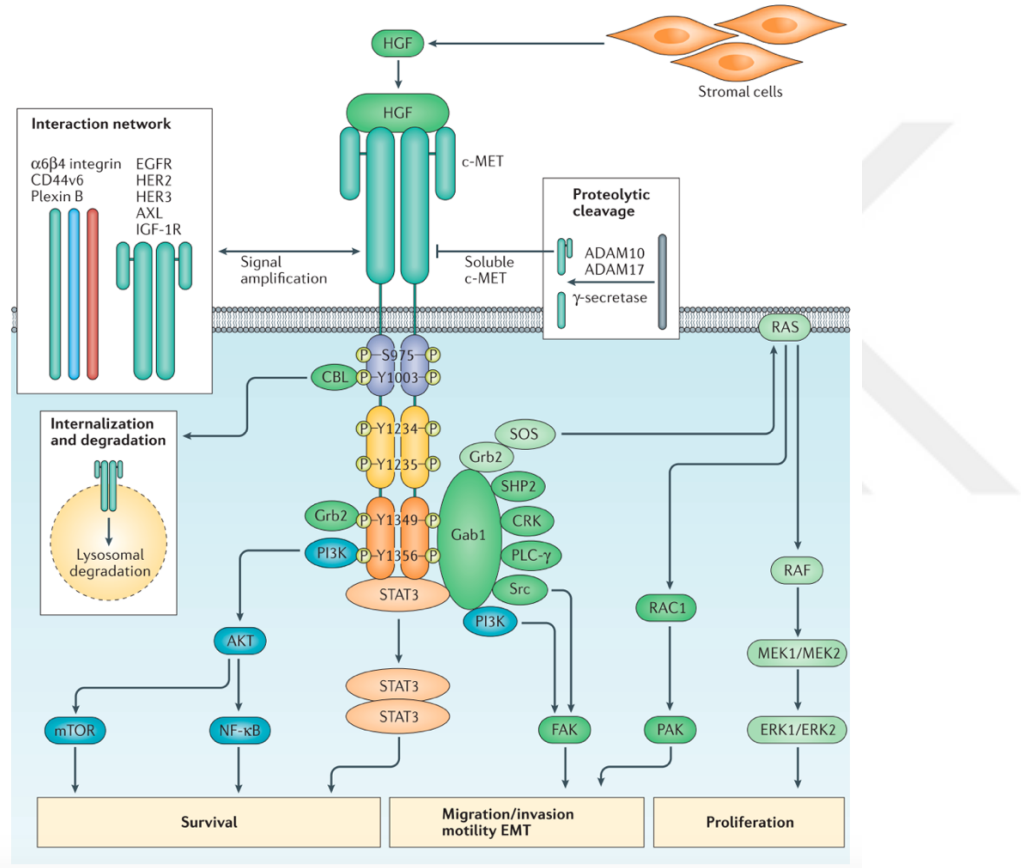

Figure 1.7. Downstream signalling pathways of HGF/c-Met activation (Bradley et al., 2017).

Another regulation of c-Met alongside of transcriptional, post-translational and ligand levels is proteolytic cleavage of c-Met extracellular domain via α-disintegrin and metalloproteinase (ADAM) protein family of metalloproteinases. Via the proteolytic activity N-terminal of c-Met is cleaved as a soluble form of domain and intracellular membrane-bound cytoplasmic portion. Cytoplasmic portion of c-Met is cleaved by γ-secretase enzymes. This decoy causes downregulation of c-Met signalling which results inhibition of HGF interaction and receptor dimerization. ADAM17 has been shown as a intermediary molecule that provides

MEK1/2 inhibition through c-Met cleavage and inhibition of c-Met/JAK1/2/STAT3 activation in KRAS-mutant colorectal carcinoma cells (Hurtz, 2017).

These regulations on HGF/-c-Met proteins and the signalling pathways is highly regulated in non-cancerous cells during wound healing, early embryogenesis and tissue regeneration during carcinogenesis of various cancer types especially in highly metastatic and treatment-resistant cells. MET amplification, mutations, transcriptional deregulation, deficient c-Met degradation and impaired HGF production and ligand activation are the mechanisms of oncogenic activation of c-Met in cancerous cells. Although, the MET mutations are rare in patients with various carcinomas; for instance only 1-5% of gastrointestinal cancer patients have these mutations. On the other hand interaction between c-Met receptor tyrosine kinase and various RTKs are vital mechanism for c-Met activation during carcinogenesis and metastasis. ErbB/HER family and EGFR phosphorylation demonstrated to provide c-Met phosphorylation through various cancers. Besides IGFR1R and AXL RTKs co-activate c-Met signalling through the similar mechanisms showing that c-Met activation through other RTKs is the one of the pivotal signalling pathways for cancer progression (Solomon, Augustine, & Yoon, 2010). These diverse activation of c-Met signalling through phosphorylation has implicated to implement epithelial-to-mesenchymal transition (EMT), inhibition of anchorage dependent cellular death anoikis and increased migration and invasion potential. In gastrointestinal cancers high HGF and c-Met expression and activation is related to increased vascular and lymphatic vessel invasion and intrahepatic liver metastasis in and invasive tumour phenotype in cancer patients and experimental models (Bradley et al., 2017).

2 MATERIALS AND METHODS

2.1 Equipments

Table 2.1. List of Equipments

Equipment	Brand	Catalogue Number
CO_2 Incubator	Memmert	Inco 153
PH Metre	HI 221	Hanna
NanoDrop	Thermo Scientific	ND2000
Vortex	Thermo Scientific	88880018
Peristaltic Pump	Watson Marlow	205U
Marprene Tube Elements for 2.79 bore Purple/White	Watson Marlow	979.0279.000
Water bath	Nüve	NB 9
Western Blotting Transfer System	Bio-Rad	1658029
Sonicator Bioruptor Pico	Diagenode	B01060001
Neubauer Hemocytometer	Marienfield Superior	0630010
Inverted Microscope for Advanced Routine	Carl Zeiss	Axio Vert.A1
mySPIN™ 6 Mini Centrifuge	Thermo Scientific	75004061
Sartorius™ Entris™ Toploading Balances	Sartorious	8221S
SimpliAmp Thermal Cycler	Thermo Scientific	A24811
Centrifuge 5810 R	Eppendorf	5810 R
Magnetic Stirrer C-MAG MS 10	IKA	0003582600
MicroCL 17R Microcentrifuge	Thermo Scientifc	75002450

2.2 Cell Culture and Splitting

Prof. Dr. Mehmet Öztürk from Izmir Biomedicine and Genome Center supplied us with all HCC cell lines.

1-Complete Dulbecco's Modified Eagle Medium (DMEM, Biological Industries) or Roswell Park Memorial Institute 1640 (RPMI 1640, Biological Industries) contained after adding 10% FBS, 1% L-Glutamine and 1%Nonessential amino acids,1% Penicilin/Streptomycin, components were used for growing HCC cell lines. The whole process took place under the laminar flow cabinet and the incubation of cells was taken place at 37^{o}C, 5% CO_2, 95% humidified incubator.

2-When the cell confluency was 70-80%, they were washed with 1X PBS then they were treated with 0.25 % Trypsin /EDTA

3-The cell culture plate was placed at 37 ^{o}C, incubator until the cells were detached. The enzyme activity of Trypsin/EDTA was inactivated by adding complete medium which contains FBS in the amount of threefold of Trypsin/EDTA.

4 Depending on the growth curve of cells and cell types, the cells were splitted into cell culture plates or flasks.

2.3 Cell Counting

1-After detachment of the cells via 0,25 % Trypsin/EDTA and inactivation of trypsin by complete medium containing FBS, cell suspension was obtained.

2-100 µl of Trypan blue solution *(0.5 %, Biological Industries),* 300 µl of 1X PBS and 100 µl cell suspension were mixed.

3- After loading of 10 µl of the mix on Neubauer hemocytometer,the cells on the five squares/areas shown on the Figure 2.1. were counted twice and calculation was shown below in detail and the cell number per 1 ml was determined.

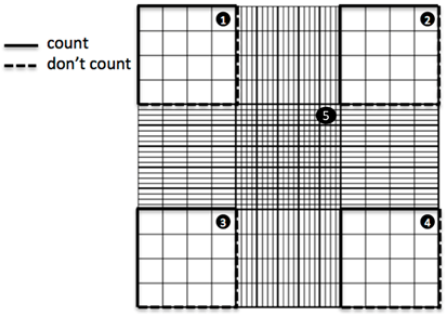

Figure 2.1. The cells on the five squares/areas on Neubauer hemocytometer were counted twice.

2.4 Thawing Cells

The cells stored at liquid nitrogen in the cryovials were thawed at water bath setting to 37°C.
1-In the laminar flow cabinet, 4 ml of complete RPMI/DMEM and 1 ml of thawing cell suspension mixed by pipeting in a 15 ml of sterile falcon.
2-The cells were centrifuged at 1500xrpm for five minutes.
3- After aspiration of supernatant, the pellet was dissolved in the complete RPMI/DMEM and seeded into the appropriate cell culture plate or flask.
4-The cell culture plate was placed at 37 °C, 5% CO2, 95% humidified incubator overnight and the cells were splitted until their confluency was reached to 70%-80%.

2.5 Freezing Cells

1-Medium on the cells was aspirated and they were elutriated by using PBS.
2- After detachment of the cells via 0,25 % Trypsin/EDTA and inactivation of trypsin by complete medium containing FBS, cell suspension was obtained.
3-Centrifugation of cells was taken place at 1500xrpm for five minutes. After aspiration of supernatant, the pellet was dissolved in the freezing medium containing 30% FBS, 10% DMSO and 60% complete medium and 1 million of cells/cryovial in 1 ml of freezing medium was put into cryovials.
4-Cryovials were placed into the Mr. Frosty including 100% isopropanol which decreased temperature 1°C/minute at -80°C overnight. Then the cells were stored at liquid nitrogen tank.

2.6 HGF and SU11274 Treatment

2.6.1 HGF Induction

1-Cells were grown until confluency reached about 70% in the complete DMEM or RPMI with 5.5 mM glucose and 10% FBS .
2- Then cells were starved in the complete DMEM or RPMI with 2% FBS for 16 hours.
3- 2% FBS containing medium was replaced with 10 ng/ml HGF containing medium (2% FBS) after 16 hours and HGF induction performed for 2 hours.

2.6.2 SU11274 Treatment

1- After cells reached to 70% confluency, the growth medium (5.5 mM glucose and 10% FBS) were replaced and cells were washed with 1 X PBS.

2-2500 nM SU11274 containing growth medium with 2- 5% FBS were added.For control groups similar amount of dimethylsulphoxide (DMSO) were added into culture medium. HGF induction was performed for 2 hours.

2.7 Fluidic Shear Stress Treatment

To apply fluidic shear stress on HCC cell lines Watson Marlow 205U peristaltic pump, Marprene Tube Elements for 2.79 bore Watson Marlow tubing and bubble traps were used.

1- After cells were grown on 70% confluency, they were were elutriated by using 1X PBS then they were treated with 0.25 % Trypsin /EDTA.

2-The cell culture plate were placed at 37 ^{o}C, incubator until the cells were detached. The enzyme activity of Trypsin/EDTA was inactivated by adding complete medium which contained FBS in the amount of threefold of Trypsin/EDTA.

3-Cells were counted with hemocytometer and three million cells were loaded in bubble traps. Peristaltic pump system turned on and placed in the 37 ^{o}C, incubator and cells were sheared under 0.5 dyn/cm^2 FSS.

4-After application of FSS at the desired amount of time the cells were collected from bubble traps and they were counted via trypan blue and hemocytometer and viability of the sheared cells detected.

5- After shear stress application cells were used for various experiments such as phalloidin dye, MTT proliferation assay, RNA and protein isolation, colony formation assays etc.

Figure 2.2. Peristaltic pump for fluidic shear stress treatment

2.8 MTT Assay (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide)

MTT assay was used for determination of cell viability and proliferation capacity of cells. The metabolically active cells converted the MTT into insoluble formazan. The color turned into purple when DMSO dissolved the formazan and the absorbance was measured by spectrophotometer.

1-After seeding of cells on 96-well plate 2,000 cells /well for HuH-7, HepG2, 1,500 cells/well for SNU-398 and 1,000 cells/well for SNU-449 in 200 µl medium and the blank wells which only contained medium without any cells for 24h, 48h, 72h.

2-Next day, the medium was aspirated before adding of 100 µl of medium and 15 µl 5 mg/ml MTT to each well.

3-Incubation of cells was taken place at 37ºC %5 CO_2 95% humidified incubator for 4 hours.

4-The solution was aspirated completely. After addition of 100 µl of DMSO per well, incubation of the plates was taken place at RT in the dark with shaking for 30 minutes in order to disperse the formazan .

5-Measurement of absorbance at 570 nm and for background absorbance at 720 nm by using Varioscan spectrophotometer device was done after 24, 48 and 72 hours incubation.

2.9 RNA Extraction From Cell Culture Samples

1-7x10^5 cells were seeded to 3 cm^2 plates.

2- When they reached to 70% confluency, they were taken from the incubator and the culture medium was removed.

3- 500 µl of Nucleozol solution has been added to the plates and pipetting has been done for cell lysis.

4- After addition of 200µl of RNase free water on the samples, their shaking was taken place for 15 seconds and they were holded at RT for five minutes.

5-Centrifigation of samples was taken place at 12.000 g for 15 minutes at RT.

6- 500 µl of supernatant containing RNA transferred to a new tube and pellet containing DNA, proteins and polysaccharides were discarded.

7- 500 µl of isopropanol has been added and holded at RT through 10 minutes for precipitation of RNA.

8- Centrifugation of samples was taken place at 12.000 g for 10 minutes at RT and supernatant has been removed.

9- RNA samples has been washed with 500 µl 75 % ethanol and centrifugation was taken place at 8.000 g for 3 minutes and ethanol was discarded.

10- RNA samples were dispersed in 30-50 µl RNase free water and vortexed shortly. RNA concentration was measured by Nanodrop and the samples with $A^{260/280}$ value nearly 2.0 accepted as good quality samples.

2.10 cDNA Synthesis

Depending on manufacturer's procedure, for cDNA synthesis Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was used. Briefly:

1-1µg template RNA placed in a sterile nuclease free tube.

2- Oligo $(dT)_{18}$ and Random-Hexamer primers has been added to RNA as 0.5 µl volume each.

3- Nuclease-free water has been added to the mix up to 12 µl total volume.

4- After addition of 5X reaction buffer (4 µl), RiboLock RNase Inhibitor (1 µl), 10mM dNTP mix (2µl), RevertAid M-MuLV RT (1 µl) to the mix and total would be 20 µl.

5- The mixture was mixed mildly and centrifugation was taken place shortly and holded 60 minutes at 42°C for Oligo $(dT)_{18}$ primed synthesis and in the sequel 5 minutes at 25 °C for Random-Hexamer primers synthesis. Termination of reaction was done by heating at 70° C for five minutes.

6- The reaction product 20 µl (50 µg/ µl) of cDNA were diluted 5 times with nuclease-free water to 100 µl (10 µg/ µl) of cDNA for qPCR reaction.

2.11 qPCR Reaction

qPCR reaction were applied with Ampliqon RealQ Plus 2x Master Mix Green Without ROX qPCR master mix solution.

1-12.5 µl of RealQ Plus 2x Master Mix was placed in a sterile tube.

2- Forward and reverse primers (1 µM stock) were used as final concentration would be 0.1 µM each.

3- The master mix was mixed up with pipetting and placed to the qPCR reaction plate instrument.

4- 20 ng of cDNA has been added in the master mix on the plate for each well and the instrument was centrifuged for 1 minute at 2.000 g to avoid the the bubbles.

5- The reaction plate was placed in the ABI FAST 7500 qPCR system device. The reaction was held with 3-step PCR programme. 1 cycle of 15 minutes 95° C and 40 cycles of 30 seconds 95° C, 30 seconds 60 ° C, 30 seconds 72 ° C.

The gene specific primers were designed from the DNA CDS (Coding) sequence found in NCBI-Gene Bank as 2-25 base pair long , annealing temperature 60-61 ° C and G-C base content.

Table 2.2. Components of qPCR Reaction

Reagent	**Stock Concentration**	**Final Concentration in Reaction**
RealQ Plus Master Mix	2X	1X
Forward and Reverse Primers	1 µM	0.1 µM

cDNA	20 ng/µl	20 ng/µl
Nuclase Free Water	Completed to 25 µl	

Table 2.3. List of Primers

Primer Name	**Forward Primer**	**Reverse Primer**
MET	TGGGCACCGAAAGATAAACCT	TCTCGGACTTTGCTAGTGCC
RPL41	TCGGCACTTAGCATCATCAC	CATTCGCTTCTTCCTCCACTT
ACTB	TGATGATATCGCCGCGCTC	CATCACGCCCTGGTGCC

2.12 Total Protein Isolation

1-1.5 x 10^6 cells were plated to 6 cm^2 plates.

2-When they reached to 70% confluency, they were taken from the incubator and placed on ice.

3- Cells were elutriated via PBS containing protease inhibitors 0.1 mM NaF and 0.1 mM Na_3VO_4.

4-Cells were scraped with sterile cell scrapers and placed in 15 ml sterile tubes.

5-Cell pellet were obtained after centrifugation at 1500 rpm at 5 minutes at 4^o degree.

6- Supernatant was detracted, pellet was dissolved in threefold RIPA solution and cells were placed on ice for 30 minutes for cell lysis and vortexed every 3-4 minutes.

7-After incubation on ice cells were sonicated for 10 seconds at 4 ^oC degree for better lysis results.

8-Centrifugation of the cells was performed at 13200 rpm for 20 minutes at 4^oC degree to remove cell debris

9-The supernatant containing proteins placed to a new sterile tube and after protein quantification they were placed at -80^o C degree fridge.

2.13 Protein Quantification

Protein measurement calculation was performed by BCA (Bradford Assay).

1-Various concentrations of BSA(Bovine Serum Albumin) were used to compose standard graphic.

2- BCA reagents A and B were mixed and added to the protein lysates and BSA standard concentrations to start the reaction.

3- The reaction was performed by incubating the proteins at 37 °C for 30 minutes or 55 °C for 15 minutes.

4- After the termination of reaction BCA/ copper mix concentrations were measuredby spectrophotometry at 562 nm wavelength.

5- The standard curve was obtained with standard BCA concentrations which used for calculation of protein concentrations.

2.14 SDS-PAGE Gel Electrophoresis

1-Resolving (10%) polyacrylamide gels of 7 ml were casted due to protocol and before they polymerized isopropanol added between 4 ml glasses to obtain smooth gels.

2-Stacking gels (5%) of 2 ml were casted on the top of resolving gels after the polymerization of resolving gels due to protocol at table 2.2. . and combs were placed on the stacking gels.

3-Protein lysates were used different concentrations for western blot due to the target protein that has been researched and 4X loading solution containing 20% beta mercaptoethanol have been mixed as all protein samples would be the same volume.

4-Protein samples were incubated at 95ºC for 10 minutes for denaturation.

5-After removing the comb, the gel was placed into the tank then 3 µl of pre-stain protein marker and denaturated protein samples were loaded into the well one by one.

6-The proteins were exposed to 70 volt firstly, the volt was set to 110 volt after loading dye passing the stacking gel.

7-After the electrophoresis run was completed, the gel was removed from the glass and holding in the 1X transfer buffer. PVDF membrane (millipore) and whattman papers were cut as 8cmx6cm and 9cmx7cm in size respectively.

8-PVDF membrane was incubated in the 100% methanol for one minute in order to open the pores. The membrane and wattman paper were hold in the transfer buffer. For wet transfer process respectively sponge, whatmann paper, gel, PVDF membrane, whatmann paper, sponge were placed to form a sandwich in the transfer buffer **(**Figure 2.3**)** . (The bubbles preventingtransfer of proteins were removed by a roller.)

9-The tray was closed and placed into the tank filled with 1X transfer buffer. Transfer took 3 hours at 400 milliAmper (mA) at RT on magnetic stirrer. In order to prevent excessive warm

due to high current, an icepack was placed into the tank and magnetic stirring bar was also placed in the tank so that current could be equally distributed through the system.

10-After transfer was completed, incubation of the membrane was performed in the 100% methanol for one minute, in the ultrapure water for 1 minute and in the 1x TBS for 2 minutes.

11- Membrane was rotated in the falcon tube for 1 hour at RT in the blocking solution as 5% BSA in 1X TBS. Blocking was done so as to prevent the binding of antibody to non-specific region.

12-Incubation of the membrane was performed by rotating in the primary antibody solution (3% BSA in the 0,2% TBS-T, 0,1% primary antibody) at +4ºC overnight in the falcon tube.

13-The membrane was washed with 0,1% TBS-T solution threefold for 5 minutes by rotating on the following day three times. Based on the primary antibody that was used, the proper infrared secondary antibody was prepared.

14-The membrane was incubated in the secondary antibody in the amber tube (SPL) having dark color to prevent light transmittance for 2 hours at RT by rotating on the Roto-shake genie *(Scientific Industries)*.

15-The membrane was elutriated with 0,1% TBS-T solution four times for five minutes, once with 1x TBS solution so as to remove the detergent on the membrane before imaging.

16-Membrane was imaged via LI-COR Odyssey Clx infrared fluorescence imaging system containing two different fluorescent channels as 700 nm and 800 nm wavelength. Finally, densiometric analysis of proteins band was performed by ImageJ programme.

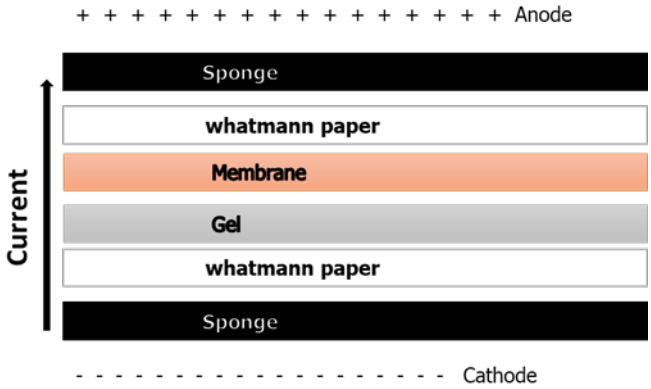

Figure 2.3. Organization of sandwich system for transfer of proteins from acrylamide gel to PVDF membrane

2.14.1 Solution for Western Blotting

1.5 M Tris pH 8,8 (100 ml); 18,165 gram Tris was dissolved in the 60 ml ddH_2O then pH was set to 8,8 via adding up HCI and volume was topped up to 100 ml by adding ddH_2O.

1 M Tris pH 6,8 (100 ml); 12,11 gram Tris was dissolved in the 60 ml ddH_2O then pH was set to 6,8 by adding HCI and volume was topped up to 100 ml by adding ddH_2O.

30% Acrylamid-Bisacrylamid Solution: 29 gram of acrylamid and 1 gram of bisacrylamid were dissolved in 60 ml ddH_2O very well then the volume was set to 100 ml by adding ddH_2O. Homogeneous mix was filtered via 0,2 µM filter and stored at +4°C in the dark.

1X TBS: 4 gram NaCI, 0.1 gram KCI, 1.5 gram Tris was dissolved in the 400 ml ddH_2O water and pH was set to 7.4 then the volume was completed to 500 ml by adding ddH_2O.

1X TBS-T : 500 ml TBS and 500 µl Tween-20 were mixed to generate %0.1 TBS-T.

Running Buffer : 14.4 gram Glisin, 3.075 gram Tris and 10 ml 10% SDS were mixed. The volume was set to 1 liter via ddH_2O. The buffer was stored at +4°C.

Transfer Buffer: 14.4 gram Glisin, 3.075 gram Tris and 2 ml 10% SDS were mixed. 800 ml of ddH_2O was added and mixed very well. The volume was set to 1 liter via adding methanol before use. The buffer was stored at +4°C.

10% APS (Ammonium Persulfate): 0.1 gram APS was mixed with 1 ml of ddH_2O.

10% NaN_3 (Sodium Azide): 1 gram NaN_3 was mixed with 10 ml of ddH_2O.

Coomassie Blue Staining Solution: 100 ml methanol, 80 ml ddH_2O, 20 ml acetic acid and 0.1 gram Coomassie R250 Blue were mixed very well and stored at RT in the light-tight bottle.

Resolving Gel (10%):. 4 ml dH_2O, 3.3 ml 30% Acylamid-Bisacrylamid solution, 100µl 10% SDS, 2.5 ml 1.5 M Tris(pH 8,8) were mixed very well. Then 100 µl 10% APS and 4µl TEMED were added.

Stacking Gel(5%):2.1 ml dH_2O, 0.5 ml 30% Acylamid-Bisacrylamid solution, 30µl 10% SDS, 380µl 1M Tris(pH 6,8) were mixed very well. Then 30µl 10% APS and 3µl TEMED were added. The mix was poured on the resolving gel.

RIPA Lysis Buffer : 50 mM Tris (pH 7.4), 150 mM NaCI, 1mM EDTA (PH 8) and 1% NP40 solutions were mixed and the volume was completed to 100 ml by adding dH_2O. Before use, 1 mM NA_3VO_4, 1 mM NaF and 1 tablet protease inhibitör coctail (*Complete ULTRA Tablets Mini, EASYpack, Protease Inhibitor Coctail Tablets, Roche)* were added to 10 ml Lysis buffer.

Primary Antibody Preparation: 3% BSA, 0.02% Tween-20 and 1:1000 primary antibody was mixed in 1X TBS.

Secondary Antibody Preparation; 3% BSA, 0.02% Tween-20, 0.01% SDS(10%), 1:20000 Secondary antibody in 1X TBS. Moreover, 0.02% NaN_3 (10%) wass added to secondary antibody.

2.15 Phalloidin Staining

1-22x22 mm Glass Microscope Slide Coverslips were autoclaved and they were placed into 6-well tissue culture plate.

2-Cells were plated on the 6-well plate and incubated overnight.

3-On the following day, the medium was detracted and cells were elutriated via PBS three times.

4-1 ml cold 4 mg/ml PFA was applied per well furthermore cells were fixed at +4°C for 10 minutes.

5-The PFA was detracted and cells were elutriated via PBS twice. 0,5% PBS-T was prepared as 10 ml of PBS is mixed with Triton-X (0,5% PBS-T) then 1 ml of 0,5% PBS-T was added per well and incubation was performed at RT for 6 minutes to permeabilize the membrane.

6-Cells were elutriated via PBS twice and primary antibody solution mixture was applied on surface completely cover the slides. Primary antibody mix was prepared as 1% BSA, 0,3% Triton-X, 2.5% 6.6 MM Phalloidin (Alexa Fluor™ 594 Phalloidin, catalogue number A12381) in 1X PBS. Excitation/Emission of phalloidin is 495/518 nm and selectively stains F-actin. Primary antibody solution mixture was applied on each coverslips and the plate is enveloped with aluminium foil and incubation was performed at RT through 45 minutes.

7-The fixed cells were elutriated via PBS three times through 3 minutes at 35 rpm. Then 0,4 µg/ml DAPI was applied on each coverslips and incubation was performed at RT for 45 seconds.

8-DAPI solution was detracted and fixed cells were elutriated via PBS once.

9-Slides were labelled with pencil and one drop of VECTASHIELD HardSet Mounting Medium was added onslides. Coverslips were inverted on mounting medium via a forceps and covered with aluminium foil and incubated at +4°C and then imaged under the upright microscope.

2.16 LOC (Lab-on-a-chip)

In thesis Project extravasation LOCs were used. In the context of TUBITAK 1003 project #115E056 Extravasation LOCs were fabricated by Assoc.Prof. Dr. Devrim Pesen Okvur and her group at Izmir Institute of Technology (IZTECH) and the validation of these LOCs for HCC cells were performed by Prof. Dr. Neşe Atabey and her group at Izmir Biomedicine and Genome Center. Extravasation LOCs were placed in the tissue culture dish and they were exposed to UV for sterilization in the laminar flow cabinet through 1 hour. Extravasation LOC was composed of 3 channels as medium channel, homing channel and circulation channel.

2.17 Coating of Lab-on-a-Chip (LOC)

Then pure Aceton-2% APTES solution was prepared freshly and the solution was loaded from the circulation channel and it should have coat all the channels. The LOCs were holded for 15 minutes in the laminar flow cabinet until all liquid evaporated. The LOCs were elutriated via PBS three times and once with ultra pure dH_2O. 0,0125 mg/ml laminin in 1X universal buffer was prepared and it was loaded to LOCs from circulation channel. The LOCs were placed into Schifferdecker staining jar (catalog number 073.03.001) as the circulation channel down in position at 37 ^oC, 5% CO2, 95% humidified incubator for 1h. Following that the channels were elutriated via 1X universal buffer, ultrapure water three times respectively. The LOCs were placed in the glass petri dishes and incubated at the vacuum desiccator which was used to completely remove water traces and dried the LOCs in a week.

2.18 Loading of Matrigel and HUVEC Cells

Matrigel was kept on the ice at +4 ^oC overnight so as to quite slowly thaw the matrigel the day before. Pipette tips were held at -20^oC the day before. Matrigel was loaded in to LOCs 1:1 ratio mixed with medium. Matrigel concentration was 8 mg/ml. LOCs were placed into Schifferdecker staining jar as homing channel position in down. Incubation of the cells took place at 37 ^oC, 5% CO_2, 95% humidify incubator for thirty minutes resulting in matrigel polymerization. HUVEC (Human umbilical vein endothelial cell) cells were used to simulate the endothelial barrier during extravasation. Centrifugation of the cells was took place at 200xg for five minutes. Supernatant was aspirated and cells were dispersed in the 8% Dextran in EGM medium as 3.8 million cells/ml. HUVEC cells were loaded into circulation channel and LOCs

were placed into Schifferdecker staining jar as medium channel down in position. Incubation of LOCs was performed at 37 ^{o}C, 5% CO_2, 95% humidify incubator overnight. LOCs were imaged under the confocal microscope whether the HUVEC cells coated the post surface or not. The image was taken as Z-stack so as to verify the 3D structure.

2.19 Loading of SNU-398 CMV GFP (MOCK) and SNU-398 MET GFP Overexpressed Cells

SNU-398 CMV GFP (Mock) and SNU-398 MET GFP Overexpressed cells were applied 0.5 dyn/cm^2 FSS for 4 hours which was described at 2.7. Then, collected cells from bubble traps were counted and they were stained with *CellTracker™ Green*.SNU-398 CMV GFP (Mock) and SNU-398 MET GFP Overexpressed cells had green color. Therefore, HUVEC cells were loaded into circulation channel without any staining. SNU-398 CMV GFP (Mock) and SNU-398 MET GFP Overexpressed Cells' colour was not so intense therefore they were stained with 5µM Green Cell Tracker *(CellTracker™ Green CMFDA Dye, catalog number C2925, Thermo Fisher Scientific*)in serum-free medium in the 6 cm tissue culture dish for 45 minutes at 37^{o}C, 5% CO_2, 95% humidified incubator. Then the dye was inactivated by adding complete RPMI. Centrifugation of cells was took place at 1500xrpm for five minutes. Supernatant was aspirated , 100,000 cells/ml SNU-398 CMV GFP (Mock) and SNU-398 MET GFP Overexpressed cells were prepared. Prepared cells suspension was loaded into circulation channel and day0 image was taken via confocal and fluorescence microscope as tile scan and 2D images. LOCs were incubated for 7 days as medium channel down Schifferdecker staining jar and the images were taken at day2,day5,day7 so as to view extravasation of SNU-398 cells to Matrigel in the form of tile scan and 2D images. The extravasated posts were determined at the end of 4th and 7th day and extravasation and extravasated post numbers and extravasated cell numbers of SNU-398 CMV GFP (Mock) and SNU-398 MET GFP Overexpressed cells were analyzed via Microsoft Excel and Graphpad Prism.

3 RESULTS

3.1 Determination of cell viability and proliferation of HCC cell lines under 0.5 dyn/cm^2 fluidic shear stress

To determine the effects of FSS on cellular behaviour on HCC cell lines three different cell lines has been chosen according to their aggressive phenotype and c-Met expression and activation levels. SNU-449 cell line which has defined as more aggressive phenotype, more mesenchymal and poor-differentiated phenotype and higher c-Met expression and constitutive activation while HuH-7 and HepG2 has mild aggressiveness, more epithelial-like and well-differentiated phenotype and lower and medium levels of c-Met expressions respectively. HuH-7 cell line does not show c-Met activation without c-Met ligand HGF induction. MTT cell proliferation assay and cell counting via trypan blue before and after 1,2,4 and 8 hours of FSS shows that SNU-449 cell line reflects higher cell proliferation and viability generally under FSS for longer time than other two cell lines (Figure 3.1. A,B).

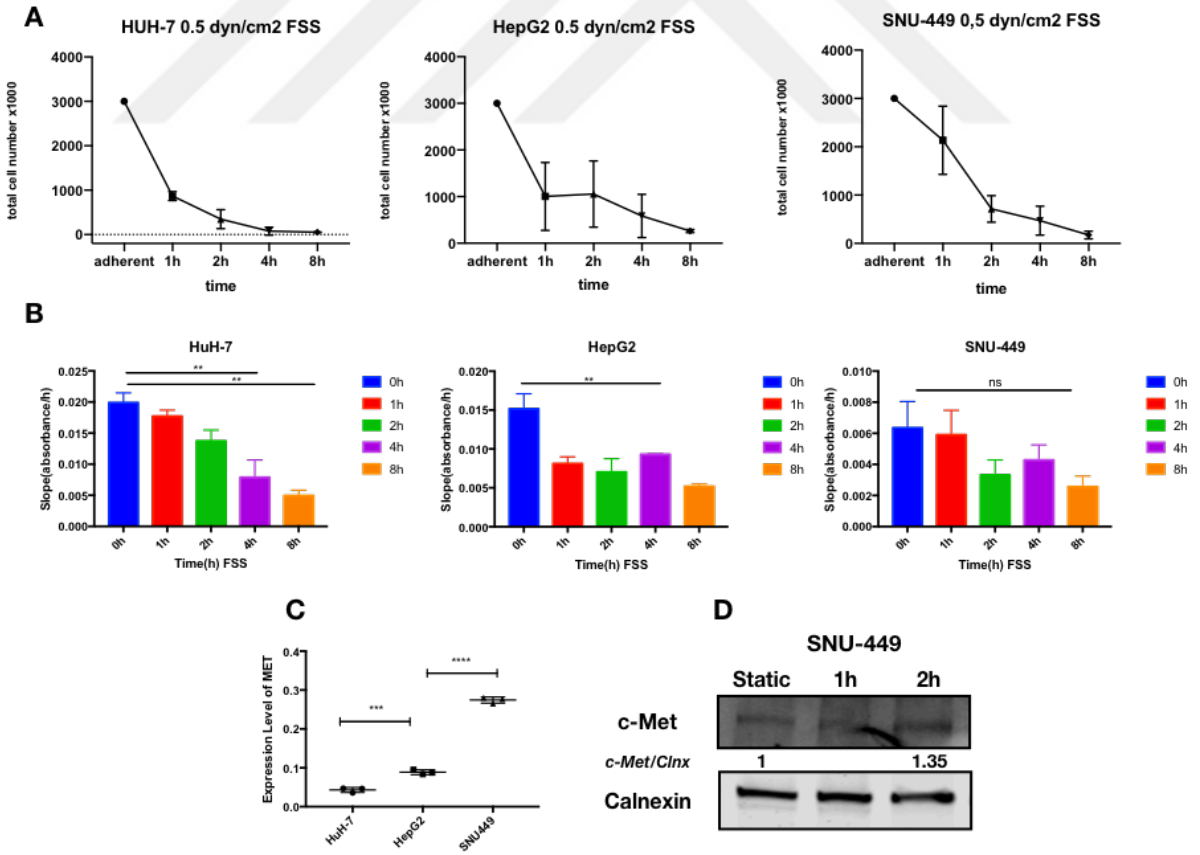

Figure 3.1. *A*- Cell viability of HCC cell lines HepG2, SNU-449 and HuH-7 Cell proliferation via MTT analysis, B- Cell proliferation via MTT analysis, C-Basal (Static) condition MET gene

expression levels of HepG2, SNU-449 and HuH-7 cell line via RT-qPCR, D-c-Met protein expression level after 1 and 2 hours of 0.5 dyne/cm2 FSS via Western Blot technique.

Basal MET mRNA levels of three cell lines has been demonstrated via RT-qPCR analysis (Figure 3.1. C). After one and two hours of FSS application c-Met protein levels were increased in time dependent manner in SNU-449 cell line (Figure 3.1. D).

3.2 Determination the effects of HGF and SU11274 on cell viability and proliferation of HCC cell line HuH-7 under 0.5 dyn/cm^2 fluidic shear stress

To determine the impact of c-Met activation and inhibition on cell proliferation and viability after FSS application we applied HGF (10ng/ml) as a ligand of c-Met receptor tyrosine kinase and SU11274 as a small molecule inhibitor of c-Met receptor to HuH-7 cell line. Results indicate that HGF induction slightly increases the cell proliferation and viability after 1 hour of 0.5 dyn/cm^2 FSS while SU11274 has decreasing effect on cell proliferation but has no significant effects on cell viability comparing to 5% FBS starvation condition (Figure 3.2.)

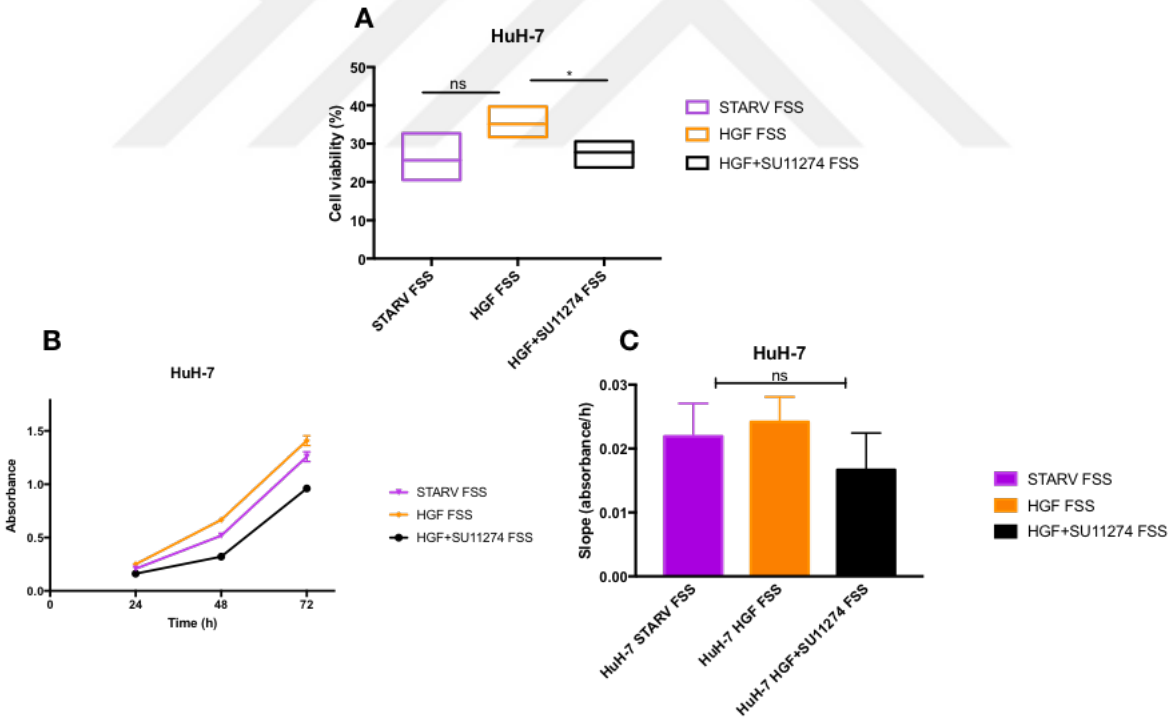

Figure 3.2. ***A***- Cell viability, B- Cell proliferation via MTT analysis through 72 hours, C- Linear regression analysis of cell proliferation via MTT of HuH-7 cell line applied HGF and SU11274 under 1h of 0.5 dyne/cm2 FSS.

3.3 Determination the effects of SU11274 on cell viability, proliferation and c-Met level of HCC cell line SNU-449 under 0.5 dyn/cm^2 fluidic shear stress

To establish the impact of fluidic shear stress on cell viability, proliferation and c-Met expression level analysis on mRNA and protein levels we performed 0.5 dyne/cm^2 FSS for 4 hours. Cell viability of SU11274 applied on SNU-449 cells which has constitutively active c-Met activation were significantly decreased under FSS. On the other hand while cell proliferation rate was slightly decreased in static SU11274 condition, FSS and SU11274 applied SNU-449 cells showed slightly increased cell proliferation rate (Figure 3.3)

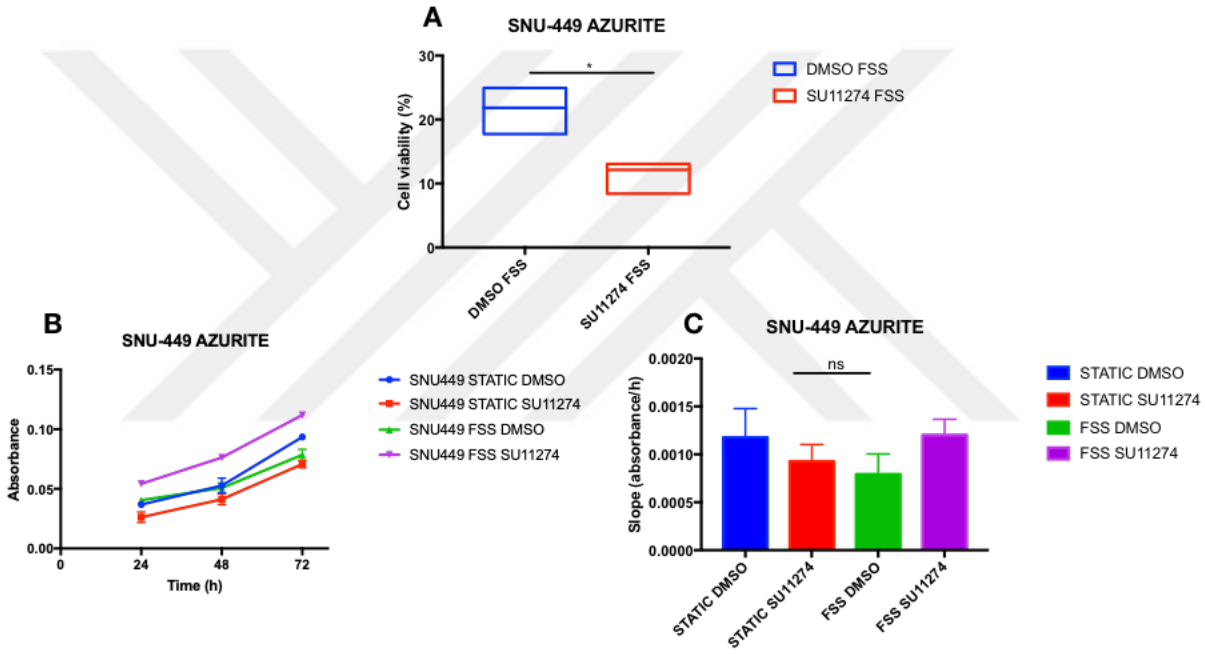

Figure 3.3. ***A***- Cell viability, B- Cell proliferation via MTT analysis through 72 hours, C- Linear regression analysis of cell proliferation via MTT of SNU-449 cell line applied SU11274 under 4h of 0.5 dyne/cm2 FSS.

RNA isolation was performed from the adherent and FSS applied samples. RT-qPCR results indicate that MET gene expression is increased due to FSS in both DMSO and SU11274 conditions comparing to static conditions (Figure 3.4.A) . c-Met protein level were decreased under static SU11274 condition while it was increased under 0.5 dyn/cm^2 FSS both in DMSO and SU11274 conditions (Figure 3.4. B)

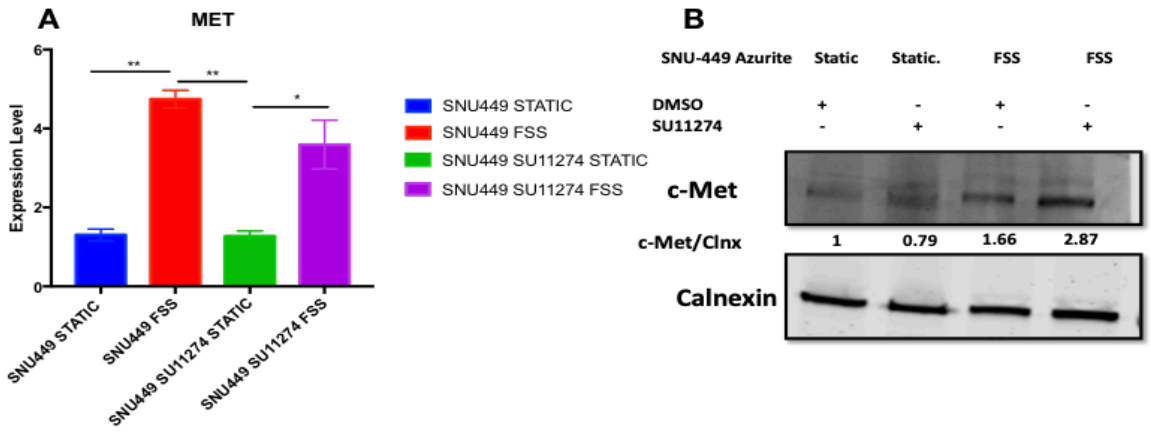

Figure 3.4. : MET gene expression and c-Met protein levels of SU11274 and FSS applied SNU-449 cell line via RT-qPCR and WB respectively.

3.4 The effects of c-Met overexpression on cell viability and proliferation under 0.5 dyn/cm^2 fluidic shear stress

To understand the role of c-Met on cellular behaviour of HCC cell lines under FSS we obtained c-Met overexpressed and MOCK SNU-398 cell lines (Figure 3.5. A-B). Cell proliferation and viability analysis under 1 and 4 hours of 0.5 dyne/cm^2 FSS application showed that c-Met overexpressed clones show higher proliferation and cell viability behaviours comparing to MOCK cell clones under FSS for one and four hours (Figure 3.5. C-E)

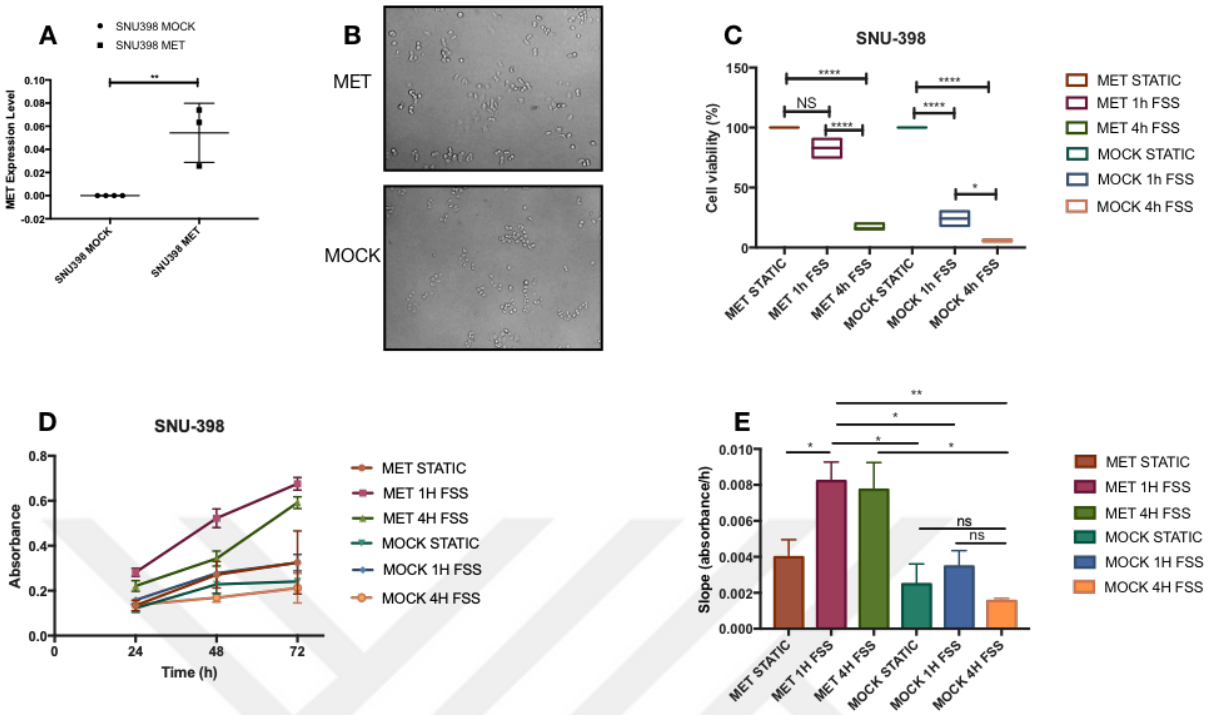

Figure 3.5. A-MET expression levels via qPCR analysis of SNU-398 MET and MOCK clones, B- Pictures of SNU-398 MET and MOCK clones under light microscopy C- Cell viability analysis, D- Cell proliferation via MTT analysis through 72 hours, E- Linear regression analysis of cell proliferation via MTT of SNU-398 overexpressed MET and MOCK cells under 0.5 dyn/cm^2 FSS.

To understand whether c-Met inhibitor SU11274 reverses these affects we applied SU11274 under 0.5 dyn/cm2 FSS to SNU395-MET and MOCK cells. Cell viability analysis showed that SU11274 applied MOCK and MET cells survived less than DMSO conditions under FSS (Figure 3.6. A).

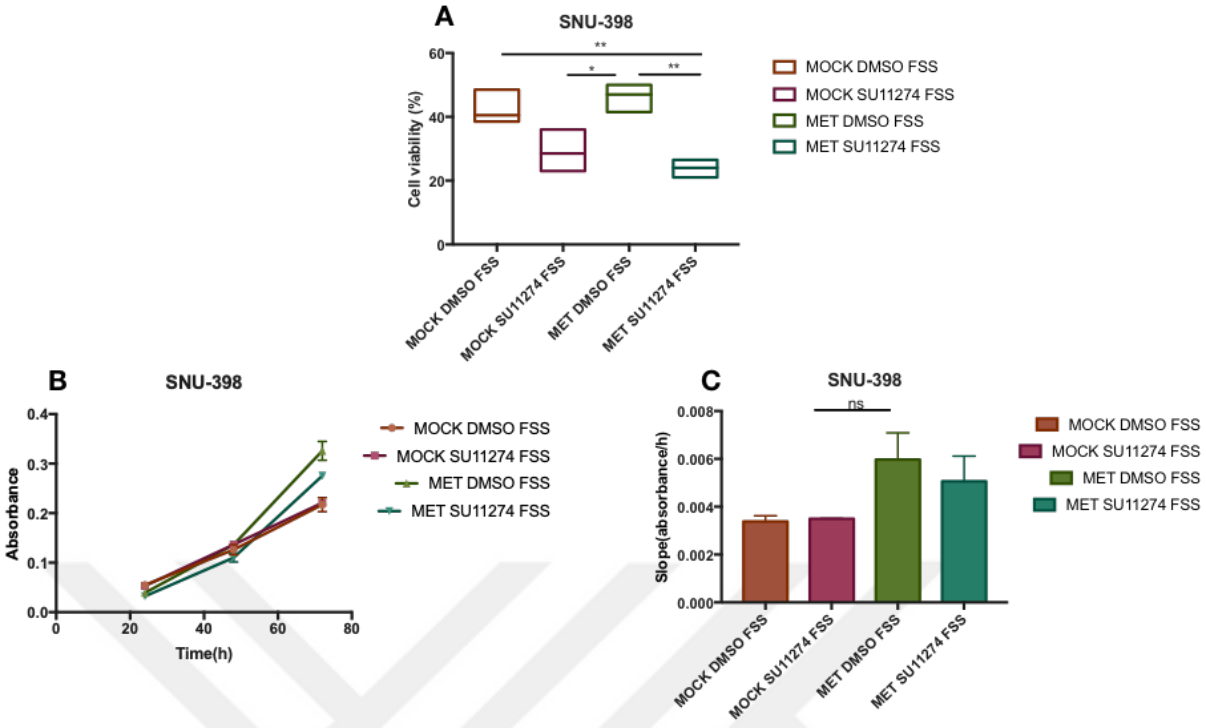

Figure 3.6. A-Cell viability analysis, B- Cell proliferation via MTT analysis through 72 hours, C- Linear regression analysis of cell proliferation via MTT of SNU-398 MET and MOCK cell lines applied SU11274 under 4h of 0.5 dyne/cm2 FSS.

On the other hand SU11274 had no significant effects on cell proliferation on SNU-398 MOCK cells although it has slight decreasing effect on cell proliferation of SNU-398 MET overexpressed cells under FSS comparing to DMSO condition (Figure 3.6. B-C).

3.5 The effects of 0.5 dyn/cm^2 fluidic shear stress on mRNA expression and protein levels of Caveolin-1

c-Met expression is elevated in mRNA and protein levels due to FSS in HCC cell lines. To understand the effect of c-Met under FSS we demonstrated Caveolin-1 which is reciprocal activator crosstalk molecule of c-Met, protein level has been increased under FSS for one and two hours in time dependent manner in SNU-449 cells (Figure 3.7. A). SU11274 application on static SNU-449 cells decreased the protein levels of Cav1 Four hours FSS alongside SU11274 treatment showed that effects of SU11274 slightly reversed under fluidic shear stress on protein levels of Cav1 in SNU-449 Azurite cell line (Figure 3.7. B). The mRNA expression level of CAV1 is also increased under four hours of FSS and SU11274 application has slightly decreased the CAV1 expression (Figure 3.7.C)

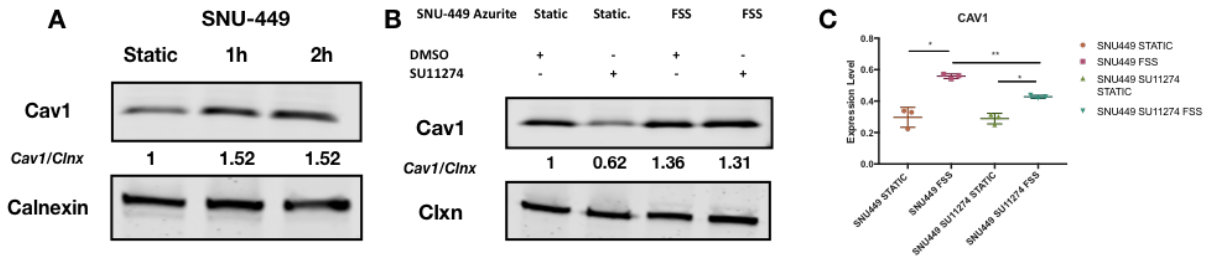

Figure 3.7. Cav1 protein and mRNA levels of FSS and SU11274 applied SNU-449 cell line via WB and RT-qPCR respectively.

3.6 The effects of Caveolin-1 overexpression on cell viability and proliferation under 0.5 dyn/cm^2 fluidic shear stress

Caveolin-1 is increased during FSS application. To understand the impact of Cav-1 on cell viability and proliferation under FSS, four hours of 0.5 dyne/cm2 FSS application was performed with HuH-7 Caveolin overexpressed (Huh-7 pCAV1) and MOCK cell lines. In basal conditions Cav-1 mRNA levels of HuH-7 clones were demonstrated by RT-qPCR in advance of FSS experiments (Figure 3.8. A). Pictures of HuH-7 clones taken by light microscopy as shown in (Figure 3.8. B.). Following the 0.5 dyne/cm2 FSS FSS application HuH-7 pCAV1 and MOCK cell viability were determined by trypan blue cell counting technique shows that HuH-7 pCAV1 cell clones slightly but not significantly survived more than HuH-7 MOCK cell clones (Figure 3.8. C). Proliferation level of HuH-7 MOCK cell line has been slightly but not significantly increased after FSS application and HuH-7 pCAV1 cell line's proliferation level is decreased slightly but not significantly again (Figure 3.8. E).

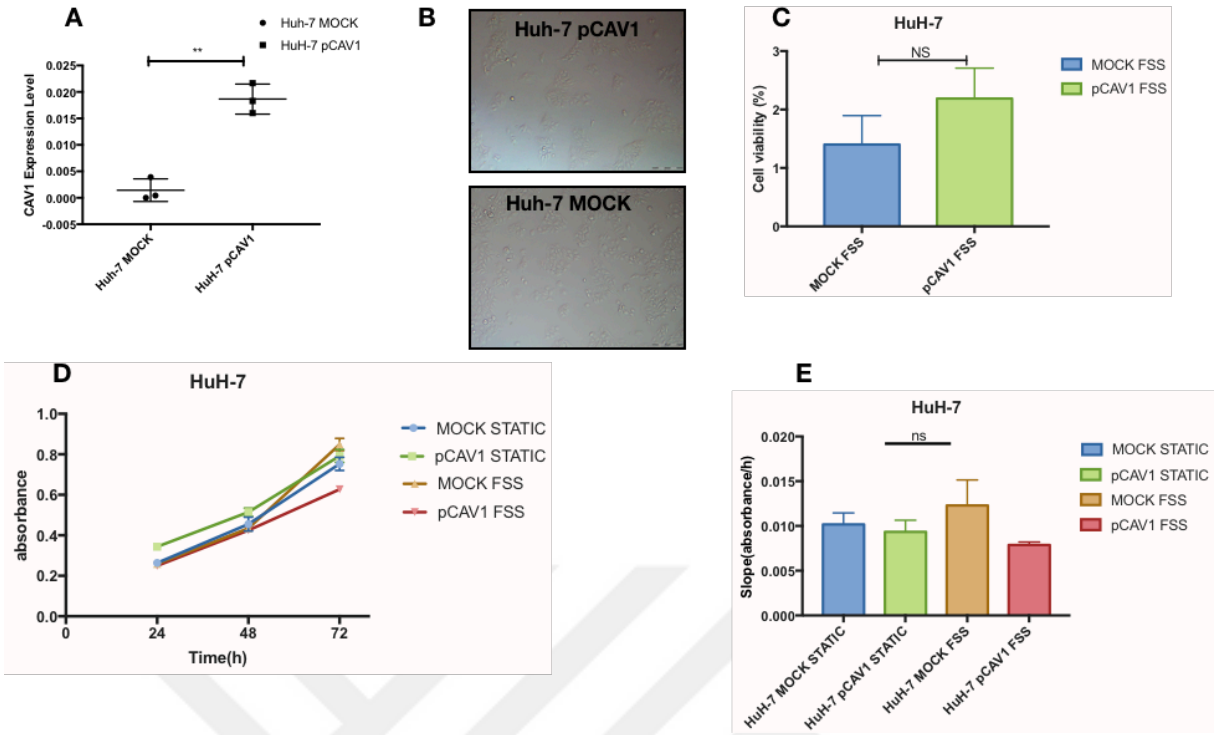

Figure 3.8. A-CAV1 expression levels via qPCR analysis of HuH-7 pCAV1 and MOCK clones, B- Pictures of HuH-7 pCAV1 and MOCK clones under light microscopy C- Cell viability analysis, D- Cell proliferation via MTT analysis through 72 hours, E- Linear regression analysis of cell proliferation via MTT of of HuH-7 CAV1 overexpressed and MOCK cells under 4h of 0.5 dyn/cm^2 FSS.

To understand the effects of c-Met inhibitor SU11274 on Caveolin-1 overexpressed cells, 0.5 dyn/cm^2 FSS experiment were performed. Due to lack of enough survived HuH-7 cells under four hours of 0.5 dyn/cm^2 FSS this experiment was performed for one hour of FSS. The results showed that SU11274 does not show reverse effects on cell viability of MOCK and CAV1 cells under FSS in addition it has slight increasing effects of MOCK cell clones (Figure 3.9. A). Similar effects of SU11274 on cell viability has been demonstrated in proliferation rates of HuH-7 MOCK and CAV1 cell lines (Figure 3.9. B-C).

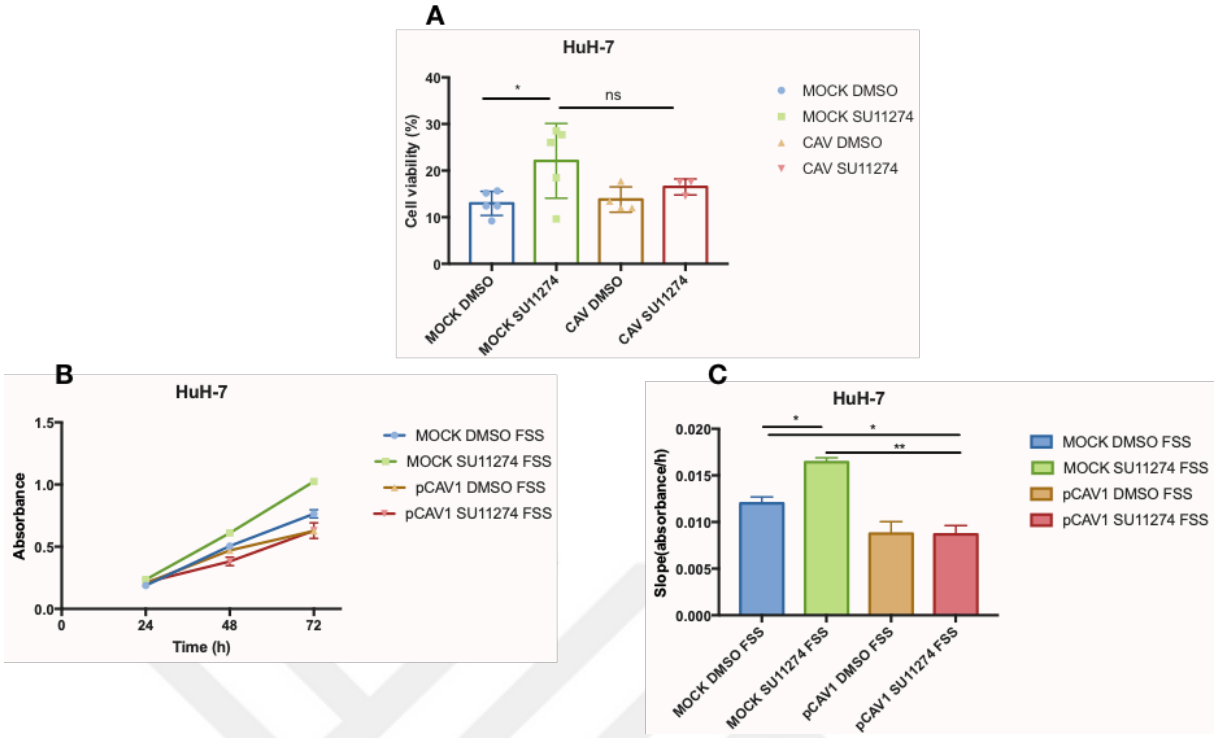

Figure 3.9. A-Cell viability analysis, B- Cell proliferation via MTT analysis through 72 hours, C- Linear regression analysis of cell proliferation via MTT of SNU-398 MET and MOCK cell lines applied SU11274 under 1h of 0.5 dyne/cm2 FSS.

3.7 The effects of c-Met overexpression on cell cytoskeleton structural alterations of SNU-398 cells under 0.5 dyn/cm^2 fluidic shear stress

F-actin cytoskeleton structures shown via Phalloidin dye (red) under the confocal microscopy after 1-4h of 0.5 dyne/cm2 FSS indicates that the time of fluidic shear stress increases the stress fibrils of the cells. On the other hand MET overexpression increases stress fibrils level of the cells in the static and FSS conditions as well.

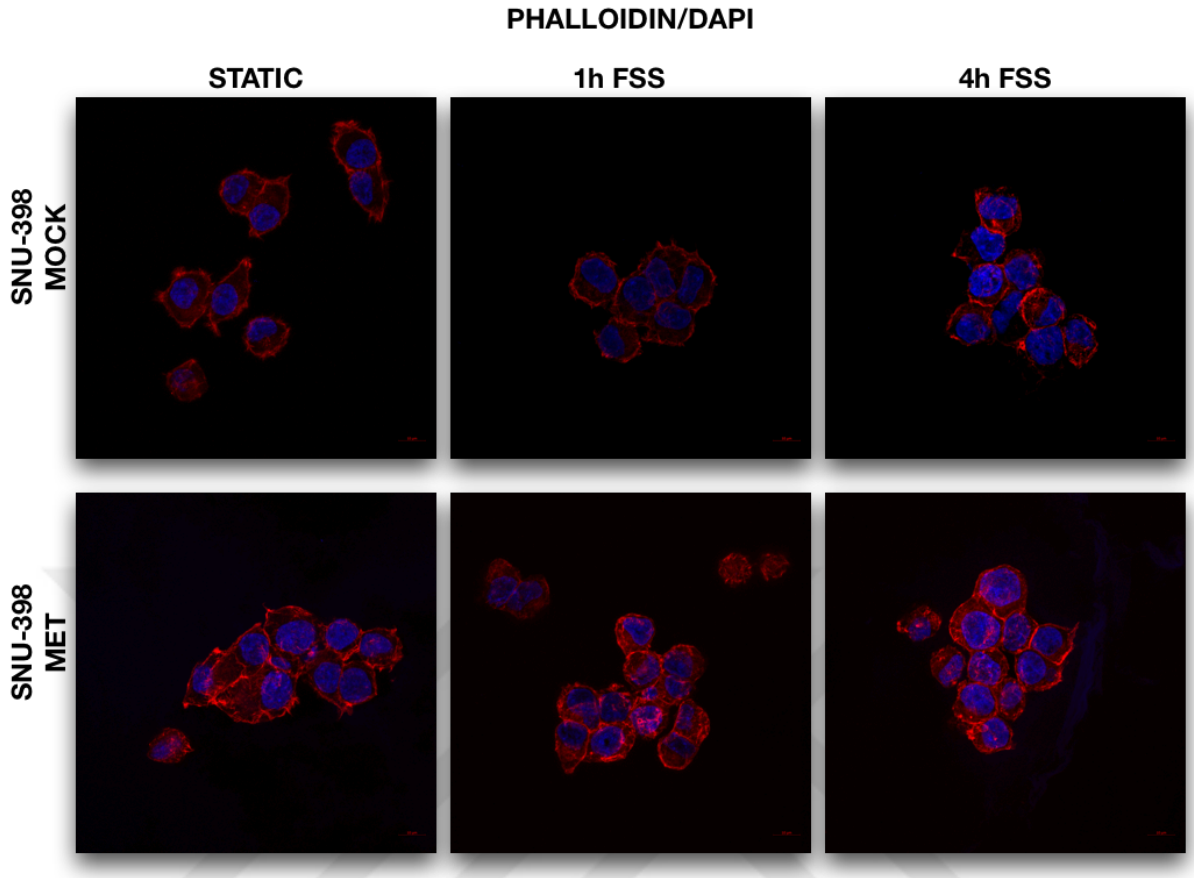

Figure 3.10. Phalloidin dye of F-actin cytoskeleton structures of SNU-398 MOCK and MET cells after 1-4h of 0.5 dyne/cm2 FSS. (Red:Phalloidin, Blue:DAPI)

3.8 The effects of c-Met overexpression on extravasation, migration and colonization of SNU-398 cells under 0.5 dyn/cm^2 fluidic shear stress

To understand the effects of c-Met overexpression on extravasation, migration and colonization of SNU-398 cells under 0.5 dyn/cm^2 fluidic shear stress we performed a Lab-on-a-Chip (LOC) experiment. Intact HUVEC (Human Umbilical Vein Endothelial Cells) dyed with green cell tracker surfacing LOC's post were analyzed via confocal microscopy through seven days (Figure 3.10. A-C). SNU-398 MET and MOCK cells following four hours of 0.5 dyn/cm^2 fluidic shear stress dyed with green cell tracker loaded onto LOCs and 4th day pictures of extravasated were shown in (Figure 3.10. A). The post numbers that SNU-398 MOCK and MET cells were extravasated indicates that MET overexpressed cells extravasated into 7 posts while MOCK cells extravasated into 4 posts after FSS application (Figure 3.10. C). Further analysis showed that extravasated MET cell number is 28 while MOCK extravasated cell number is 16 after FSS application (Figure 3.10. D).

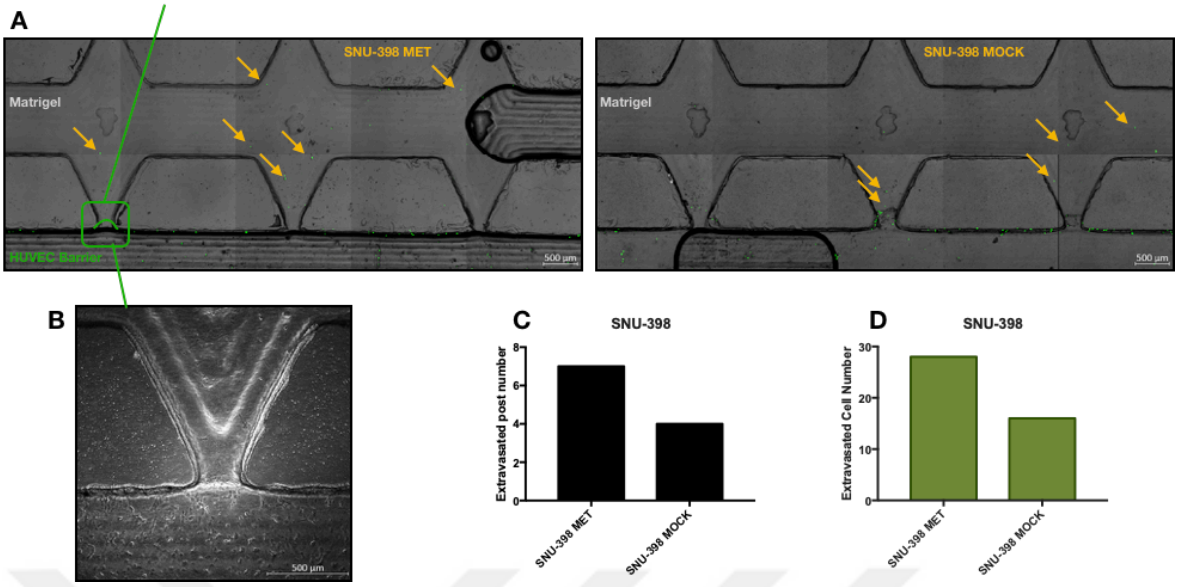

Figure 3.11. Lab-on-a-Chip (LOC) analysis of SNU-398 MET and MOCK cell clones. A- HUVEC cell barrier confocal microscopy images and LOC images, B- HUVEC cell barrier LOC post image, C- Extravasation post number analysis, D- Extravasation cell number analysis of SNU-398 MET and MOCK cell lines.

4 DISCUSSION

Tumour metastasis is the spearheading reason of cancer associated mortality and lately hepatocellular carcinoma has been arisen the second leading reason of cancer associated mortality worldwide (Sun, Luo, Liu, & Song, 2018). HCC has been characterised with high malignancy, fast progression, poor curative effect and short survival time. Intrahepatic and extrahepatic metastasis plays crucial role in bad prognosis. In early stages HCC treatments vary from local ablation and resection to liver transplantation. Although after transplantation and resection intrahepatic metastasis is one of the major bad prognosis factor leading to bad prognosis of HCC patients (K. Wang et al., 2019). During the cancer development tumour migration through blood circulation from primary to secondary tumour site to from new tumour colonies is amongst the vital steps of metastasis process. Throughout this journey cancer cells are applied to blood flow forces such as fluidic shear stress. This tumour fluidic microenvironment particularly the fluidic shear stress plays a pivotal role in tumour progression and metastasis. Therefore more advanced studies on the role of FSS to circulating tumour cells and the mechano-transduction signalling pathways needs to be done to understand tumorigenesis, metastasis and develop nouveau strategies and emergent targets for tumor treatment (Huang et al., 2018). Fluidic shear stress has been shown to be involved in drug resistance in few studies. These research strongly suggest that tumour cells develop molecular mechanisms that is induced by fluidic shear stress to survive apoptosis caused by antitumor drugs such as ABCG2 and P-gp activation to develop drug resistance under shear stress (Ip et al., 2016). Understanding the molecular mechanisms through fluidic shear stress provide the insight on tumour biology to overcome drug resistance, metastasis and recurrence and translate these research for more effective treatments.

c-Met receptor has been widely studied and been strongly shown in various cancer types inducing motility, extravasation, proliferation and survival of cancer cells leading to bad prognosis of cancer patients. It is one of the pivotal deregulated molecular signalling pathway in HCC and it has been recently considered as a bad prognostic marker in clinic-pathological studies. There are few research on the effects of fluidic shear stress on HCC metastasis through cellular responsive behaviours. One of them shows fluidic shear stress induces autophagy which is a mechanism provides cancer cells high level of migration and invasion capacity (X. Wang et al., 2018). One of few studies shows that low-level shear stress induces migration of liver cancer stem cells via ERK1/2 signalling which is c-Met downstream molecule (Sun et al.,

2018). Another study indicates that fluidic shear stress regulates HCC cell line HepG2 cell migration through integrin signalling cascade (Yu et al., 2018). There are studies in various cancer models like breast cancer cell mechano-sensor regulation via Caveolin-1 molecule under fluidic shear stress (Yang et al., 2016). Since previous studies showed that integrin and c-Met signalling molecule cooperation provides cell survival (Barrow-McGee et al., 2016)and reciprocal activating crosstalk between c-Met and Caveolin-1 fosters invasive phenotype in HCC (Korhan et al., 2014) published by our lab, these regulatory mechano-sensing molecules and their survival role in FSS alongside their regulatory crosstalk with c-Met molecule thereby c-Met molecule, its function in HCC metastasis brings the idea that c-Met can be highly possible protective mechano-transduction molecule under fluidic shear stress. Although there are a great number studies on c-Met signalling pathway and its essential role in HCC metastasis and fluidic shear stress is one of the metastasis limiting rate during metastasis process there has been no research on the effects of c-Met signalling regulation in circulating tumour cells which exposed to fluidic shear stress. Our study on the effects of c-Met regulation in HCC cells under fluidic shear stress provides an insight of HCC metastasis and c-Met signalling perspective.

Since our study focuses on effects of c-Met signalling cascade on cellular behaviour of HCC cell lines under fluidic shear stress mimicking liver sinusoidal vein blood shear stress (0.5 dyn/cm^2) the results of the experiment were performed with three HCC cell lines which has been chosen according to their c-Met expression and activation demonstrate that the HCC cells which have higher c-Met gene and protein levels and activation levels (SNU-449) survived and proliferate in higher levels than the HCC cells which doesn't have (HuH-7 and HepG2).

Fluidic shear stress experiments that were performed with HGF induction through c-Met pathway activation indicate that c-Met activation can be the survival factor and provide HCC cell lines from apoptosis and anoikis under shear stress. c-Met constitutively active SNU-449 cells were applied SU11274 c-Met activation inhibitor under FSS. The results demonstrates that c-Met inhibition decreases the cell viability and proliferation under sinusoidal fluidic shear stress. Furthermore c-Met expression in mRNA and protein levels are increased in SNU-449 cells after 1-2 and 4 hours of FSS exposure showing that c-Met can be the survival factor that protects surviving cells from apoptosis due to FSS.

To obtain deeper understanding of c-Met regulation under FSS we sheared SNU-398 MET overexpressed cell line. Results support that MET overexpressed HCC cell lines might have higher survival and proliferation rates comparing to MOCK cells which has no c-Met

expression. Furthermore data shows that fluidic shear stress even increases the proliferation rates of MET overexpressed cells under shear stress comparing to static condition. Data from the experiments which performed with SU11274 to reverse these effects of c-Met promoted survival and proliferation shows that protective effects of c-Met from cell death and promotion of increased proliferation rate can be reversed.

Previous studies indicate that cav1 can regulate the cellular responses on cell membrane and may act as a mechano-sensor under shear stress. Our previous studies show that there are reciprocal interactions between c-Met and Cav1 through cell signalling To have deeper understanding how c-Met regulates these cellular behaviour in molecular level we hypothesized Caveolin-1 can be the cooperative molecule alongside of c-Met signalling pathway. To demonstrate if HCC cells under fluidic shear stress has higher Cav-1 expression levels we performed FSS experiments and showed that Cav-1 is increased through FSS in time dependent manner and this increasing pattern can be reversed by SU11274 c-Met inhibitor.

To advance the understanding on the role of Cav-1 in HCC cells under FSS we used HuH-7 Cav-1 overexpressed clones. Under fluidic shear stress HuH-7 pCav-1 cells did show minor but not significant alterations in terms of cell viability and proliferation. The experiments were performed with c-Met inhibitor SU11274 and FSS showed the similar results. Although it was demonstrated that Cav-1 can be a vital molecule in the response of fluidic shear stress in breast cancer cells and our experiments showed that HCC cell line SNU-449 cells Cav-1 expression levels are increased during FSS we could not see any pivotal effect of Cav-1 on cell survival and proliferation in HuH-7 MOCK and Cav-1 overexpressed cell clones.

To demonstrate the biological responses of SNU-398 MET overexpressed cells to fluidic shear stress we performed microfluidic 3D device to mimic blood circulation and endothelial barrier to understand the extravasation capacity of MET overexpressed cells. In the metastasis process the capacity of survival under FSS, extravasate to secondary tissue and colonization in the target tissue are crucial steps for cancer metastasis. LOC experiments showed that SNU-398 MET clones survived in higher rate under FSS than MOCK cell clones, extravasate in to 9 post while MOCK cell clones extravasate in to 4 posts, and colonization capacities showed that MET cell clones colonized in the post as a number of 28 while MOCK cell number is 16.

5 CONCLUSION

In the light of our data we have established that HGF/c-Met pathway plays crucial role on cellular behaviours such as cell survival, proliferation, cytoskeleton reorganization, extravasation and aggressive phenotype in HCC cell lines under 0.5dyn/cm^2 fluidic shear stress. The hypothesis was supported by several experiments in 5 different cell lines and MET overexpressed cell clones. MET clones showed significance difference comparing to MOCK cell clones under fluidic shear stress. Supporting that endogenous c-Met expression level HCC cell lines showed the similar pattern on cellular behaviours under FSS. Caveolin-1 seems to be a candidate mechanism of cellular behaviour response to FSS alongside c-Met signalling pathway which needs further research. 3D microfluidic study expanded our understanding on the cellular responses and aggressive phenotype in terms of cell survival, extravasation and proliferation capacity after FSS application in HCC. Besides fluidic shear stress application system peristatic pump can be used for further metastasis research.

6 PERSPECTIVES

Further analysis to enlighten the molecular mechanism of the role c-Met signalling under FSS are needed. To understand whether c-Met is the upstream mechano-sensor receptor in shear stress conditions or downstream surviving molecule of another mechano-sensor receptor such as Caveolin-1, integrin family etc. More molecular signal transduction analysis, protein conformational regulation studies and in-vivo metastasis model experiments such as zebrafish, mice models need to be considered for advanced understanding of the mechanism of c-Met regulation through fluidic shear stress and metastasis process.

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8 APPENDIX

8.1 CIRRICULUM VITAE (CV)

Contact Information

Address	6756 Sokak Yüksek Vadi Evleri B-101 Daire: 9 Karsiyaka/Izmir
Phone	(0090) 555 723 9592
E-mail address	dehancomez1@gmail.com

Personal information

Date of Birth/ Place	02.01.1992/ Izmir, Turkey
Nationality	Turkish

Languages

Turkish (Native speaker)
English (IELTS: 6.5/9)
Italian (Beginner)

Education and Training

2016-2019	M.Sc. in Molecular Biology and Genetics (GPA: 3.50/4.00) Dokuz Eylül University, Izmir International Biomedicine and Genome Institute
2014-2016	M.Sc. in Molecular Life Science (Incompleted) Friedrich Schiller University, Jena, Germany

2011- 2014	B.Sc. in Molecular Biology and Genetics (GPA: 3.47/4.00) Department of Molecular Biology and Genetics, Istanbul University, Istanbul, Turkey
2010-2011	BSc in Biology (GPA:3.67/4.00) Department of Biology, Istanbul University, Istanbul, Turkey
2006-2010	Izmir Karsiyaka Atakent Anatolian High School (GPA:78/100)

Research Experience

April 2018- July 2019	Izmir Biomedicine and Genome Center Cancer Biology and Signalling Lab, Associate Researcher Izmir, Turkey Supervisor: Prof. Dr. Nese Atabey
2016-2019	Potential Regulatory Effects of Fluidic Shear Stress Through c-Met Signalling on Biological Behaviours of Hepatocellular Carcinoma Cells (MSc Thesis) Supervisor: Prof. Dr. Nese Atabey, Izmir Biomedicine and Research Institute
2016-2018	Project: Molecular Mechanism of High Glucose Induced Hepatocellular Carcinoma Progression: c-Met Activation and New Players Thioredoxin Interacting Protein, Sestrin2 and lncRNA HOTAIR. Dokuz Eylul University Supported by: The Scientific and Technological Research Council of Turkey (TUBITAK) Responsibilities: Performing all experiments of the project.
2016-2019	Project: A Novel Lab-on-a-Chip Device for Early Diagnosis of Metastasis Dokuz Eylul University

	Supported by: The Scientific and Technological Research Council of Turkey (TUBITAK) Responsibilities: Performing flow circulation and HCC/breast cancer CTC experiments.
2016-2019	Investigation of the Relationship of c-Met Signalization and lncRNA HOTAIR in Epithelial-to-Mesenchymal Transition Process of Hepatocellular Carcinoma Dokuz Eylul University Supported by Scientific Research Project Committee of Dokuz Eylul University
January 2015-June 2015	Leibniz Institute on Aging- Fritz Lipmann Institute K. Lenhard Rudolph Lab- HiWi (Student Assistant) Jena, Germany
March 2014- June 2014	Graduation Project- Funded by TUBITAK (The Scientific and Technological Research Council of Turkey) Department of Molecular Biology and Genetics Istanbul University, Istanbul ,Turkey Supervisor: Ass. Prof. Semian Karaer Uzuner
October 2013 – March 2014	Erasmus Student (Lab work) Department of the Biotechnology and Bioscience, The University of Milano-Bicocca, Italy Supervisor : Ass. Prof. Andrea Becchetti
July- September 2013	Internship :The Wellcome Trust Centre for Cell Biology, Adrian Bird's Lab The University of Edinburgh, Edinburgh, UK
January- March 2013	Internship: The Institute for Ageing and Health, Gabriel Saretzki's Lab The University of Newcastle, Newcastle, UK
March- July 2012	Internship :Department of Neuroscience, Experimental Medicine Research Center Istanbul University, Istanbul, Turkey

Organisation of Conferences, Meetings and Workshops

4 May 2018	Bridging Biobanks in MENA Countries to Promote Research and Healthcare, Izmir Biomedicine and Research Center, İzmir, Turkey Funding: BBMRI-ERIC-ADOPT, Horizon 2020
2-3 May 2018	International Workshop on Biobanking for Rare Diseases Izmir Biomedicine and Research Center, İzmir, Turkey Funding: BBMRI-ERIC-ADOPT, Horizon 2020
7-9 March 2014	Istanbul University, IUGEN Molecular Biology and Genetics Winter School XI.- Istanbul University, Istanbul, Turkey
8-9-10 March 2013	Istanbul University, IUGEN Molecular Biology and Genetics Winter School X-,Istanbul University, Istanbul, Turkey
13 May 2012	1.International Students' Stem Cell Congress of Turkey Faculty of Medicine, Istanbul University, Turkey
24-26 February 2012	Istanbul University IUGEN Molecular Biology and Genetics Winter School, Istanbul University, Turkey
24 December 2011	Istanbul University Department of Molecular Biology and Genetics Meeting of Graduates Career Day, Turkey
4-6 March 2011	Istanbul University, IUGEN Molecular Biology and Genetics Winter School VIII, Istanbul, Turkey

Certifications

March 2017	Certificate of Experimental Animal Usage for Researchers Animals Testing Committee of Local Ethics Testing Animal Utilization of Dokuz Eylul University

Poster/Oral Presentations

21-23 February 2019	(Oral) **Comez D**., Bagci G., Topel H., Bagirsakci E., Yılmaz Y., Gunes A., Atabey N. The Effects of c-Met Activation on Survival and Metastatic Capacity of Hepatocellular Carcinoma Cells Under Fluidic Shear Stress

	Bagci G., **Comez D.,** Topel H., Bagirsakci E., Yılmaz Y., Gunes A., Ayaz G.B., Pesen-Okvur D., Atabey N. Investigation of the Role of c-Met Pathway on Organ Specific Metastasis of Hepatocellular Carcinoma Cells via Lab-on-a-Chip 3. Cukurova Hepatocellular Carcinoma Congress,2019, Adana, Turkey
30 June-3 July 2018	Topel H, **Comez D**, Atabey N. HGF/c-met signalling pathway downregulates lncRNA HOTAIR to induce adhesion independent growth in HCC by increasing caveolin-1 expression EACR25: 25th Biennial Congress of the European Association for Cancer Research, 2018,Amsterdam, Netherlands.
30 June-3 July 2018	Topel H, Yilmaz Y, Gunes A, Bagirsakci E, Gizem BA, **Comez D**, Bagci G, Kahraman E,Pesen Okvur D, Atabey N. Predicting homing ability of hepatocellular carcinoma cells by using a lab-on-a-chip system, EACR25: 25th Biennial Congress of the European Association for Cancer Research, 2018, Amsterdam, Netherlands.
23-26 April 2017	Topel H, Bagirsakci E, Gunes A, Kahraman E, Yilmaz Y, **Comez D**, Atabey N. Effects ofc-Met on Glucose Metabolism of Hepatocellular Carcinoma, EMBO Workshop on Metabolic Disorders and Liver Cancer, 2017, Palma de Mallorca, Spain.
10-14 September 2017	Topel H, **Comez D**, Yilmaz Y, Atabey N. Reciprocal Interaction of c-Met Receptor Tyrosine Kinase and lncRNA HOTAIR in Hepatocellular Carcinoma, 42nd FEBS Congress, 2017,Jarusalem, Israel
7-10 September 2017	Oral: Topel H, **Comez D**, Yilmaz Y, Atabey N. Reciprocal Interaction of c-Met Receptor Tyrosine Kinase and lncRNA HOTAIR in Hepatocellular Carcinoma, FEBS Young Scientist Forum, 2017,Jerusalem-Israel.

26-29 October	Topel H, **Comez D**, Yilmaz Y, Atabey N. Hepatoselüler Karsinomda c-Met Reseptör Tirozin Kinaz ve lncRNA HOTAIR İlişkisi, XV. Ulusal Tıbbi Biyoloji ve Genetik Kongresi, 2017, Türkiye

Laboratory Skills

Basic Molecular Biology and Genetics Techniques (WB, qPCR, etc.)
Cell Culture Techniques
Flow (Circulating) and Lab-on-a-chip Applications in Cell Culture
Biological Assays (Proliferation, Motility, Invasion, xCELLigence proliferation/motility/invasion applications etc.)
Cell Viability Assays (MTT,SRB etc)
Cloning Techniques
Confocal and Fluorescence Microscopy Techniques and Imaging
Immunohistochemistry and immunofluorescence
CTC(Circulating Tumor Cells) Isolation and Enrichment