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FUNCTIONAL CHARACTERIZATION OF ADIPOCYTES IN REGULATION OF LIVER CANCER STEM CELLS

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MPhil

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FUNCTIONAL CHARACTERIZATION OF ADIPOCYTES IN REGULATION OF LIVER CANCER STEM CELLS

Shilpa Gurung

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Philosophy
August 2018

CERTIFICATE OF ORIGINALITY

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Shilpa Gurung (Name of student)

ABSTRACT

Functional characterization of adipocytes in regulation of liver cancer stem cells

Submitted by

Shilpa Gurung

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at The Hong Kong Polytechnic University

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Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world. An evident increase in HCC incidence is observed in both developing and developed countries as the consequence of rise in obesity, which is considered to be one of the major risk factors for development of non-alcoholic fatty liver disease (NAFLD). Given cancer stem cells (CSCs) are the root of tumor development, we hypothesize that adipocytes, one of the key cellular factors within the tumor microenvironment, may play a critical role in HCC pathogenesis via regulation of liver CSCs.

Various reports on the interaction between adipocytes, abundant cellular factor in obese tumor microenvironment and cancer has been reported. However, the role of adipocytes in the regulation of liver CSCs has not been studied. In this study, we aim to dissect the crosstalk between adipocytes and HCC in regulation of liver CSC properties. For this purpose, we employed human visceral preadipocytes and differentiated it into functional adipocytes. Upon confirmation of the functionality of adipocytes, we examined the potential CSC enhancing effect of adipocytes on HCC cells in a co-culture system in which adipocytes and HCC cells were physically separated. Upon functional analysis, we first found that adipocytes enhanced self-renewal and tumorigenicity properties of HCC cells via paracrine secretion. Concurrently, condition medium (CM) of adipocytes enhanced liver CSC

properties including self-renewal, tumorigenicity, migration, invasion, drug resistance and expression of liver CSC markers. Our results confirmed interaction between adipocytes and HCC aided in amelioration of liver CSC, and such CSC enhancing effect was further augmented by reciprocal interaction between adipocytes and HCC.

To better understand the mechanistic mode of communication between adipocytes and HCC, we analyzed the adipocyte CM by orbitrap liquid mass spectrometry and identified fatty acid binding protein 4 (FABP4) to be critical player in this communication. Quantification of secreted FABP4 by enzyme-linked immunosorbent assay (ELISA) confirmed preferential secretion of FABP4 in adipocytes, while its secretion was further increased (~5-fold) upon stimulation by HCC cells. Recombinant FABP4 (rhFABP4) enhanced CSC properties of HCC cells; while FABP4 inhibitor (BMS309403) abolished the CSC enhancing effect of adipocytes CM. Clinically, we found that overexpression of FABP4 in non-tumor samples was significantly correlated with poor prognosis of HCC patients.

We next deciphered the downstream mechanism by which adipocytes derived FABP4 regulates liver CSC properties. We carried out RNA sequencing and compared the genetic profiles between rhFABP4 treated PLC/PRF/5 cells and control counterparts, followed by pathway analysis using Database for annotation visualization and integrated discovery (DAVID). DAVID highlighted deregulation of genes involved in PI3K-Akt signaling pathway upon administration of rhFABP4. Western blot analysis confirmed the phosphorylation of Akt within 60 minutes of administration of rhFABP4. Further investigation on the downstream pathway of PI3K-Akt signaling identified the activation of wnt/β-catenin signaling pathway by rhFABP4.

In conclusion, our study characterized the functional role of adipocytes on regulation of liver CSCs and elucidated a novel mechanism via FABP4/Akt/wnt-β-catenin signaling pathway. Targeting this signaling cascade may be a potential therapeutic strategy against NAFLD-induced HCC.

An abstract of exactly 499 words

PUBLICATIONS

1. **Gurung S**, Chung KPS, Lee TK. Emerging role of fatty acid binding proteins in cancer pathogenesis. Histol Histopathol 2018 June; 18014.
2. Cheng BY#, Lau EY#, Leung DH#, Ho NP, **Gurung S**, Cheng LK, Lin CH, Lo RC, Ma S, Ng IO, Lee TK**.** IRAK1 augments cancer stemness and drug resistance via the AP-1/AKR1B10 signaling cascade in hepatocellular carcinoma. Cancer Research 2018 May; 78(9):2332-2342.

CONFERENCE PRESENTATIONS

1. **Gurung S**, Lee TK. The pivotal role of adipocytes on regulation of cancer stemness and drug resistance in hepatocellular carcinoma. Annual meeting of American Association for Cancer Research, 14-18 April 2018. (Poster Presentation)
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AWARDS

1. **Miss Shilpa Gurung** (MPhil)-Travel Award, The Liver Week 2018, The Korean Association for the Study of the Liver (KASL), The Liver Week 2018 Annual meeting of The Korean Association for the study of Liver, June 22-24, 2018, Incheon, Korea.
2. **Miss Shilpa Gurung** (MPhil)-Best Poster Oral Presentation Award, The Liver Week 2018, The Korean Association for the Study of the Liver (KASL), The Liver Week 2018 Annual meeting of The Korean Association for the study of Liver, June 22-24, 2018, Incheon, Korea.

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LIST OF ABBREVIATIONS

Ab	Antibody
Akt	Protein kinase B
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
ATCC	American type culture collection
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAA-CM	Stimulated adipocyte CM
CAF	Cancer associated fibroblasts
cDNA	Complimentary DNA
CM	Conditioned medium
CRP	C-reactive protein normal range
CSC	Cancer stem cell
CSF1	Colony stimulating factor 1
CT	Threshold cycle
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTSB	Cathepsin B
CXCL	Chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
DDT	Dichlorodiphenyltrichloroethane
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphates

Dox	Doxorubicin
ECL	Enhanced chemiluminescence
ECOG	Eastern Cooperative Oncology Group
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
FABP	Fatty acid binding protein
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3β	Glycogen synthase kinase 3 beta
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HSC-hTERT	Hepatic stellate cells
IAA	Indole-3-acetic acid
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin

LAMC1	Laminin subunit gamma 1
LGMN	Legumain
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NID1	Nidogen 1
NK	Nature killer
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
OLFL3	Olfactomedin-like protein 3
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein-1
PDGF	Platelet-derived growth factor
PDTX	Patient-derived growth factor
PFA	Paraformaldehyde
PLTP	Phospholipid transfer protein
PI3K	Phosphoinositide 3-kinase
PI	Propidium Iodide
PMSF	Phenylmethylsulphonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
qPCR	Quantitative polymerase chain reaction
rh	Recombinant
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid

RT	Room temperature
SORA	Sorafenib
TACE	Transarterial chemoembolization
TAE	Transarterial embolization
TAM	Tumor associated macrophages
TBS-T	Tris-buffered saline-tween 20
TGF-β	Transforming growth factor beta
Th	T helper cells
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

Section 1.1 An introduction to hepatocellular carcinoma

1.1.1 Hepatocellular carcinoma (HCC)

Liver is one of the busiest organs in living body responsible for maintaining homeostasis and preventing accumulation of toxins in the body. Although regenerative in nature, liver cancer is one of the prevalent cancers in the world with rising incidence worldwide. Reports have claimed an astonishing rise in liver cancer by 75% between 1990 to 2015 (Global Burden of Disease Liver Cancer). Yearly, nearly 800,000 cases of liver cancer are reported (Gravity, 2012). Unless diagnosed earlier, liver cancer at the later stage has less than 10% 5-year survival rate making it one of the deadliest cancers owing to its extremely aggressive nature and lack of effective therapy (El-Serag, 2012). Pathologically, liver cancer comprises of four different kinds- HCC, cholangiocarcinoma, liver angiosarcoma, hepatoblastoma in which HCC accounts for approximately 80% of the diagnosis worldwide (El-Serag, 2012). The incidence of HCC varies worldwide geographically. Reports have found higher incidence of HCC in mostly east and south east Asia and Sub- Saharan Africa. Notably, China itself accounts for 55% of the total incidence of HCC worldwide (Lai et al., 2003). Although much research on HCC has been conducted identifying various preventive measures and drugs for HCC with the incidence remaining stable in some countries except Hong Kong and Singapore where the incidence of HCC in both sexes is on the rise (Zhu et al., 2016).

In the context of Hong Kong, the Hong Kong Cancer registry (2015) identifies liver cancer as the fifth most common cancer incidence in the city and the third most common cancer related deaths in the city. In both sexes, liver cancer falls within top 10 commonest cancer diagnosed and cancer related deaths. In Hong Kong, the cancer incidence increases with age and is reported to be highly diagnosed in

patients in their seventh decades and later. Previous reports have claimed the rise in HCC is due to the HBV endemic in the city with the numbers declining upon institutionalization of HBV vaccination in 1998 in the city (Chang et al., 1997). The incidence of HCV related HCC is found to have increased from 3% to 6.3% from 1992 to 2006 (Zhu et al., 2016). In addition to HCV, NAFLD has been found to be highly prevalent in Hong Kong Chinese population forecasting the possible rise in incidence of higher NAFLD to HCC in the coming future (Fung et al., 2014).

HCC, like many other cancers is not caused by a single condition. Several risk factors contribute to the development of HCC. Some of the etiologies of HCC are hepatitis B (HBV), hepatitis C (HCV), non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease, obesity, intake of dietary aflatoxin, iron overload, diabetes and smoking (Zhu et al., 2016; Ghouri et al., 2017; Oliveira et al., 2017). HBV infection is found to be endemic in various regions such as South-East Asia and sub-Saharan region leading to a high prevalence of HCC in these area (Bosch et al., 2005). Since the development of HBV vaccination and proper enforcement of vaccination, the risk of HBV-HCC has been found to be lowered (Chang et al., 1997; Chang et al., 2009). Although anti-viral vaccination looks promising, the other etiologies of HCC has been compensating the number for HBV-HCC. New reports indicate the rise of HCC incidence due to HCV and metabolic syndrome like diabetes, NAFLD and alcohol abuse (Balogh et al., 2016).

1.1.2 Stem cell model for multi-step hepatocarcinogenesis

HCC is a heterogeneous model of cancer. Due to its diverse population and diverse risk factors, no robust step wise model is created. It is widely believed that hepatocarcinogenesis is a multistep development of HCC attributed to the predisposition of various risk factors leading to cirrhosis and eventual development

of cancer. The multi-step development of the cancer could last for decades until the development of cancer in both HBV positive, HCV positive and metabolic syndrome altered risk factors with varying time for development of cancer. The changes brought in by the risk factors overtime, alters the genetic and microenvironment of cancer resulting in mutations in various common cancer related genes such as TP53, MYC, CTNNB1 and others as well as genes related to telomere stability (TERT promoter) and epigenetic mechanisms (IDH1 and IDH2) and genes responsible for chromatin remodeling (Castelli et al., 2017).

The heterogeneous population of liver cancer is believed to recapitulate fetal liver development upon liver environment damage due to deposition of risk factors resulting in emergence of cells expressing stem cell like markers and initiating signaling pathway resulting in possible development of liver stem cell like properties (Yamashita et al., 2013; Dhanasekaran et al., 2016). These epidemiological data indicating over 80% of the HCC arising from inflammation and chronic injury of liver, it is wise to postulate the modification of liver environment and predisposition of various risk factors resulting in genetic alteration mark the journey of development of HCC. The liver stem cell so generated upon injury of liver is thought to be transformed transforming it into liver cancer stem cells (CSCs). Similar to normal stem cell, cancer stem cells are found to exhibit properties such as self- renewal and expression of markers. This transformation could possibly be affected upon continuous inflammation and regeneration process of liver. Although this is one of the hypothesized theory of development of liver cancer stem cells in HCC, liver CSCs is also hypothesized to be developed through various liver cells such as hepatocytes and biliary cells by initiating stem cell regulating pathways (Holczbauer et al., 2013). Another theory is the

dedifferentiation of the mature cells which has been reported in many solid cancers (Nio et al., 2017). Although, a complete and robust picture on the stepwise development of liver CSCs in HCC is not confirmed yet, recent research indicate the predisposition risk factors that result in changes in tumor microenvironment resulting in genetic mutations and stability of both liver stem cells and mature cells to undergo both genetic and molecular changes eventually leading to promote alteration of HCC development pathways such as wnt/β-catenin, PI3K/Akt-mTOR, TP31 cell cycle, MAPK, IL-6/STAT, TGF-beta, oxidative stress, TERT promoter activating telomerase expression and hepatic differentiation and hence consequently resulting in development of small population of liver cancer cells capable of stem cell properties (Castelli et al., 2017).

1.1.3 Current therapies for HCC

Based on cancer stage, various cancer treatments are available for HCC therapies. Protocol on different therapies for different stages are presented. Barcelona Clinic Liver Staging and Treatment Strategy (BCLC) as illustrated in Figure 1.1 is used to follow protocol to recommend various therapies for HCC diagnosed patients. In addition to cancer stage, physiological condition of the patient is also considered so a step wise protocol staging is followed to recommend various options for the patient. In this section, we review the various current therapies available for different liver cancer stages.

1.1.3.1 Early stage diagnosis- Surgical treatments

A) Liver Resection (partial)

The first line of treatment is the removal of affected areas. Liver, being a regenerative organ can regrow itself if resected. Nearly ~80% of the affected liver could be resected. However, this is only in case the cancer is detected at an early

stage where the cancer has not spread and metastasized to other sites. Although an invasive process, there lies a risk of post-operative infection, blood loss, operative malpractice, operation time and length of time for recovery, with the latest advancement in surgical procedures in liver laparoscopy, the risk is lowered significantly (Cheung et al., 2013). Prior to the surgery, in addition to the stage of the cancer, patient condition and biological parameters such as hepatic volumetry is to be assessed (Kubota et al., 1997; Hemming et al., 2001). The 5- year survival can extend up to ~70% following the procedure if the cancer is detected early (Poon et al., 2002; Nathan et al., 2009). Adjuvant therapies after surgery usually follows to eradicate any remaining cancer cells in the liver. Although a definitive adjuvant therapy is not yet defined, sorafenib, a newly approved FDA approved drug has reached phase III stage for resected patients however no difference in the recurrence free survival (Bruix et al., 2014). Another promising candidate was interferon which showed decrease in recurrence in HCV related HCC resection within the first two years of operation (Breitenstein et al., 2009), however a long-term follow-up did not replicate the result (Chen et al., 2012). In HBV-related HCC, anti-viral adjuvant therapy with nucleotide analog after surgery is showing promising result in recurrence free survival (Wu et al., 2012). Although various adjuvant therapies are in trials to minimize the recurrence post operation in HCC, most of the diagnosis of HCC has passed the early stage eliminating the resection option.

B) Liver Transplantation

Unlike partial resection of liver, liver transplantation aims to completely changing the liver hence getting rid of the virus infected cells which is most likely to contribute to development of recurrence. Mazzaferro et al., has listed the criteria for eligibility for transplantation which comprises of patients having single tumor

of <3cm and no more than three tumors and no hepatic disease (Mazzaferro et al., 1996). The 4-year survival post transplantation was found to be 85% to 92% in HCC patients. Although a promising result, research on expanding the eligibility criteria is being discussed however the main limitation of liver transplantation is bounded by the availability of healthy livers for transplantation and the waiting time for organ. Furthermore, the possibility of donor rejection if of primary concern.

1.1.2.2 Intermediate/ Advanced Stage

A) Transarterial Embolization (TAE) and Transarterial Embolization chemotherapeutic drugs and embolic particles (TACE)

The rationale of this therapy is to directly supply drugs and starve cancer cells off by intervening with the supply of nutrients via the important arteries and vasculature network. TAE primarily blocks the vasculature nature and TACE further supplies chemotherapeutic drugs such as cisplatin and doxorubicin in addition to embolization (Lanza et al., 2017). A meta-analysis study on TAE and TACE has confirmed survival benefits with unresectable HCC (Llovet and Bruix, 2003). In a large cohort with TACE, Takayasu et al., reported 26% of the patients reached 5-year survival after TACE (Takayasu et al., 2006). However, recent studies have shown the benefits of TAE and TACE is highly dependent on the patient condition and the result has a wide standard deviation with poor response in patients including cirrhosis, portal vein thrombosis, tumor size and vessel invasion (Lanza et al., 2016).

Measures in improvement of embolization method and modification of embolized particle has provided better options with positive results in different scenarios of treatment. Recently, radioembolization has been introduced in TACE field where radioactive microspheres Yttrium-90 (Y-90) is introduced and so along with chemotherapy, in-situ therapy is also administered to the site (Memon et al., 2013).

Clinical studies with Y-90 with unresectable hepatocellular carcinoma was found to be relatively more tolerable than traditional therapies with better survival (Bhangoo et al., 2015; Kallini et al., 2016). Randomized clinical trials with sorafenib and other chemotherapeutic agent is undergoing to better understand the efficacy.

B) Molecular-targeted therapies

Regardless of various treatment options available for HCC patients, the survival rate of HCC remains low until now. This could primarily be attributed to the spread of cancer to intra and extra hepatic regions suggesting the need of a systemic intervention to eradicate cancer in other parts of the body to prolong remission state. With the aberrant nature of cancer, various molecules targeting specific group of receptors and pathways dysfunctional in cancer cells are identified and has undergone clinical trials. Most of the molecular targeted therapies is designed to target range of tyrosine kinase receptors and kinases which is found to be upregulated in cancer cells resulting in uncontrolled growth and invasion (Paul et al., 2004). The only FDA approved systemic molecule for HCC patient is Sorafenib- an oral kinase inhibitor which targets serine/threonine kinase regulating the Raf/MEK/ERK pathway, common downstream signal transduced by VEGFR1, VEGFR2, VEGFR3 and PDGFR-α/β and inhibit angiogenesis by suppressing tyrosine kinases (Wilhem et al., 2004; Chang et al., 2006). Clinical studies with sorafenib in SHARP trial and Asia pacific trial has shown positive correlation to better survival hence approving it as the only FDA approved systemic molecular intervention for HCC (Llovet et al., 2008; Cheng et al., 2009). Other molecular target targeting kinases such as brivanib, sunitinib, linifanib which are oral kinases has been tested for clinical trials for HCC patients however the results has not been

too promising as of now as no significance difference in overall survival was observed (Kudo, 2017). Another promising candidate is peretinoin, a molecular target that inhibits carcinogenesis (Kudo., 2015). A 2017 paper also demonstrated effective activity of peretinoin hepatocarcinogenesis by downregulating expression of sphingosine kinase 1 expression (Funaki et al., 2017). Peretinoin is administered to remove any precancerous lesions upon radiotherapy and resection and is under clinical trials for Phase III (Kudo, 2017).

Combinatorial therapy with sorafenib and other kinases along with TACE was also tested however the result has not been quite promising indicating the urgent need of discovery of more specific molecular targets for HCC.

1.1.3.2 The future- immune checkpoints

In 1995, James Allison demonstrated the blockade of CTLA-4, T cell surface glycoprotein result in inhibition of T cell proliferation thus modulating immune cell response (Krummel and Allison, 1995). The implication of this result was pronounced in cancer research when Leach et al, a year later demonstrated the blockade if CTLA-4 resulted in rejection of tumor that was preestablished as well attained immunity to secondary exposure to immune cells providing a substantial evidence of the potential of immune cells in cancer eradication (Leach et al., 1996). Thus, the field of immune check point molecules for cancer therapy was born which led to the field of immunotherapy today. Immunotherapy is basically based on the hypothesis that cancer cells evade immune cells by expressing signals that inhibit immune cells invasion to cancer cells hence by blocking those signals, immune cells can identify cancer cells and eradicate pre-existing cancer cells and attains immunity similar to in response to infection (Disis, 2014). The initial trials with immunotherapy in various cancers such as melanoma, lung has been quite

promising as the cytotoxicity was found to be lower than that of traditional therapies (Voena and Chiarle, 2016). In case of HCC, immunotherapy has been explored recently. Dysregulation of immune cells has been previously reported with upregulation of anti-inflammatory/immunosuppressive Th-2 like cytokines and downregulation of pro-inflammatory/immunogenic Th-1 cytokines in adjacent cells to HCC which indicate poorer prognosis are is key indicator of HCC metastasis (Budhu et al., 2006). Given to the etiologies of HCC comprising of chronic inflammation, viral infection and cirrhosis, the role of immune system in HCC development could be quite substantial. The liver itself is immunosuppressive by nature due to its primary function is to get rid of toxins and a gateway for exit of foreign particles (Cantor et al., 1967; Khan et al., 2014). Therefore, immunotherapy seems quite promising in tackling HCC.

Cancer vaccines against HBV has already been widely introduced to lower the most common cause of HCC in Asian population which has dramatically lowered the number of development of chronic liver disease and HCC eventually (Chang et al., 1997).In terms of immune check point blockade, clinical trials with CTLA-4 blockade molecule-tremelimumab has been studied in HCC in HCV cirrhosis which showed disease control in 76% and the viral load decreased in most patients with no adverse effects related to immune system observed (Sangro et al., 2017). Apart from CTLA-4, another immune check point is PD-1 comprising of two ligands – PD-1 and PD-2. Expression of PD-1 in cancer was found to induce apoptosis and thus evade cancer cells from immune surveillance in body and thus the blockade resulted in cancellation of tumor induced immune suppression by cancer cells hence a potential molecular target in cancer therapy (Okazaki et al., 2007). PD-1 inhibitor Nivolumab has been studied in HCC patients which achieved disease control of

64% with 1% showing complete response. Nivolumab not only demonstrated less side effects to antiviral activity but the response was independent of viral infection or sorafenib treatment (Sangro et al., 2017). The overall survival with Nivolumab was found to be 28.6 months in non-sorafenib administered patients and 15 months among sorafenib treated patients, following this phase I/II trial, Nivolumab was approved by FDA for second line treatment for HCC (Ikeda et al., 2018). Another PD-1 inhibitor- Pembrolizumab has been studied for HCC treatment (Finn et al., 2017) which is in its global phase III trial. Other clinical trials with immune check point inhibitor and molecular targets is being assessed currently for the possibility of enhanced effects in HCC eradication.

1.1.4 Remarks

Although new therapies for HCC is undergoing clinical trials, HCC is one of the complex diseases with more complexity added due to its heterogeneity and its interactions with its microenvironment. This attributes to its low survival rate and high mortality rate among cancer patients. Furthermore, the regenerative nature of hepatocytes, it makes it more challenging for researchers to predict response for HCC patients and also identify highly tumorigenic population in liver. Therefore, it is very important we invest on identification of population that could result in regeneration of tumors and the tumor biology of HCC along with its microenvironment that is found to modulate not only their own physiology but that of the surrounding organs hence making it difficult for systemic intervention of therapies for HCC patients.

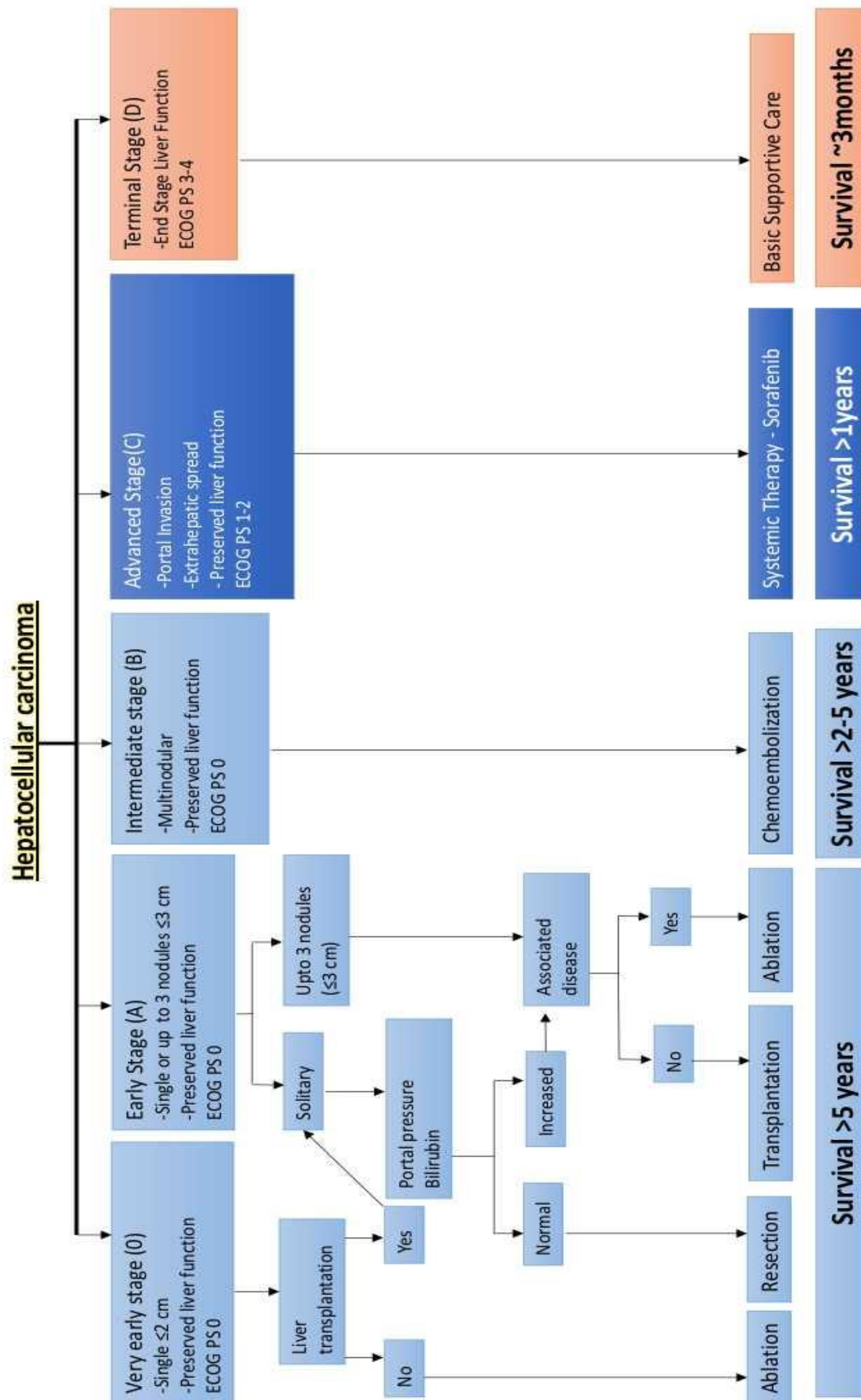


Figure 1.1: Barcelona Clinic Liver Staging and Treatment Strategy (BCLC). Varying treatment strategies adopted upon diagnosis of liver cancer. The chart is designed with reference to the chart provided by Forner, 2012.

Section 1.2 Cancer microenvironment

1.2.1 An introduction to cancer microenvironment

Cancer cells are very resistant to various therapies that is targeted toward and its evolving nature makes it even more difficult to eradicate. Apart from its chemical differences to that of the normal cells, one of the key attributes of cancer cells is its ability to manipulate the non-cancerous cells and thrive in harsh conditions and hence survive in harsh conditions as posed by traditional therapies- radiotherapy and chemotherapy. In addition to its protective mechanism against traditional therapies, the environment is expected to play a critical role in development cancer cells as epidemiological report suggests the higher incidence of particular cancers such as hepatocellular cancer in *Helicobacter pylori* prevalent regions (Wu et al., 2006) and gastric cancer (Wroblewski et al., 2010), inflammatory bowel syndrome to colon cancer (Kim and Chang, 2014) where it is evident the role of environment in development of cancer. The niche so developed by cancer cells that helps them to survive, evolve to a more aggressive form is called the cancer microenvironment. The cancer microenvironment comprises of various types of non-cancerous cells such as endothelial cells, fibroblasts, adipose cells, neuroendocrine cells and immune cells. The role of cancer microenvironment in tumorigenesis has been recently explored with the adaptation of ecology therapy (Pienta et al., 2008). It is widely believed that the state of the cancer microenvironment can either accomplice with the therapy or cancer that is if the cancer microenvironment is healthy, it will aid in cancer therapy however aberrant features in cancer environment will help cancer cells to thrives and hence result in relapse (Wang et al., 2017). As the tumor environment comprises of different kinds of cells and the interaction mechanism between these cells among themselves and with cancer cells is poorly understood. The interaction has been both reported to be direct physical contact to indirect

paracrine and autocrine with signals administered with the release of cytokines, proteins, interleukins, chemokines and growth factors (Balkwill et al., 2012). As cancer is described to be a heterogeneous in nature, the complexity of the diversity is further complicated by the involvement of its interactions with its environment and hence providing higher chances of diversity and eventually leading differences in response. Among the different kinds of cells in tumor microenvironment, stromal fibroblasts, commonly called cancer associated fibroblasts (CAFs) has been widely studied in various cancers like hepatocellular carcinoma (Lau et al., 2016). Schematic drawing of the existence of cancer microenvironment is illustrated in Figure 1.2.

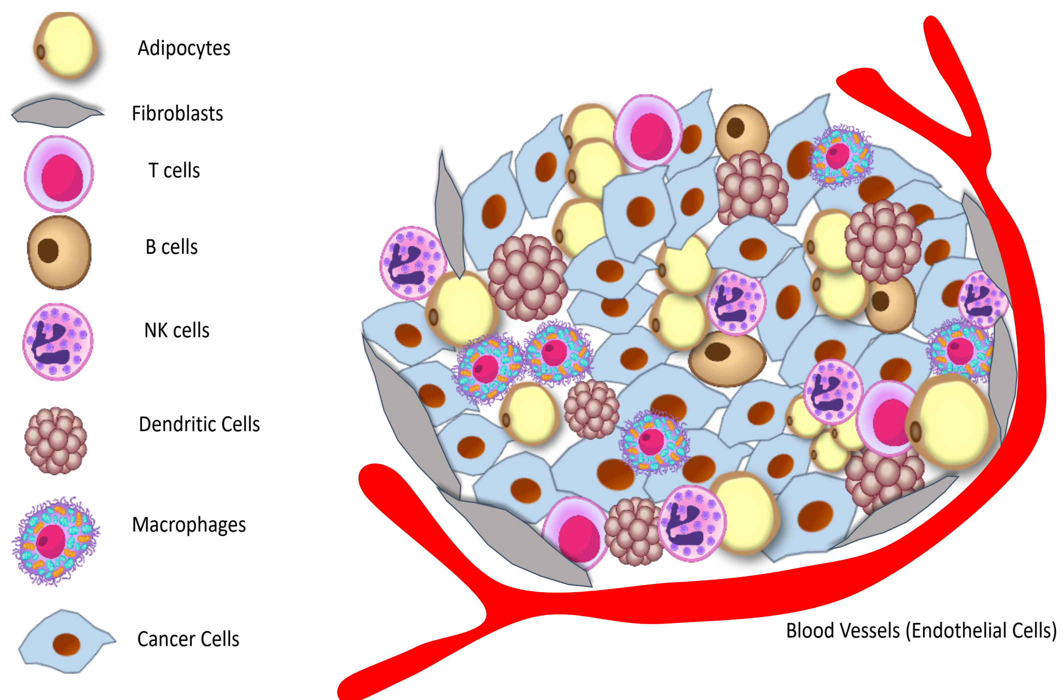


Figure 1.2: Schematic drawing of the composition of a typical cancer microenvironment surrounded by various non-cancerous cells. Cancer cells are usually found to exist with different kinds of non-cancerous cells such as stromal cells-fibroblasts, adipocytes, preadipocytes, stellate cells (in case of liver), immune cells-macrophages, monocytes, natural killer (NK) cells, lymphocytes and neuronal cells-dendritic cells and others. These cells are hypothesized to be modulated by cancer cells and aid in cancer development and progression.

1.2.2 Adipocytes and cancer

One of the important cell types found in cancer microenvironment is adipocytes. The fat cells previously thought to only function as lipid storage and supplier in response or organ signals is re-introduced more as an endocrine system found to influence physiological and chronic metabolic changes systematically. Adipocytes are found to be more than just a storage organ but the source of various kinds of adipokines, proteins and other signaling factors influencing not only cancer cells but also other components of the cancer microenvironment. The critical role of adipocytes in cancer is pronounced with the strong epidemiological data analyzed by Calle et al., which suggests the poorer prognosis in cancer patients with higher adiposity (Calle et al., 2003). World Health Organization expects, obesity to rise in the coming decades and the rising incidence in cancer diagnosis, it is timely we study the influence adipocytes has on cancer prognosis. However, the complexity in crosstalk between adipocytes and cancer cell is also noted as adipocytes secrete more than one kind of secretomes influencing broad range of cells therefore exists the potential to have both direct and indirect influence on cancer cells.

However, preliminary research in this field has provided some positive outlook in this matter as adipocyte secretomes have been found to promote cancer progression in various cancers by promoting epithelial mesenchymal transition (Carbone et al., 2018), radio-resistance (Coelho et al., 2017), resistance to antibody therapy (Duong et al., 2015), drug resistance (Sheng et al., 2016). Adipose rich cancers such as breast and prostate cancer have been widely studied which showed the detrimental role of adipocytes in these cancers both directly and in-direct contact with the cancer cells. Adipocytes in the microenvironment have been reported to provide paracrine signaling mediators, bioactive substrates which have been found to help cancer cells to thrive and model the microenvironment for their benefits and hence

enhance migration and invasive characteristics of cancer cells (Laurent et al., 2016; Hoy et al., 2017). In addition to the supply of key cytokines and modulate the tumor microenvironment, Adipocytes also perform their primary role by supply fatty acids as energy source to cancer cells. This supply of energy source has been a key phenomenon in metastasis of cancer as reported in various cancers such as ovarian (Nieman et al, 2011), prostate (Laurent et al., 2016), colon (Wen et al., 2017), breast (Hoy et al, 2017), leukemia (Shafat et al., 2017) and gastric (Xiang et al., 2017). Given these reports, it is very interesting to note that adipocytes confer cancer progression in both direct and in direct manner which is interesting however this further makes it tedious to decipher the crosstalk between adipocytes and cancer. Furthermore, a reciprocal relationship between adipocytes and cancer has been established in which the cancer cells are found to educate adipocytes to release more of the essential components or indulge in lipolysis for the abundant supply of fatty acids for cell metabolism (Duong et al., 2017). This reciprocal stimulation of adipocytes highlights the physiological remarks of cachexia on cancer patients at later stage of life as well as the stronger correlation between obesity and cancer related death.

Moreover, apart from progression of cancer, adipocytes have been linked to development of cancer given to its role in different metabolic syndromes like diabetes, hypoxia, dyslipidemia and its link to development of cancer (Braun et al., 2011). Chronic inflammation is widely accepted to the cause of development of various cancers and the role of adipocytes in inflammation indicates the developmental role of adipocytes in cancer development. The supply of fatty acids enhances the beta-oxidation pathway resulting in upregulation of inflammation in cells and the elevated release of inflammatory cytokines like IL-6, TNF-α, CRP

provide an inflammatory environment and thus could result in chronic inflammation and is hypothesized to provide an environment for cancer development. Recently, adipocytes are also found to secrete colony stimulating factor 1 (CSF1) which is found to mature macrophages and hence could also play a role in educating macrophages and monocytes to modulate the immune system and aid in cancer development (Weisberg et al., 2003). As illustrated in Figure 1.3, various researches on the roles of adipocytes and cancer highlighted the multiple roles of adipocytes could play in progression of cancer and play a detrimental role in cancer prognosis and therapy however also highlights the complexity of this network that requires to be further investigated.

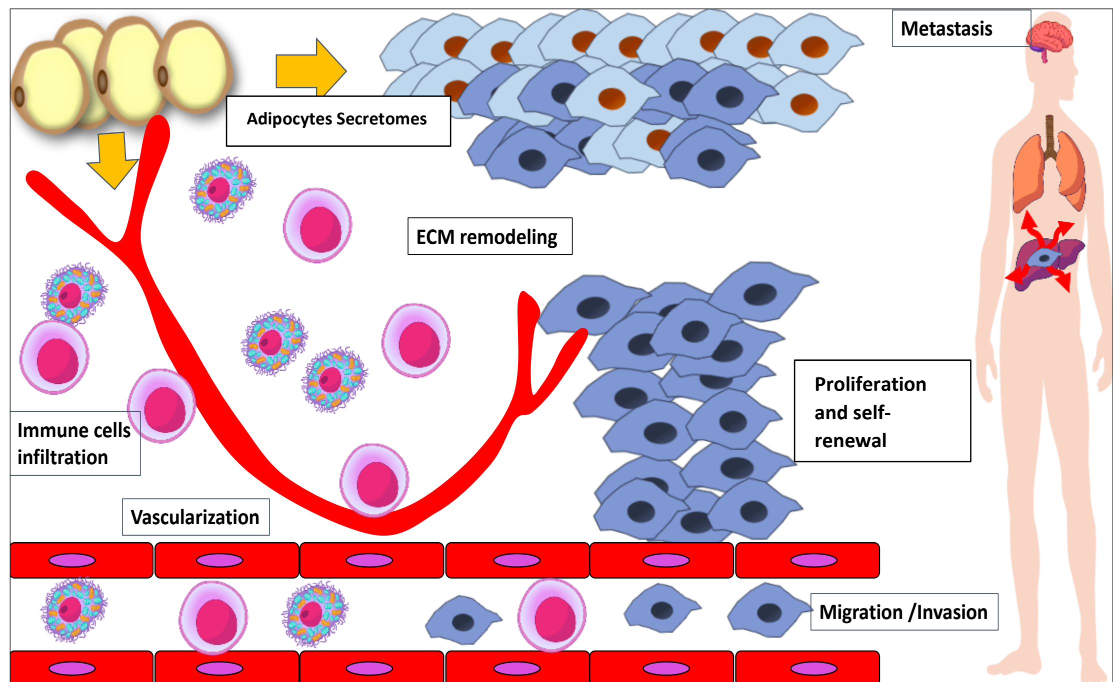


Figure 1.3: Schematic drawing demonstrating the possible effects of adipocytes in cancer progression. Adipocytes have been reported to promote cancer progression by enhancing its proliferation, migration and invasion abilities and modulate the cancer microenvironment to help to evade traditional therapies and hence thrive in adverse conditions.

1.2.3 Cancer stem cells and adipocytes in tumor microenvironment

Cancer stem cells are group of cells found in the heterogeneous population of cancer cells which is thought to be highly resistant to traditional therapies such as radio-therapy and chemo-therapy and possess self-renewal property thus contributing to relapse of cancer. The strong correlation developed between obesity and cancer related mortality signifies the possible role of development of this small population of cancer stem cells which could play a role in poor prognosis and recurrence of cancer. Several pathways as demonstrated in Figure 1.4 were found to be activated in these cells including wnt, Sonic Hedgehog pathway, JAK-STAT, PI3K/PTEN and NF-κB signaling (Matsui, 2016). Therefore, the role of adipocyte microenvironment could also play a role in cancer stem cell development.

For adipocyte enriched cancers including breast and prostate, researchers have provided evidence of the promoting role of adipocytes in cancer stemness properties by enhancing their self-renewal properties and upregulating cancer stem cell pathways (Wolfson et al., 2016; Tang et al., 2016). In leukemic stem cells, adipocytes niche is found to help stem cells to maintain its existence by aiding to evade chemotherapy by supplying metabolic essentials and thus helping to adapt to harsh condition developed by chemotherapy (Ye et al., 2016). Although much research on the effects of adipocytes on cancer stem cells is not fully explored however the initial research on adipocytes rich environment cancers like breast and prostate and result provides substantial evidence of the role of adipocytes in development of cancer stem cell like properties. Furthermore, research on adipocytes secretomes have also been studied which shows the role of adipocytes secretomes like leptin, adiponectin, Interluekin-6 has been found to regulate cancer properties which further emphasizes the vital role adipocytes and adipocytes

microenvironment could play in regulating cancer stemness properties in various cancer types (Coelho et al., 2016; Himbert et al., 2017).

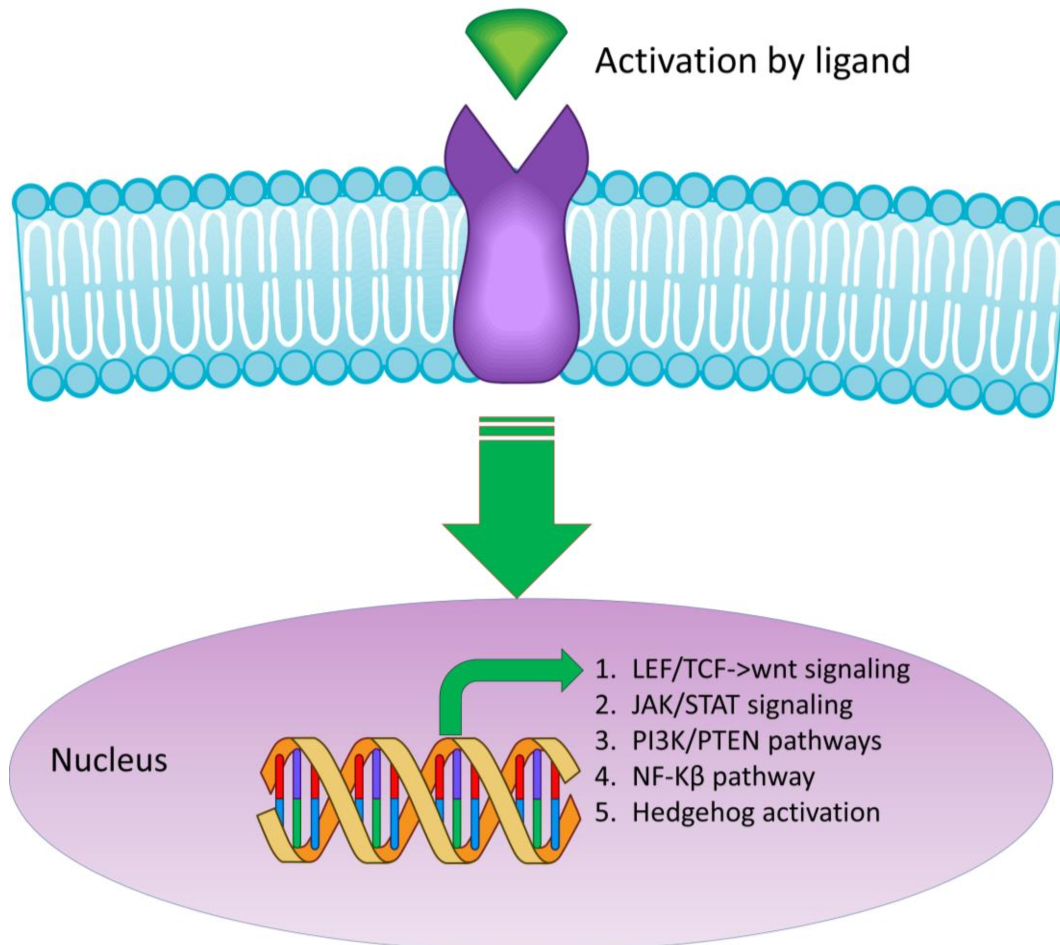


Figure 1.4: Schematic drawing of the development of CSC properties upon upregulation of key signaling pathways. Constitutive expression of pathways mentioned above have been reported to govern CSC properties and the expression has been linked to development of key CSC properties such as self-renewal, drug resistance, migration, invasion and tumorigenicity in various cancer types.

1.2.4 HCC and adipocytes-The relationship

The relationship between adipocytes and HCC has been recently been in limelight as the major cause of HCC was previously dominant with hepatitis virus infection. Liver, however, is a very busy organ with constant supply of secretomes from visceral adipocytes and the source of lipids to be supplied to other organs. Although the abundance of adipocytes in liver is not as prevalent as in prostate and breast cancer, the effect of adipocytes on HCC cannot be underestimated. Thus, the role of fatty acids and adipocytes secretomes could be of some importance. Relationship between adipocytes and HCC could be more pronounced as a benign condition of liver is in high surge recently known as non-alcoholic fatty liver disease (NAFLD)- the condition of accumulation of fats in liver. NAFLD mostly is a benign condition which could be reversed by following lifestyle changes however the incidence of developing HCC from NAFLD previously thought to be minimal is on rise in recent years (Masuzaki et al., 2016). Epidemiological data suggests a stronger correlation between NAFLD and obesity in HCC indicating the possible role of adipocytes on HCC progression (Younossi et al., 2015). Particularly in reference to the global context today, NAFLD is found to be develop to HCC without progressing into cirrhosis in 30-40% of NAFLD to HCC related cases in Asia (Fan et al., 2017). Schematic drawing on the progression of NAFLD to HCC is illustrated in Figure 1.5.

Research on the effects of fatty acids, supplied by adipocytes on HCC progression has been reported which suggests fatty acids playing a crucial role in EMT transition (Nath et al., 2015). Adipocyte secretomes such as leptin has been found to play a role in development of HCC via interaction with the tumor microenvironment (Stefanou et al., 2010). On the other hand, higher level of adiponectin in serum level has been suggested to use for reference for poor

prognosis in HCC patients (Shen et al., 2016) and was also found to play a role in hepatitis B virus infected cohort too indicating apart from metabolic syndrome, adipocytes could also play a role in HCC progression in HCC which further strengthens the role of adipocytes and their secretomes in HCC (Chiang et al, 2013). NAFLD is thought to progress to NASH and cirrhosis to develop into HCC (Issa et al., 2017). The progression of NAFLD to NASH is governed by inflammation. Supply of fatty acids is abundant in liver with fat rich environment in NAFLD and also the supply in obese population, therefore it would not be too wrong to assume fatty acid playing a role in inflammation in liver (Calder, 2011). Oxidative stress is another key factor of development of carcinogenesis particularly with the given epidemiological data on chronic hepatitis C and hepatocarcinogenesis (Ivanov et al., 2013). Fatty acids are metabolized via oxidation in cells resulting in production of reactive oxygen species (ROS). ROS is widely known to play crucial role in cancer development and therefore, the fatty acid rich environment could make liver cells more prone to oxidation and subsequently lead to HCC development (Takaki and Yamamoto, 2015). Metabolic changes brought in by fatty liver condition is also found to alter the oxygen homeostasis in liver creating an oxygen deprived environment (Suzuki et al, 2014). Hypoxia along with ROS rich environment and inflammation are molecular etiologies which are found to be associated with the development of HCC. Given, the alteration of oxygen homeostasis in fatty liver condition, adipocytes, the prime cellular factor in fatty liver could also play a role in HCC development. As it is widely known that adipocytes not only directly assist in cancer initiation and progression but can also aid in the process by modulating the microenvironment of tumor, it is important to understand the influence of adipocytes and adipocytes rich environment on hepatic microenvironment-stellate

cells, hepatocytes, immune cells in cancer initiation stage and progression stage. Hence, this complex study of cross talk between these cells is urgently required to provide alternative therapeutics options to HCC.

In HCC, cancer stem cells have also been identified in liver cancer cells which are characterized to by its unique properties of self-renewal, migration, invasion, tumorigenicity and drug resistance and are identified by various membrane proteins that has been discovered such as CD24 (Lee et al., 2011), CD47 (Lee et al., 2014), CD133 (Ma et al., 2008), and EpCAM (Yamashita et al., 2009). Since adipocytes are found to modulate cancer properties, the possibility of modulation CSC properties by adipocytes is also possible. Identification of stem cells specific markers provides a new horizon to therapy against cancer by targeting the main source of recurrence. Although much research has been conducted on therapeutic measures of HCC the survival rate remains quite low making HCC one of the deadliest cancers in the world with the third highest mortality rate in amongst cancer demo graphs (McGlynn et al., 2011). Despite identifying the different markers that help to differentiate CSCs, we are still unaware about the origin of CSC and as cancer survives through interaction with the environment and modulating our body system, the full understanding of the cancer invasion has not been explored much.

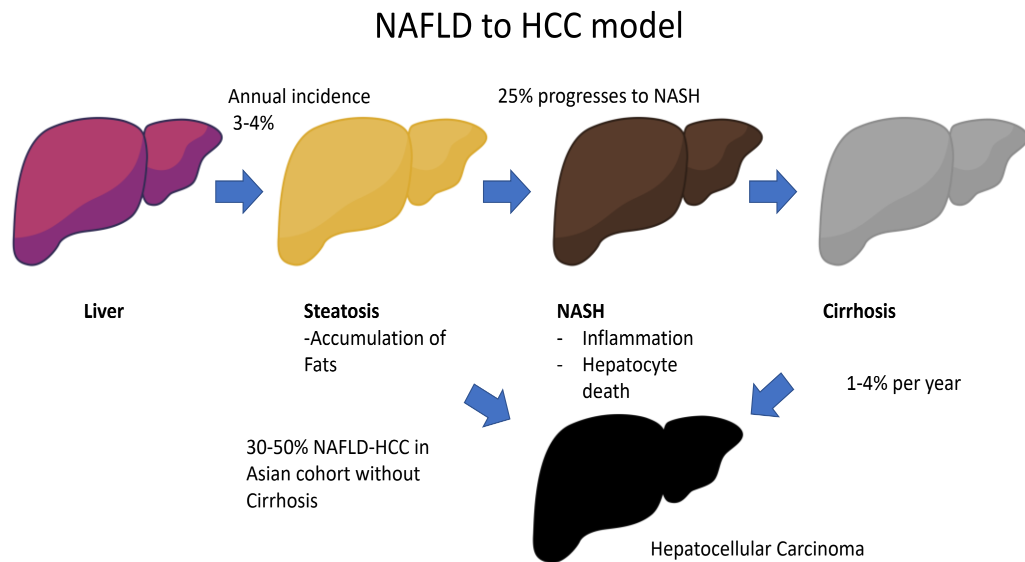


Figure 1.5: Representative drawing illustrating the phenotypical changes observed upon development of NAFLD to HCC. The relative incidence of the progression is also listed as reported by Fan et al., 2017. Accumulation of fatty acids either by dietary supply or dysregulation of adipogenesis in liver which is reversible upon lifestyle changes however the progression to NASH resulting in cell death and inflammation will eventually develop into HCC. Recently, the incidence of direct development of HCC from NAFLD is on the rise which is quite alarming, yet the molecular mechanism involved is not yet known.

Section 1.3. Cancer stem cells

1.3.1 Overview of cancer stem cell (CSC) models

Accumulating evidence have suggested that tumor is composed of heterogenous population of tumor cells within the tumor bulk. This variance in cancer population refers to both genetic and phenotypic differences among cancer cells which redefines the one therapy targets all cancer cells theory. The differences could be marked by the variance in the properties of cancer cells such as expression of cell surface markers, proliferation rate, response to therapy and self-renewal property, migratory and invasive abilities (Kreso and Dick, 2014). These differences in cancer population make it difficult to achieve 100% effective therapy therefore various combinational therapies have been recently proposed and used widely as cancer therapy options (Bayat et al., 2017). However, to study the population of cancer cells, two cancer models are proposed- hierarchical cancer model and stochastic cancer model. Schematic drawing of the two models is illustrated in Figure 1.6.

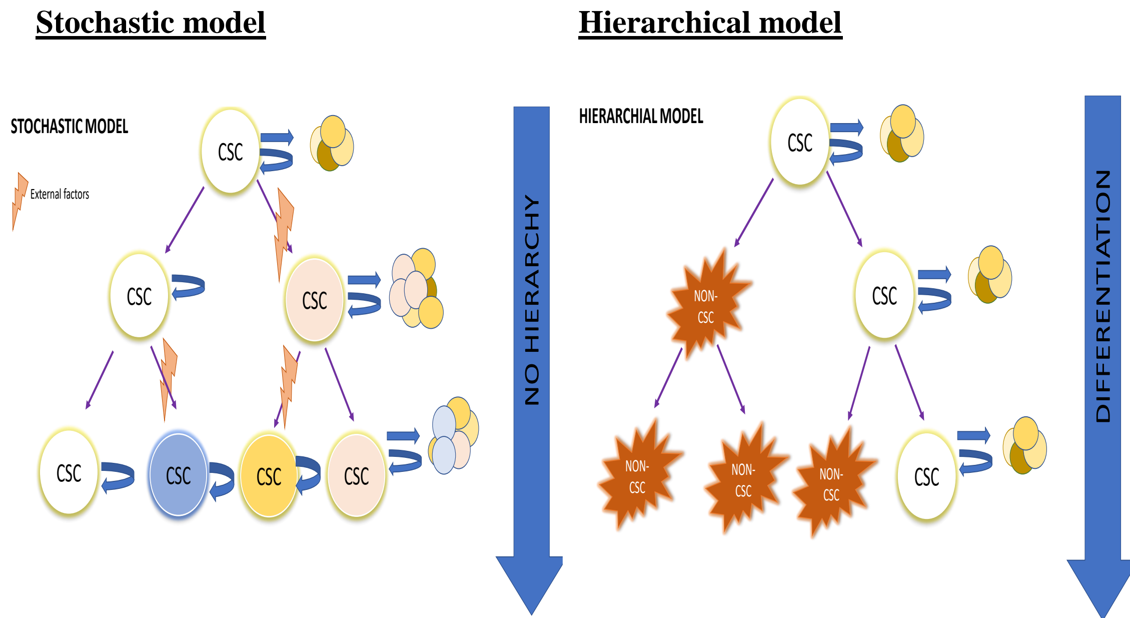


Figure 1.6: The stochastic and hierarchical models of CSCs. In the stochastic model, each cell has equal potential to develop into cancer stem cells and thus initiate tumors with heterogeneity and variance acquired due to external factors and upon sequential divisions. As for the hierarchical model, the tumor initiating cancer stem cells resides in the apex of the hierarchy and upon division, only a subset of cells will acquire the stemness properties. The level of differentiation increases with intrinsic accumulation of genetic mutation upon subsequent division.

1.3.1.1 Hierarchical model

Hierarchical cancer model postulates that cancer stem cell follow a functional hierarchy in which the capacity of self-renewal and differentiation is only limited to a subset of cancer cells. Mirroring the progression to that of normal stem cell, it is proposed that upon subsequent generation, the tumor initiating cells develop epigenetic changes similar to differentiation of normal cell differentiation losing its tumorigenic potentials along the way. A subset of cells at the apex of this model, which results in dysregulation of growth process and hence forms clinal tumor. The population in this clonal bulk consists of differentiated forms of cancer stem cells that are phenotypically different to each other as it comprises of differentiated cancer stem cells capable of proliferation of self-renewal. This model provides an explanation as to why tumor bulk once removed by chemo or radiotherapy result in relapse as it is hypothesized the remaining small population of cancer stem cells drive tumor initiation.

1.3.1.2 Stochastic model

Stochastic model is the traditional thought of tumor initiation in which cancer cells are physiologically and phenotypically homogenous in nature which have equal potential to self-renew and proliferate and form tumor in a randomized manner. The potential to self-renew and initiate tumor formation in a randomized manner. Intrinsic factors mostly include acquired genetic mutations upon DNA damaging stimulus. Extrinsic factors include exposure to tumor microenvironment and response accordingly resulting in accumulation of genetic changes. As the cancer progresses, subsequent acquisition of genetic changes provides growth and survival advantage to cancer cells which could result in higher proliferative properties making it more tumorigenic than the rest of the cancer cells in the pool hence

providing heterogeneity in properties. Most of the current therapies are based on this cancer model where the bulk is targeted having the tumor initiating potential however the model cannot explain why patients with same cancer type respond differently as some patients undergo full remission however some relapse.

1.3.2 Identification of CSCs

The developmental process of a single cell zygote to a fully-grown living being comprising of tissues are and organs with specific function itself is something that has fascinated scientific world with the discovery of developmental process in living being. Zygote, a single cell able to perpetuate and differentiate into different cell type and tissues itself is a fascinating work of nature. Interest in stem cell research ignited with the discovery of subset of cells in living beings upon the discovery of hematopoietic stem cells in bone-marrow reconstitution in 1917 which provided the evidence of existence of stem cells capable of differentiation after development of zygote (Pappenheim, 1917). The existence of stem cells with the ability to proliferate and differentiate was further supported with the result demonstrated by Furth and Kahn in 1937 where they demonstrated inoculation of single leukemic cells was able to initiate leukemia in the recipient mice (Furth et al., 1937). This was the first evidence to confirm the role of stem cells in formation of tumors which is further exploited in cancer therapy today.

Upon confirmation of existence of group of cells which is able to proliferate and differentiate giving rise to tumors, the CSCs were first identified based on their markers in acute myeloid leukemia by Bonnet and Dick in 1997 where they were able to isolate a subset of AML cells from the peripheral blood of patients which were able to initiate tumors in recipient NOD/SCID mice (Bonnet and Dick, 1997). The cells were separated based on this surface markers $CD34^{+}CD38^{-}$ antigen

phenotype. Interestingly, as hypothesized they reported the tumor initiating ability remained consistent upon serial transplantation into secondary mice conserving their phenotypic and morphological heterogeneity as their original tumor. This was an important finding in cancer stem cell research as the result provided evidence of the existence of group of cells that expressed markers similar to regular stem cells which preserved its self-renewal and tumor initiating abilities upon subsequent generation despite providing heterogeneity in the pool as described in stochastic model of tumor initiating cells. Upon this discovery, other cells surface markers in leukemia was identified such as $CD90^-$, $CD123^+$, $CD71^-$, HLA^-DR^-, $CD117^-$, $CD44^+$ and $CD96^+$ characterizing leukemia-initiating cells (Blair et al., 1997; Blair et al., 1998; Blair and Sutherland, 2000; Jordan et al., 2000; Jin et al., 2006; Hosen et al., 2007; Majeti et al., 2009). This led to search of potential tumor initiating cells in other solid tumors. However, unlike circulating leukemia cells, solid tumors are difficult to isolate and characterize. Michael Clarke and his team were able to identify tumor cells expressing $CD44^+/CD24^{-/low}$ $Lineage^-$ of breast cancer cells as breast cancer CSCs in 2003 (Al- Hajj et al., 2003). Similar to the properties exhibited by leukemia CSCs, the subset was able to initiate formation of tumors when human breast cancer cells were injected into mouse mammary fat pad. Interestingly, histological study of the tumors formed by the subset contained malignant cells whilst the other set were not able to form tumors or malignant type. This confirms the hierarchical model of CSCs which supports the existence of only a subset of cells in tumor capable of initiation of tumors. The data further confirmed the common property of CSCs in solid tumor similar to that of circulating tumor in which the subset is able to self-renew, ability to initiate tumors and differentiate providing heterogeneity among tumor population.

With the identification of CSC markers in AML, research on the possible identification of CSC markers tumors in other solid tumors was immensely investigated. Discovery of CSC markers in various cancer types was summarized in Table 1.1.

Types of Cancer	CSC Markers	References
	Leukemia	
Acute Myeloid Leukemia	$CD34^{+}CD38^{-}CD90^{-}$, $CD123^{+}$, $CD71^{-}$, $HLA^{-}DR^{-}$, $CD117^{-}$, $CD44^{+}$, $CD96^{+}$, $CD47^{+}$	(Blair et al., 1997); (Bonnet and Dick, 1997); (Blair et al., 1998); (Blair and Sutherland et al., 2000); (Jordan et al., 2000); (Jin et al., 2006); (Jaiswal et al., 2009)
Acute Lymphoblastic Leukemia	$CD34^{+}CD10/CD19^{-}$, $CD34^{+}CD4^{-}/CD7^{-}$	(Cox et al., 2004); (Cox et al., 2007)
	Solid Cancers	
Breast Cancer	$CD44^{+}CD24^{-/low}$ $EpCAM^{+}$ $Lineage^{-}$, $CD49F^{+}$ $DLL1^{high}DNER^{high}$, $ESA^{+}CD44^{+}$ $CD24^{-/low}$, $ALDH^{-}1^{+}$, $CD133^{+}$	(Al-Hajj et al., 2003); (Ginestier et al., 2007); (Wright et al., 2008); (Pece et al., 2010)
Brain Cancer	$CD133^{+}$, $CD49f^{+}$, $CD90^{+}$	(Hemmati et al., 2003); (Singh et al., 2003); (Gilbert and Ross., 2009)

Colorectal Cancer	$CD133^+$, $CD44^+$, $CD166^+$, $EpCAM^+$, $CD24^+$	(O'Brien et al., 2007); (Yeung et al., 2010)
Gastric Cancer	$CD44^+$, $CD24^+/CD44^+$, $CD54^+/CD44^+$, $CD44^+/CD166^+/ALDH^+$, $CXCR4^+$	(Chen et al., 2012); (Fujita et al.,2015); (Nguyen et al., 2016)
Head and Neck Cancer	$CD44^+$, $CD44^+$ $ALDH^+$	(Prince et al., 2007); (Krishnamurthy et al., 2010)
Hepatocellular Carcinoma	$CD133^+$, $CD133^+ALDH^+$, $CD133^+$ $CD44^+$, $CD90^+$, $CD90^+CD44^+$, $EpCAM^+$ AFP^+; $OV6^+$, $CD24^+$, $CD47^+$	(Ma et al., 2007); (Ma et al., 2008); (Yamashita et al., 2009); (Yang et al., 2008); (Yang et al., 2008); (Yang et al., 2008); (Zhu et al., 2008); (Lee et al., 2011); (Lee et al., 2014)
Lung cancer	$CD133^+$, $ABCG2^{high}$	(Ho et al., 2007) (Eramo et al., 2008);
Melanoma	$CD20^+$	(Fang et al., 2005)
Ovarian Cancer	$CD44^+CD117^+$, $CD24^+$	(Zhang et al., 2008); (Gao et al., 2010)
Pancreatic Cancer	$CD133^+CXCR4^+$; $CD44^+CD24^+EpCAM^+$	(Hermann et al., 2007); (Li et al., 2009)
Prostate Cancer	$CD133^+$, $CD44^+/\alpha_2\beta_1^{high}/CD133^+$	(Collins et al., 2005)

Table 1.3 Summary of CSC markers identified in various cancer types including both leukemic and solid cancers.

1.3.3 Properties of CSCs

CSCs as mentioned above in section 1.3.1 are hypothesized to be small populations in tumors that can initiate tumors hence result in tumor formation. With increasing number of studies on the characteristic of such populations has been governed by not only in tumorigenicity property but also its ability to self-renew, invade through the extracellular matrix to metastasize and found to be resistant to traditional therapies as summarized in Figure 1.7. In addition to identification of CSCs in tumor cells, with the understanding of tumor microenvironment in cancer prognosis, it is important we study the interaction of various cells in tumor microenvironment in order to understand the influence of adjacent cells in tumor behavior. Hence, researchers assess CSC properties in cancer upon interaction with other cell types or in response to drugs to study the effect of the external stimuli to CSC properties which could determine cancer response and help to predict prognosis or could be a biomarker for better therapeutic options. Therefore, some of the key characteristics assessed for CSC properties are tumorigenicity and differentiation, self-renewal, drug resistance, metastatic potential and angiogenesis. We will be looking at the properties and experiments in the following section.

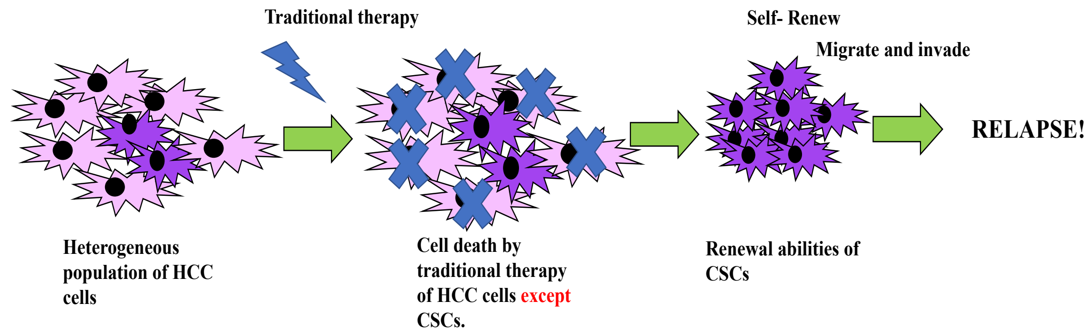


Figure 1.7: Distinct characteristics of CSCs in tumor heterogeneity. CSCs are hypothesized to survive through traditional therapies and have the ability to self-renew, migrate, invade through organs and forms tumors resulting in relapse of cancer.

1.3.3.1 Tumorigenicity and Differentiation

Both CSC models highlight the key characteristic of CSCs that is its ability to form tumors and also differentiate upon successive division which is the basis of heterogeneity in cancer tumors. In order to study this characteristic, tumorigenicity is assessed by in vivo transplantation of cancer cells by either inoculating it subcutaneously or orthotopically in immunocompromised mice. Tumor formation is observed, and the size and incidence calculated to upon termination of the experiment (Clarke et al., 2006). Prior to *in vivo* studies, preliminary data on tumorigenicity could be assessed using a soft agar assay to evaluate anchorage - independent formation of colonies which would suggest the trend in formation of tumors of treated cells. Differentiation markers are used to assess the degree of differentiation in tissues (Mohanta et al., 2017).

1.3.3.2 Self-renewal

Self-renewal is key to CSC properties as to form tumors by itself, the cell can grow and self-renew itself to maintain its population. Self-renewal property of CSCs is tested in an *in vitro* setting where single cells are suspended in methylcellulose medium in low adherent plates where individual cells cannot settle to the bottom

and differentiate but form colonies upon acquiring self-renewal property. The growing environment for this experiment is serum starved and only essential elements is provided for growth. Cells with self-renewal property forms spheroids within a week's time and based on the number of spheres so formed and the size, the self-renewal property assessed (Shaheen et al., 2016). Secondary spheres could also be formed to assess the self-renewal property of CSCs models and the expression markers of the spheroids formed was also assessed using flow cytometry.

1.3.3.2 Drug resistance

One of the biggest hurdles in cancer research has always been the ineffectiveness of current therapies in eliminating the small group of cancer cells that are resistant and have the property of tumor formation. CSCs are believed to be highly resistant to the current therapies therefore survive through the therapy. Therefore, it is important to study the response of CSCs to drug and other therapies. Like normal stem cells CSCs confer resistance to external toxicities like drugs by upregulating ATP-binding cassette transporter (Dean, 2009), activating DNA damage response (Blanpain et al., 2011) and upregulating anti-apoptotic pathways (Ma et al., 2008). It is important to assess the effect of drugs and chemotherapy while assessing CSC characteristics in cancer cells.

1.3.3.3 Metastatic potential

Cancer metastasis is the prime cause of cancer mortality. CSCs are also believed to be highly metastatic in nature as they are concurrently found to be more invasive in nature. However, all CSCs are not found to be equally metastatic in nature. For instance, only $ALDH^{+}$ breast CSCs were found to be highly metastatic both in *in vivo* intra-cardiac mouse model (Korkaya et al., 2008). Similarly, $CD133^{+}$ $CXCR4^{+}$ colon cancer cells were found to induce metastasis (Pang et al., 2010). The

metastatic potential can be examined *in vivo* using mouse models while invasion and migration abilities of cells could be assessed using 3D migration and invasion assays or wound healing assay before proceeding to animal studies.

1.3.3.4 Angiogenesis

The tumor associated environment is found to be very much modulated by tumor and CSCs in a reciprocal manner where both cells co-exist for the benefit of cancer cells. Tumor vasculature is a key modification in cancer microenvironment which is essential for survival of cancer cells. Research has demonstrated that compared to its other counterpart, CSCs are found to express high levels of pro-angiogenic factors such as VEGF which subsequently induce endothelial cell proliferation and blood vessel formation thus forming the vasculature architecture in cancer microenvironment (Bao et al., 2006; Tang et al., 2012). Apart from secreting itself, cancer cells have also been reported to secrete proteins that modulate the secretion of VEGF, PDGF by stromal cells (Brogi et al., 1994; Tsai et al., 1995).

Section 1.4 Hypothesis of the study

Study in liver cancer and adipocytes is of high significance because liver is an organ where visceral fats is directly delivered via intrahepatic system that increases its access to excess fats. The rising incidence of NAFLD has been attributed to obesity and fatty acids being delivered to liver due to various metabolic abnormalities (Fabbrini et al., 2010). NAFLD is a metabolic syndrome resulting in accumulation of fats in liver and has been linked to inflammation and development into steatohepatitis and cirrhosis and found to develop to HCC. With more convincing reports between adipocytes and cancer and the availability of adipocytes secretomes via intrahepatic system and non-alcoholic fatty liver, the influence of adipocytes and adipocytes secretomes on development of HCC could be of high significance and further aid in enhancing the stemness of liver cancer cells which needs to be urgently addressed.

With strong epidemiological evidence illustrating the strong correlation between obesity and NAFLD and to HCC, the influence of adipocytes on HCC requires to be urgently studied as HCC is one of the most common cancers with high mortality rate. Nath et al., have demonstrated the promoting effect of fatty acid derived from adipocytes on EMT in HCC. (Nath et al., 2016). Based on these findings, together with the rising epidemiological data, suggest the possible role of adipocytes on HCC development. In this study, we hypothesize that adipocytes could enhance CSC properties in HCC either directly or indirectly. For this purpose, we first differentiated the preadipocytes to functional adipocytes. After successful establishment, we study the effects of adipocytes on HCC cells in a co-culture set up barring the physical contact between the two cells but allowing exchange of soluble materials. Further, we characterize and elucidate the role of adipocytes on

regulation of liver CSCs. Schematic drawing of our hypothesis is presented in Figure 1.8.

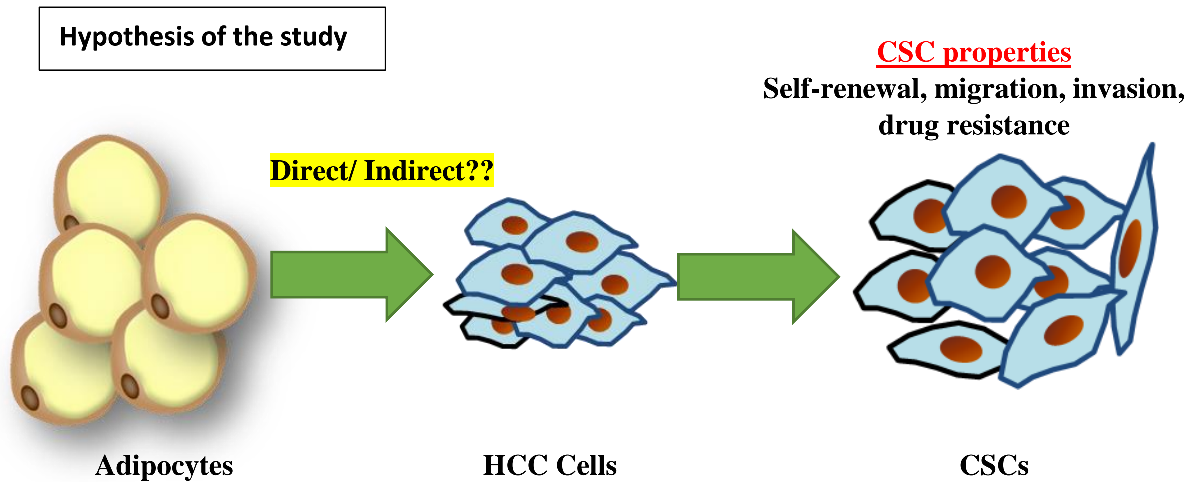


Figure 1.8: Hypothesis of the study. We hypothesize adipocytes could enhance liver CSC properties- self-renewal, migration, invasion, tumorigenicity and drug resistance either directly or indirectly resulting in aggressiveness of the disease. For this purpose, we have employed visceral adipocytes to understand the interaction of HCC cells with adipocytes and assessed the effect of adipocytes using various functional assays to study its effect on CSC properties.

1.4.1 Objectives of the study

1: To functionally characterize the effect of adipocyte secretomes on regulation of liver CSCs.

2: To identify the crucial factor in adipocyte secretomes that critically involved in regulation of liver CSCs.

3: To delineate the molecular mechanism of how adipocyte secretomes regulates liver CSC properties and its clinical significance.

CHAPTER 2: MATERIALS AND METHODOLOGY

Section 2.1 Materials

2.1.1 Cell lines

Cell lines	Characteristics	Source
Bel-7402	HCC	SIBS, Chinese Academy of Sciences
PLC/PRF/5	HCC	ATCC
Huh7	HCC	JCRB, JCRB0403
Hep3B	HCC	ATCC
MHCC-97L	HCC	LCI, Fudan University
PT5005	Visceral Preadipocytes	Lonza
HSC-hTERT	Activated Hepatic Stellate cells	ATCC

Table 2.1 List of cell lines.

2.1.2 Cell culture reagents

Reagents	Role	Source
DMEM- HG	Culture	GIBCO
DMEM/F-12	Culture	GIBCO
Fetal bovine serum	Culture	GIBCO
B-27 supplement	Supplement for sphere formation	GIBCO
Insulin	Supplement for sphere formation	Sigma
TrypLE Express	Culture	GIBCO
Penicillin G and streptomycin	Culture	GIBCO
Antimycotic	Culture	GIBCO
PT8202	Preadipocytes and adipocytes culture	Lonza

L-Glutamine	Preadipocytes and adipocytes culture	Lonza
Gentamicin sulfate and amphotericin-B	Preadipocytes and adipocytes culture	Lonza
Recombinant human insulin	Adipocytes culture	Lonza
Dexamethasone	Adipocytes culture	Lonza
Indomethacin	Adipocytes culture	Lonza
IBMX	Adipocytes culture	Lonza
Oil red O	Lipid staining	Sigma
Hematoxylin	Lipid staining	Sigma

Table 2.2 List of cell culture reagents.

2.1.3 Antibodies

Antibodies	Source	Dilution used/ Isotype control
For flow cytometry		
FITC-conjugated mouse anti human EpCAM	BD Pharmingen	1:10/Mouse IgG1k
PE-conjugated mouse anti human CD90	BD Pharmingen	1:4/ Mouse IgG1k
FITC-Annexin V	Biovision	1:100
Propidium Iodide	Life technologies	1:10
For western blotting		

Rabbit monoclonal anti-FABP4	Cell Signaling	1:1000
Rabbit polyclonal anti-phospho-Akt (Ser 473)	Cell Signaling	1:500
Rabbit polyclonal anti-Akt	Cell Signaling	1:2000
Rabbit monoclonal GSK3β (Ser9)	Cell Signaling	1:2000
Rabbit monoclonal total GSK3β	Cell Signaling	1:2000
Rabbit monoclonal β-catenin	Cell Signaling	1:2000
Mouse monoclonal anti actin	Sigma	1:4000
HRP-linked mouse IgG (from sheep)	GE Healthcare	1:5000
HRP-linked rabbit IgG (from donkey)	GE Healthcare	1:5000

Table 2.3 List of antibodies.

2.1.4 Primers

Human Gene	Forward primer (5'-3')	Reverse Primer (5'-3')	Accession No.
FABP4	ACTGGGCCAGGAATTTGACG	ACGCATTCCACCACCAGTTTA	NM_001442.2
PPARγ	GCAAACCCCTATTCCATGCTG	GTGTCAACCATGGTCATTTCTTGT	NM_015869
GAPDH	CCGGGAAACTGTGGCGTGATGG	AGGTGGAGGAGTGGGTGTCGCTGTT	NM_002046

Table 2.4 List of primers.

Section 2.2 *In vitro* studies

2.2.1 Human visceral adipocytes

Visceral human preadipocytes (PT5005) were purchased from Lonza. Visceral human preadipocytes were extracted from visceral sites from non-diabetic patients. Preadipocytes so bought were maintained in preadipocytes basal medium supplemented with fetal bovine serum and L glutamine, all obtained from Lonza (PT8202). Preadipocytes were grown in six wells based until 80% confluency and then the medium replaced to differentiation medium obtained from Lonza. Differentiation medium was prepared as instructed by Lonza and the ratio of adipocytes differentiation to preadipocytes growth medium was optimized to 3:1. Morphological changes in adipocytes were observed within a week of differentiation and incubation with differentiation medium for nearly 20 days resulted in 70% of differentiation of preadipocytes to adipocytes. Lipid accumulation visibly observed under microscope and further assays were conducted to confirm the viability of adipocytes.

2.2.2 HCC cell lines and hepatic stellate cells

All the experiments were conducted using Bel-7402 and PLC/PRF/5. PLC/PRF/5 were purchased from American Type Culture Collection; Bel-7402 was gifted from Shanghai Institute of biological sciences. All the cells were grown in Dubecco's modified Eagle minimal high glucose essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin and streptomycin (GIBCO). Immortalized Hepatic stellate cells (HSC- hTERT) were kindly provided by Dr. Stephanie Ma's lab (HKU, Hong Kong) and were cultured according to lab's protocol in pre-warmed DMEM supplemented with 10% FBS, 2% penicillin and streptomycin and 0.1% Antibiotic- Antimycotic (GIBCO). All cell cultures were

kept in incubator at 37°C with 5% CO_2 and culture medium refreshed every two days.

2.2.3 Patient derived xenograft

Patient derived xenograft (PDTX) were sub-cultured by subcutaneously injecting into the flanks of nude mice. Fresh HCC samples were washed with serum free DMEM/F-12 medium and finely minced into small pieces of around 1mm X 1mm in size. The tissues were then transferred into a gentleMACS C tube (Miltenyi Biotec GmBH) containing 20µg/ml DNase I (Roche) and 4µg/ml liberase (Roche) in DMEM/F-12 medium. The tissues were then homogenized with gentleMACS dissociator (Miltenyi Biotec GmbH) using the built-in protocol for human tumor homogenization according to manufacturer's instruction. The homogenized tissues were then passed through a 100µm cell strainer (BD Biosciences), washed twice with DMEM/F-12 medium and centrifuged at 700rpm for 5 minutes. The cell pellets were resuspended with DMEM/F-12 and the viability of cells evaluated by trypan blue (Sigma) staining. The live cells were then resuspended and mixed with Matrigel™ Matrix (BD Biosciences) in 1:1 ratio and subcutaneously injected into the mice for further culturing. The live cells were used for further experiments.

2.2.4 Cell counting

Cells upon reaching confluency and also for normalizing the cell numbers in experiments, cells were trypsinized with 0.5- 4ml of 0.25% TrypLE Express (Life Technologies) depending on the culturing dish area. Upon addition of TrypLE Express, cells were incubated at 37°C for 3 minutes. After the cells were detached from the dish, equal volume culture medium containing 10% FBS was added to inactivate the TypLE Express. The cells were centrifuged at 1500rpm for 3 minutes. The cells were re-suspended in fresh culture medium. For counting, cell suspension

was mixed with 0.4% trypan blue solution (Sigma) in 1:1 ratio and 10µl of the mixture loaded onto hematocytometer. Average of the cells from the four quadrants were calculated and the concentration of cells per ml were calculated using the following equation:

$$\text{Number of cells in suspension/ml} = \frac{\text{Total no. of cells in all 4 quadrants}}{4} \times 2 \times 10^4$$

2.2.5 Co-culture setup

In order to imitate the tumor microenvironment of liver cancer with surrounding adipocytes which may not involve physical contact between the two cells, a co-culture set up was established which allows the interaction between the cells without physically contacting each other. For this purpose, adipocytes were co cultured with cancer cells in 12 well plate. Adipocytes and preadipocytes were trypsinized and seeded on the insert at around 4×10^4 cell density in a serum free medium. Millipore 0.4µm (Millipore) inserts were used that allows secretomes to be transferred from one system to another and prevent the transfer of cells through hence imitating the natural state of delivery of secretomes from adipocytes to liver through the intra hepatic portal. The cells were incubated in serum free medium for 72 hours before proceeding towards other functional assays. For sphere formation assay, this set up was continued until the end of the experiment.

2.2.6 Collection of conditioned medium (CM)

Adipocytes, Preadipocytes, HSC-hTERT and HCC cells were seeded in 6 well plate until 70-80% confluency was attained. Their respective serum containing medium was removed and was washed twice with serum free medium in order to remove serum contents prior to collection of CM. A total of 1400µl of serum free medium was added per well and incubated for 72 hours at 37°C. The CM so obtained were centrifuged at 3000rpm at 4°C for 5 minutes to remove any cells and debris. A total

of 1ml of medium was collected per well. CM used for experiments was stored at -80°C for a maximum of three days. All cells were washed with their respective fresh culture medium prior to adding its respective medium to replenish. Cell conditions were highly monitored prior to collection of CM.

2.2.7 Oil red O staining

Adipocytes upon full differentiation becomes fatter and rounded compared to its predecessors- preadipocytes. This was observed while the differentiation process was on the way with rounded lipid like vacuoles were observed in the cells while undergoing differentiation. Lipophilic dye- oil red o was used to stain lipid vacuoles to confirm the differentiation of adipocytes. Oil red O was conducted as described (Hashimoto et al., 2010). Briefly, adipocytes were washed twice with PBS. Fixed with 4% paraformaldehyde for 30 minutes. The cells were washed again, incubated with isopropanol for 5 minutes and stained with oil red o working solution for 15 minutes and washed with PBS and observed under microscope. The cells were also stained for hematoxylin for 5 minutes to stain nucleus. Photos at 20× and 10× magnifications were taken using light microscope with nucleus stained blue and lipid stained red.

2.2.8 Spheroid formation assay

Single cells were seeded at 300 cells (unless stated otherwise) per well in non-adherent 24-well plate (unless stated otherwise) pre-coated with 1% poly (2-hydroxyethylmethacrylate) (Sigma). Each well was plated with 300µl DMEM/F-12 medium (GIBCO) containing 0.25% methyl cellulose (Sigma), supplemented with 1× B27 (Life Technologies) and 4µg/ml insulin (Sigma). Spheres were supplemented with 70µl of supplemented medium every other day with the same ratio of supplements (unless stated otherwise). Spheroid formation was observed

on a daily basis and the experiment terminated upon formation of spheres larger than 100μm at 20X magnification and with clear difference between groups. The spheroids so formed were counted and their image captured under light microscope.

2.2.9 Transwell assay-Migration and invasion assays

Migration and invasion assays were carried out using a 24 well transwell hanging inserts (Millipore) with PET membrane of 8.0μm pore size in which cells in the insert were incubated in serum free medium and the lower compartment with full medium. Parental cells at 3×10^4 were seeded on top of the wells in a serum free medium with CM supplemented with 10% serum as a chemo attractant. Cells were allowed to migrate for 24 hours or otherwise stated) and the transwell cells were fixed with paraformaldehyde for 15 minutes and stained with crystal violet for 30 minutes followed by washing with double distilled water. Remaining stains were removed using a cotton swab. Similar to migration, invasion was carried out in a Boyden chamber transwell system with serum supplemented CM in bottom. For invasion however, the inserts were coated with a thin layer of Matrix gel diluted with DMEM/ F12 at 1:3 ratio.

Minimum of three fields were photographed at 4× magnification and the migrated and invaded cells counted, and the average calculated to study the relative migration between different conditions.

2.2.10 Annexin V/propidium iodide apoptosis assay

Double staining for Annexin V-FITC and propidium iodide (PI) was performed for analyzing cell viability after treating with chemotherapeutic drugs. 1×10^5 cells for PLC/PRF/5 and 1.5×10^5 for Bel-7402 were seeded in 12 well plate, 1-2μg/ml doxorubicin (EBEWE pharma) was applied for 24 hours along with CM. After incubation, both floating and attached cells were collected and re-suspended in 1×

Annexin V binding buffer (BD Pharmingen), 1µl of FITC-Annexin V(MBL) and 10µl of PI (Sigma) and incubated at room temperature for 15 minutes in dark. Following incubation, 400µl of 1X binding buffer was added to stop the reaction and the cells were subjected to flow cytometry analysis using Accuri C6 flow cytometry. The data was analyzed using Flow Jo software to generate graphs and quantification of data. Unstained, single stain with Annexin V and PI were used as controls.

2.2.11 Liver CSC markers analysis

1×10^5 cells were incubated at 4°C for 30 minutes with direct fluorochrome-conjugated primary antibody in 1×PBS supplemented with 2% FBS. The experiment setting was protected from light. Samples were analyzed using BD FACS Accuri flow cytometer and Flowjo, LLC software. Samples were consecutively incubated with fluorochrome-conjugated, isotype- matched mouse or rabbit immunoglobins for respective controls. The antibodies, their respective source and working dilutions are listed in Table 2.3.

2.2.12 Total protein extraction

Total proteins from cell lysates were extracted using RIPA buffer containing 50mM Tris-HCL pH 7.4, 1% NP-40, 0.05% SDS, 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM PMSF, 1× complete protease inhibitor (Roche) and 1× phosphoSTOP phosphatase inhibitor (Roche). The lysate with the cocktail of lysis buffer was re-suspended using pipette and incubated on ice for 15 minutes. The protein lysates were then centrifuged at 13000 g for 15 minutes at 4°C. The supernatant was collected, and the protein concentration determined by Bradford assay (Bio-Rad Laboratories). Standard curve using known concentration of BSA was used to create standard curve using Bradford reagent. Protein was diluted in

1×Bradford solution in 1:500 ratio and the absorbance measured at 595nM using a spectrophotometer.

2.2.13 Western blot assay

20-60µg of protein samples were mixed with 6× SDS loading buffer (0.35M Tris-HCL pH6.8, 30% glycerol, 21.4% ß- mercaptoethanol, 10% SDS and 0.05% bromophenol blue) and boiled at 95°C for 5 minutes. Based on the molecular weight of the proteins to be evaluated, resolving gel from 6-15% SDS polyacrylamide gel were made. Pre- stained standard marker (Bio-Rad) was loaded parallel to the protein lanes. Gel electrophoresis was performed at constant voltage of 100 volts for 1.5-2.5 hours. Following electrophoresis, proteins were transferred from gel to methanol activated PVDF membrane (Millipore) at constant voltage of 100 volts for 1.5 hours.

The membrane was then washed with TBS-T and blocked in 5% non-fat milk in TBS-T or 5% BSA (based on the respective primary diluting reagent) at RT for 1 hour. The blocked membrane was then incubated with respective primary antibody, diluted in either 5% BSA or 5% non-fat milk overnight at 4°C. After primary antibody probing, the membrane was washed at least three times with TBS-T, each for 10 minutes. The membrane was then incubated with HRP-conjugated secondary antibody, diluted in their respective diluent for an hour. The membrane was then washed for three times, each for 10 minutes. Protein signal was developed using enhanced chemiluminescence (ECL) system (GE Life Sciences) and then exposed on X-ray film in dark. The antibodies used, along with the source and working dilutions were listed in Table 2.3.

2.2.14 Extraction of RNA

Total RNA was extracted using TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. Cells were homogenized with 1ml of Trizol medium and incubated at RT for 5 minutes. Upon incubation, 200µl of chloroform was added to the sample. The samples were mixed vigorously and incubated at RT for 5 minutes. The mixture was then centrifuged at 12000 g at 4°C for 15 minutes. The RNA mixture is thus separated into protein, DNA and aqueous RNA solution which is transferred to a new tube. In order to precipitate RNA, 500µl of isopropanol was added and the tube inverted 7-8 times and then incubated at RT for 20 minutes. The solution was then centrifuged at 12000 g at 4°C for 15 minutes. The RNA pellet was then washed with 1 ml of 70% ethanol and ethanol discarded upon centrifugation at 7600 g for 5 minutes. The pellet was then air dried at RT. RNA was dissolved using DEPC-treated water and the quantity and quality of RNA were assessed using BiodropµLITE with A260/280>1.8 and A260/230>2.0.

2.2.15 Complementary DNA (cDNA) synthesis

cDNA was synthesized from total RNA extracted using GeneAmp Gold RNA PCR Core Kit (Applied Biosystems) according to the manufacturer's instructions. 1µg of RNA was used for cDNA synthesis. RNA was mixed with 5µM oligo dT and 1mM dNTPs, 2× buffer with a final volume of 10µl with DEPC-treated water. Synthesis proceed in PCR machine with a cycle of 15 minutes of heating at 37°C for 15 minutes followed by 85°C for 5 seconds and cooling down at 4°C. The cDNA so synthesized were diluted with DEPC-treated water in 1:4 ratio and incubated at 4°C for 5 minutes prior to usage.

2.2.16 Quantitative polymerase chain reaction (qPCR) analysis

Reaction mixture comprising of 1× SYBR Green (Applied Biosystem) and 0.2µM forward and reverse qPCR primers with a total volume of 10µl with DEPC-treated water is used per well in an optical 96-well plate. 2µl of the diluted cDNA is added in each well. The reaction was performed in ABI 7900HT Fast Real Time PCR System (Applied Biosystem) and was monitored real time with SDS 1.9.1 software (Applied Biosystems). The amplification plots were analyzed using RQ manager 1.2 software (Applied Biosystems). All qPCR reactions were performed in triplicate, and the amount of target gene expression was calculated by relative quantification method with respective housekeeping gene as the normalization. The primer sequences used are listed in Table 2.4.

2.2.17 Mass spectrometry analysis of CM

To identify the possible components of the secretomes of the adipocytes, we analyzed the CM obtained after incubating adipocyte in serum free medium for 72 hours using mass spectrometry. The CM was centrifuged at 3000 rpm for 5 minutes and a total of 1 ml of CM was analyzed using Orbitrap mass spectrometry. For this purpose, phenol free, serum free DMEM HG supplemented.

2.2.18 Organoid assay

All organoid assay was conducted using serum free, supplement free Ad++ medium. CM for organoid assay was collected by incubating adipocytes in serum free, supplement free Ad++ medium for 72 hours. The medium was then centrifuged at 3000rpm for 5 minutes, aliquoted in eppendorf tube and frozen at -80°C for future use.

2.2.18.1 Culturing of organoids

Patient derived organoids were established in Dr. Stephanie Ma's Lab. All the organoids were supported by matrix gel for the formation of organoids. A full formation of organoids was observed in about 10 days. Organoids were supplemented every three days. All the experiments were conducted twice.

2.2.18.2 Invasion assay-organoids

Invasion assay for organoids were performed using a pre-coated insert. A total of 1 x 10^5 cells were seeded onto the insert supplemented by essential supplements and the lower compartment filled with Ad++ medium incubated in Adipocytes for 72 hours and supplemented with essential supplements and 20% FBS. The cells were incubated for 9 days with supplementation and also in the lower chamber.

Invaded cells were fixed using paraformaldehyde as mentioned in invasion assay for cells and stained with crystal violet prior to observing the trend and taking pictures and the invaded cells counted per picture and the average calculated.

2.2.19 Recombinant human fatty acid binding protein 4 (rhFABP4)

rhFABP4 was purchased from Biovision (BioVision) and diluted in milli Q water as per manufacturer's instructions. The proteins were stored at -20°C and the working concentration was prepared freshly before adding to cells. The concentration of rhFABP4 was adjusted based on in house ELISA experiment.

2.2.20 FABP4 inhibitor (BMS309403)

Recombinant Human FABP4 protein specific inhibitor were purchased from Cayman (Cayman) and diluted in DMSO as per the manufacturer's instructions. Working vials were stored at -20°C and stock stored at -80°C. Control groups involving the inhibitor had Control medium diluted with same volume of DMSO to

prevent alterations in result because of DMSO. The concentration of DMSO was kept lower than 1% in all the assays.

2.2.21 RNA sequencing

RNA sequencing of the CM incubated and recombinant proteins incubated cell lines were subject to RNA sequencing by external sequencing company- HKU CGS (Hong Kong) and Novogene (Novogene Inc.) to analyze commonly de-regulated pathways. Purified RNA was extracted from CM treated Bel-7402 and recombinant FABP4 treated PLC/PRF/5 and Bel-7402 were extracted using TRIzol reagent (Life Technologies) according to manufacturer's protocol. The quality of the total RNA was checked by Thermo Scientific Bioanlayzer (Thermo Scientific) to have OD260/280 ratio of between 1.8-2.0. RNA integrity was further assessed by running the samples in an agarose gel prior to sending samples for Noveogene analysis. The RNA samples were then subjected to Illumina Solexa Sequencing using Hiseq 1500 sequencer (Illumina). Specific library construction, HiSeq sequencing run and data analysis were performed by Solexa Sequencing service team at Centre for Genomic Sciences in University of Hong Kong. All data was expressed as fragments per kilobase of exon per million fragments mapped (FPKM) values and fold changes in transcript levels relative to the respective controls. All data were then further shortlisted with $p<0.05$ and with relative fold change of greater than 1.3. The shortlisted list was then fed to publicly available pathway analysis – DAVID Bioinformatics (DAVID).

2.2.22 Enzyme linked immunosorbent assay (ELISA)

The concentration of FABP4 in the CM were measured using DuoSet ELISA (R&D Systems) supplemented with DuoSet ancillary Reagent Kit (R&D Systems). In order to determine the concentration of FABP4 in various cancer cell lines –

PLC/PRF/5, Bel-7402, adipocytes, preadipocytes, HSC-hTERT and also in the co-culture set up, the CM were collected as described in Section 2.2.6. The concentration of FABP4 were measured using DuoSet ELISA Human FABP4/A-FABP (R&D Systems) according to manufacturer's instructions. Human FABP4 standard were provided with the kit and seven standards of decreasing concentration from 4000pg/ml to 62.5 pg/ml was prepared by serial dilution using reagent diluent provided in DuoSet ancillary Reagent Kit. CM was 50-fold and 20-fold diluted with assay buffer. 100µl of the standard and samples were added per well. The plates were pre-coated with capture antibody diluted in coating buffer as recommended. As per the protocol, the coated wells were blocked using Reagent diluent for an hour and half and then the samples added and incubated for next 2 hours. Detection Antibody diluted in reagent diluent were then added to the wells and incubated for 2 hours at room temperature followed by incubation with Streptavidin- HRP for 20 minutes. The wells were washed a total of three times with wash buffer before proceeding to next step. The wells were then added with substrate solution and incubated in dark for 20 minutes. Stop solution of 50µl was added to each well to stop the reaction and the optical density measured using clariostar (Clariostar) at 450nm and 570nm. The standard curve was plotted by using the standards and the concentration of FABP4 calculated accordingly.

2.2.23 β-catenin TCF binding assay (TOP/FOP assay)

β-catenin activity was examined using luciferase reporter assay of TCF/LEF dependent transcription. 600ng of Firefly luciferase pSuper8XTOPflash or pSuper8XFOPflash constructs (gifted from Dr. Moon R, University of Washington, USA) together with 20ng of renilla luciferase pRL-CMV construct from Promega was transfected into HCC cells using lipofectamine®2000. Luciferase activities

were assayed using Dual- Luciferase® Reporter Assay system (E1910, Promega) as per manufacturer's instructions and the signal measured with clariostar. Results expressed as relative luciferase activity (firefly/renilla luciferase).

Section 2.3 *In vivo* studies

2.3.1 Animals

All the experiments and experiment protocols were approved by and in full accordance with the committee of centralized Animal Facilities (CAF) at The Hong Kong Polytechnic University. Non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice aged between 4-8 weeks were used for experiments. Mice were housed in centralized Animal Facilities at The Hong Kong Polytechnic University.

2.3.2 *In vivo* tumorigenicity experiments

Various numbers of HCC cells were re-suspended in 50μl medium mixed with 50μl Matrigel™ Matrix (BD Sciences) were injected subcutaneously into the flanks of NOD/SCI mice using 27-gauge needle insulin syringes (Terumo). Mice were sacrificed between 1-4 months post injection and the tumors harvested. For those mice injected with tumor cells. Size and number of tumors were recorded with a photograph and the incidence was calculated.

Section 2.4 Statistical analysis

To study the correlation between the control subjects and the conditioned subjects, statistical analysis was performed applying independent student t-test using Prism software. All error bars in the graphs represent standard deviation. To study the correlation between the relative expression of FABP4 and the survival of patients, Kaplan-Meier survival analysis was used to determine both disease- free survival and overall survival, and the statistical significance calculated by log-rank test.

CHAPTER 3: ADIPOCYTES ENHANCED CSC PROPERTIES IN HCC VIA A PARACRINE SIGNALING

Section 3.1 Introduction

The first line of treatment is resection of the tumor followed by chemotherapy with targeted agents such as sorafenib and doxorubicin or radiotherapy. Although these therapies have been proven to be effective in some cases, the survival rate remains unsatisfactory over the past decades because HCC acquires chemo-resistance against these traditional therapies and subsequently develops an aggressive and metastatic phenotype, resulting in a poor prognosis of these patients. Recently, scientists in cancer research fields have been exploring the influence of cellular factors within the microenvironment including stromal cells, adipocytes, and immune cells on the cancer phenotype. Specifically, researchers are trying to dissect how cellular factors within the tumor microenvironment affect the plasticity of CSCs, which are believed to be at the root of tumor recurrence and therapeutic resistance. Several studies have reported that cells within the tumor microenvironment enhance the stem cell-like properties of various cancer types, including prostate, breast and gastric cancer (Kise et al., 2016). Most recently, increasing numbers of studies are being focused on the interaction between cancer cells and immune cells, which may lead to the development of immunotherapy against cancer. One of the potential directions for the study of the cancer microenvironment and its influence on CSCs has been found to be stromal intervention.

For instance, the secretomes from cancer associated fibroblasts (CAFs) have been found to regulate the plasticity of liver CSCs via paracrine signaling (Lau et al., 2016). Apart from CAFs, adipocytes, being one of the major cellular factors within the microenvironment, may also play a major role. This hypothesis is supported by the recent reports on the role of adipocytes secretomes on the regulation of cancer

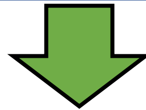
phenotypes such as proliferation, invasiveness, tumorigenicity and chemo- and radio- resistance. Furthermore, with the strong correlation reported by Calle in 2003 regarding the correlation between obesity and cancer-related mortality a decade ago, research on the influence of adipocytes in cancer has gained increasing attention in recent years (Calle et al., 2003). With the establishment of this relationship, the association and influence of adipocytes in various cancer types such as breast (Hoy et al., 2016), prostate (Laurent et al., 2016), gastric (Xiang et al., 2017), leukemia (Behan et al., 2009), pancreatic (Meyer et al., 2016), ovarian (Nieman et al., 2011) and others has been widely studied. The influence of adipocytes on CSCs has only been reported for prostate cancer, which showed enhancement of the self-renewal ability of prostate cancer cells via the paracrine effect of adipocytes (Tang et al., 2015).

With our aim to shed light on the relationship between liver CSC properties and adipocytes and the possible promoting effects conferred by adipocytes, in this chapter, we first functionally characterize the effects of adipocytes on HCC cells using HCC cell lines, patient-derived tumor xenografts (PDTX), and *in vitro* organotypic *ex vivo* human HCC clinical samples. We will mainly focus on the effects of adipocytes on the modulation of CSC properties using various *in vitro* and *in vivo* functional assays. This chapter will be the root of our experimental hypothesis to delineate the functional mechanism of adipocytes on regulation of liver CSCs and is therefore of high importance.

Section 3.2 Experimental scheme

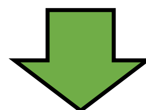
Differentiation of human visceral adipocytes

Differentiation of preadipocytes to adipocytes upon administration of differentiation medium containing a cocktail of IBMX, indomethacin, insulin and dexamethasone for 14–20 days



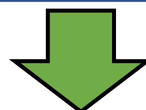
Characterization of differentiated adipocytes

- Observation of morphological change from elongated spindle-like preadipocytes to a relatively rounded structure with a lipid-like intracellular structure
- Oil red O staining of intracellular lipids
- Expression of genes specific to adipocyte differentiation—PPARγ and FABP4

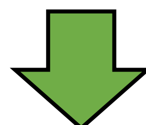


Investigation of the mode of communication between adipocytes and HCC cells and their effects on tumorigenicity

- Co-culture sphere formation set up between adipocytes and HCC along with preadipocytes as a control
- Subcutaneous injection of adipocytes and preadipocytes co-cultured cells into NOD/SCID mice



Adipocytes, not preadipocytes, enhance abilities of tumorigenicity and self-renewal in HCC cells in a paracrine-dependent manner



3.2 Experimental scheme (cont'd)

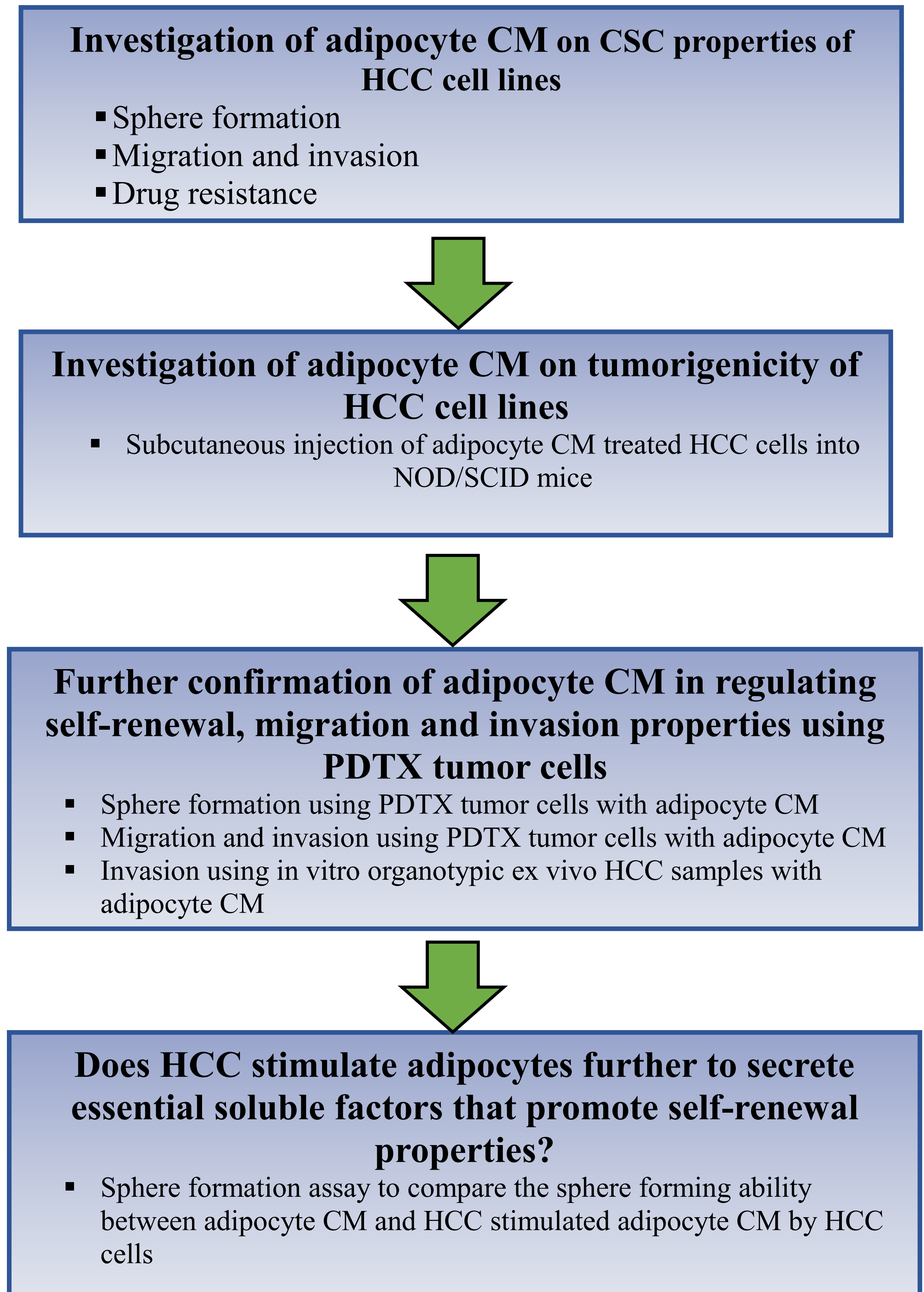


Figure 3.2 Experimental scheme of chapter 3.

Section 3.3 Results

3.3.1 Successful differentiation of functional visceral adipocytes in the presence of lipids

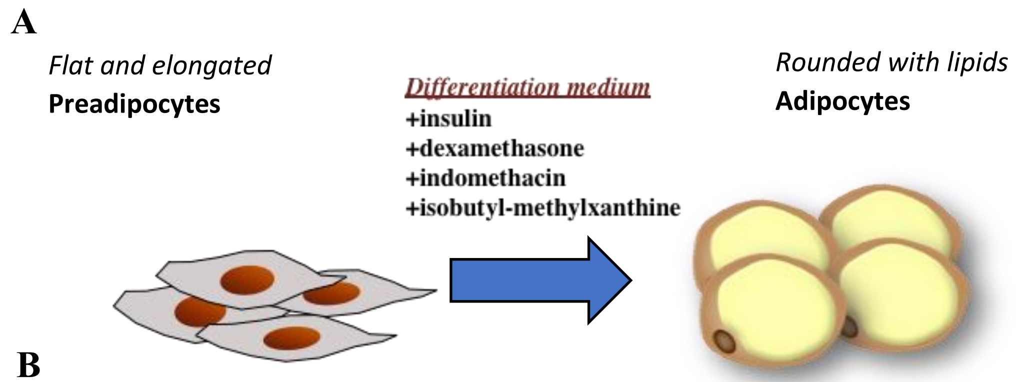
Adipocytes are a special cell type exhibiting features different from their precursors (preadipocytes and mesenchymal stem cells) and are widely known for their ability to store fats, hence the striking feature of the accumulation of intracellular lipids. Therefore, prior to starting our experiments on the effects of adipocytes in HCC cells, we had to differentiate human adipocytes from their precursors and well characterize them. Since the liver is an internal organ surrounded by visceral adipocytes, we purchased primary human visceral preadipocytes (PT5005) from Lonza. Based on the information provided by the manufacturer, the cells were obtained from nondiabetic normal donors and were not immortalized, and thus the cells were only used for differentiation until passage 5 at the most. As per the manufacturer's recommendation, we cultured the preadipocytes until they were 70–80% confluence before proceeding with differentiation. A differentiation cocktail with a mixture of IBMX, insulin, dexamethasone, and indomethacin were prepared with the base-preadipocyte growth medium supplemented with 10% FBS, GA-1000 and glutamate. Upon initiation of differentiation, morphological changes in the preadipocytes could be observed as shown in Figure 3.3.1 (A) with obvious changes in morphology from flat, spindle-like fibroblasts to more rounded differentiated adipocytes. Lipid-like vacuoles could be observed after approximately 5 days of incubation in differentiation medium.

Before proceeding with the experiments, it was important to confirm successful establishment of differentiated adipocytes in addition to observation of morphological changes. To confirm the lipid-like vacuoles were indeed lipid deposits, we stained the differentiated adipocytes with Oil red O, a fat-soluble dye

used for staining neutral lipids and triglycerides (Kraus et al., 2016). As shown in Figure 3.3.1 (B), the vacuole-like structures stained positive for Oil red O. Unlike the differentiated adipocytes, the preadipocytes did not contain the vacuole-like structure and showed no obvious Oil red O dye staining intracellularly, confirming the accumulation of lipids were only present in differentiated cells. These features confirmed that the differentiated cells were adipocytes. In addition, specific genes related to adipocyte differentiation including PPARγ and FABP4 (Ntambi and Young-Cheul, 2000; Ullah et al., 2013) were also examined in the differentiated adipocytes and their preadipocyte counterparts. FABP4 is a well-known lipid chaperone that is in high demand during adipogenesis when more lipids are generated intracellularly, while PPARγ is a well-known transcription factor in adipocytes known to regulate adipogenic genes involved in maintaining mature adipocytes (Ullah et al., 2015). Therefore, to functionally characterize the adipocytes, determining the expression of these genes was important.

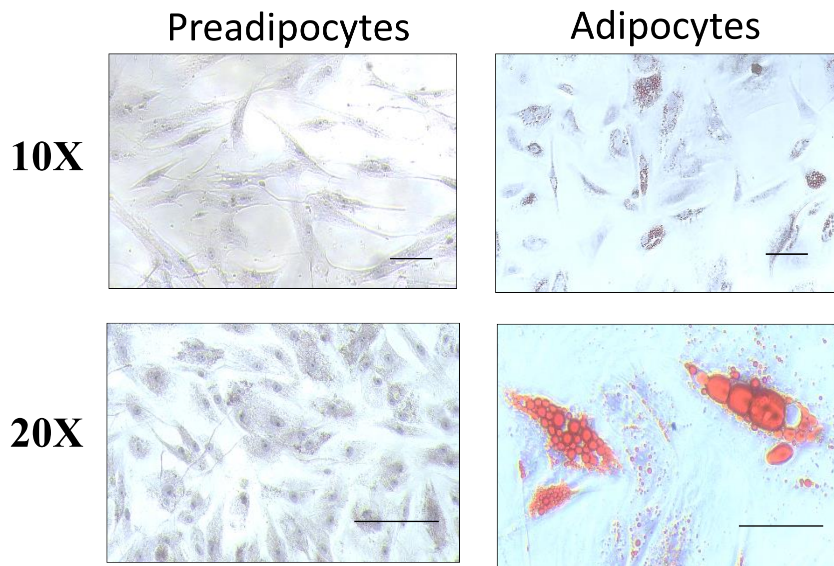
To examine the expression levels of these two genes, we extracted RNA from the differentiated adipocytes and preadipocytes and analyzed the relative expression of these genes using qPCR. In line with the morphological changes from preadipocytes to adipocytes, the expression levels of both FABP4 and PPARγ were found to be markedly upregulated as demonstrated in Figure 3.3.1 (C), indicating that the differentiated cells are indeed adipocytes and functional and thus can be used for further experiments. Collectively, these experiments confirmed the successful differentiation of preadipocytes into functional adipocytes.

A



B

Oil red O staining



C

Expression of adipocyte-specific genes

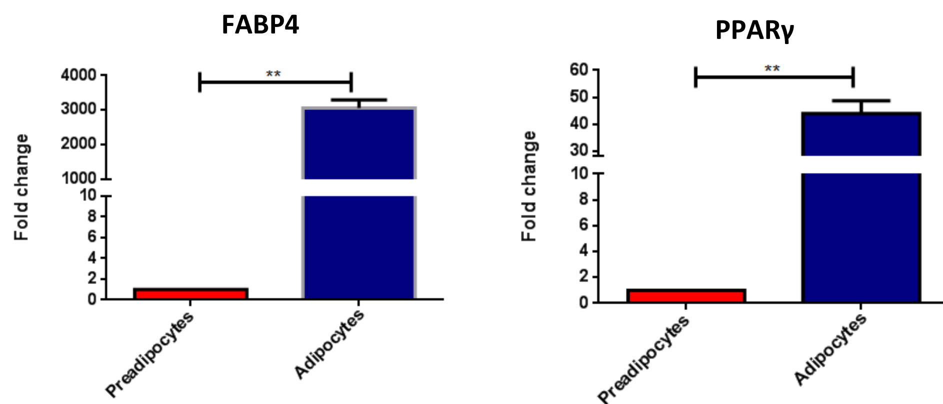


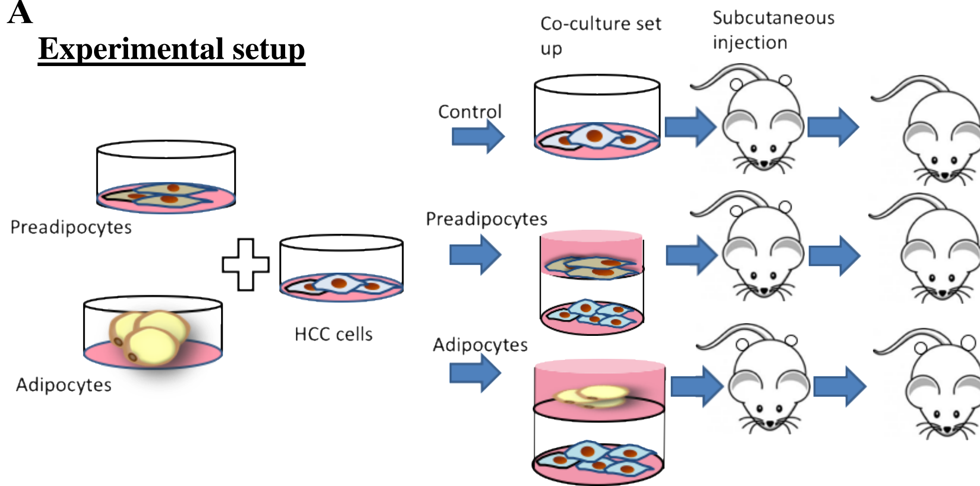
Figure 3.3.1 Characterization of functional visceral adipocytes differentiated from visceral preadipocytes. A) Schematic diagram demonstrating the morphological changes observed in preadipocytes from elongated to the more rounded morphology of adipocytes upon administration of differentiation cocktail to the medium. B) Oil red O staining of intracellular lipids (red) in adipocytes (a signature mark of differentiation into adipocytes) compared to their preadipocyte counterparts. Photos were taken using a light microscope at 10× and 20× magnification, respectively. The scale bar is 100μm. C) Relative expression of adipocyte specific functional genes (PPARγ and FABP4) is shown for both differentiated adipocytes and their preadipocyte counterparts. These results confirmed the successful establishment of differentiated adipocytes and hence they can be used for subsequent experiments with HCC cells.

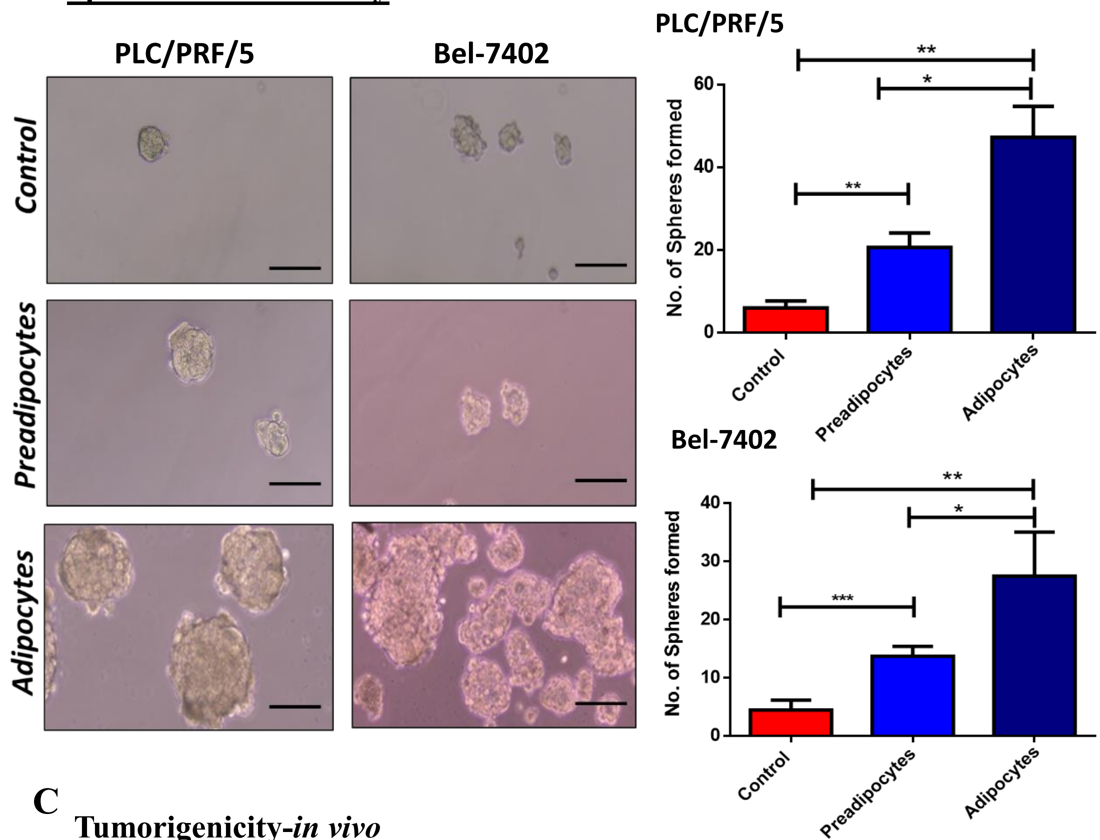
3.3.2 Adipocytes, not preadipocytes, enhanced self-renewal and tumorigenicity properties in HCC cells in a paracrine manner

For functional characterization of the adipocytes, we had to determine the effect of adipocytes on liver CSC properties. One of the key CSC properties is its ability to self-renew, thus having the ability to grow and form colonies. Therefore, we first investigated whether adipocytes confer any enhancement on the self-renewal property of HCC cells. Since we hypothesized that the tumor microenvironment containing adipocytes played a detrimental role in conferring CSC properties in HCC, we elucidated the method of communication to determine whether the cells are required to physically be in contact with each other or if they communicate in a paracrine manner where the cells do not necessarily need to be in physical contact and therefore need stimulation from cancer cells. The effects of adipocytes on cancer cells with physical contact and in a paracrine manner has been previously reported in other cancers; however, in HCC, we had to confirm the mode of communication first. Paracrine communication provides greater support because the liver is surrounded by adipocytes in the form of visceral adipocytes and omental adipocytes. To mimic paracrine communication between the two cell types we set up a co-culture setting with adipocytes in the upper chamber insert with a pore size of 0.4 μM and HCC in the lower chamber as shown in Figure 3.3.2 (A). The cells were cultured in the same medium, allowing the transfer of soluble parameters across the membrane but no physical contact between the cells was possible. The self-renewal properties of HCC under the influence of adipocytes was then assessed in this setup. Similar to the adipocytes, another set with preadipocytes placed in the upper chamber was also established to confirm the effect observed was exclusive to the effect of adipocytes on the microenvironment. Since adipocytes are derived from preadipocytes and the microenvironment contains various stromal cells,

including preadipocytes, we had to verify that the effect is indeed from adipocytes. Self-renewal was assessed based on the spheroids so formed, and relative size reflected the self-renewal property of HCC cells. Differences in sphere number and size were already observed on the third day after setting up the experiment, and, as demonstrated in Figure 3.3.2 (B), adipocytes co-cultured with HCC cells developed larger spheroids compared to both control and preadipocytes; in addition, a higher number of spheroids were found in adipocyte co-cultured HCC cells. Compared to preadipocytes, adipocytes had twice the effect on spheroid formation and the size of the spheroids was found to be more than doubled. The setup and the results confirmed it is indeed adipocytes that significantly promote the self-renewal properties in HCC cells without the need of physical contact.

Upon confirmation of self-renewal properties enhanced by adipocytes, we wanted to investigate whether this enhancement in property could result in tumorigenicity or not. Bel-7402 incubated with adipocytes and preadipocytes were then subcutaneously inoculated into NOD/SCID mice at a density of 10,000, and the tumor formation was observed over a span of two months. As noted in Table 3.3.2 and demonstrated in tumors extracted from the mice in Figure 3.3.2 (C), adipocyte-incubated HCC clearly formed more and larger tumors in comparison to the control and preadipocytes, thus confirming adipocytes indeed conferred both self-renewal properties as well as tumorigenicity upon HCC cells. Using this experimental setup, we confirmed that adipocytes and HCC cells communicate in a paracrine manner, and thus, for further experiments, we decided to use adipocyte CM to study the role of adipocyte secretomes in the development of CSC properties.


A
Experimental setup
Co-culture set up
Subcutaneous injection
Control
Preadipocytes
Preadipocytes
HCC cells
Adipocytes
Adipocytes


B
Sphere formation assay
PLC/PRF/5
Bel-7402
Control
Preadipocytes
Adipocytes
PLC/PRF/5
No. of Spheres formed
60
40
20
0
**
*
**
Control
Preadipocytes
Adipocytes
Bel-7402
No. of Spheres formed
40
30
20
10
0
**
*
***
Control
Preadipocytes
Adipocytes

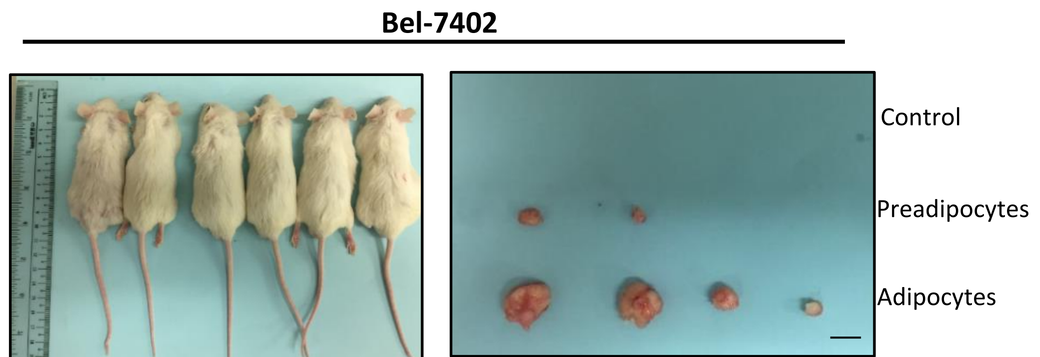

C
Tumorigenicity-in vivo
Bel-7402
Control
Preadipocytes
Adipocytes

Figure 3.3.2 Adipocytes enhanced self-renewal and tumorigenicity in HCC cells in a paracrine manner. A) Schematic drawing of the experimental setup for the sphere formation assay and *in vivo* subcutaneous injection for tumorigenicity to study the direct or indirect communication between cancer cells and adipocytes vs preadipocytes. A co-culture setup with adipocytes or preadipocytes placed in an insert of 0.4 µm preventing physical contact between two cell cultures. B) Representative spheres formed in three different conditions: control, preadipocytes and adipocytes co-cultured with both HCC cell lines (PLC/PRF/5 and Bel-7402) with their respective sphere numbers demonstrated in graphs. The results show cells cultured with adipocytes formed more and larger spheres than the cells cultured with the control or preadipocytes (* $p<0.05$, ** $p<0.01$, t-test). The scale bar is 100 µm. C) Co-cultured Bel-7402 cells were injected subcutaneously into NOD/SCID mice with two mice in each group: control, preadipocytes and adipocytes. As shown in the picture, larger tumors were found to grow from the adipocyte cultured cells, thus confirming that adipocytes play a pivotal role in the self-renewal properties of HCC cells. Furthermore, this also confirms the existence of paracrine communication between cancer cells and adipocytes. The scale bar is 10 mm. Hereafter, adipocyte CM was collected to study the communication between cancer cells and adipocyte differentiation of adipocytes and hence can be used for further experiments with HCC.

1×10^4 HCC cells injected	No. of tumors
HCC cells in serum free medium only	0/8 (0%)
HCC cells co-cultured with preadipocytes	2/8 (25%)
HCC cells co-cultured with adipocytes	4/8 (50%)

Table 3.3.2 Tumor incidence rate of co-cultured HCC cells. Bel-7402 treated in a co-culture setup with adipocytes and preadipocytes and in serum-free medium. The cells were co-cultured for three days prior to injection to NOD/SCID mice subcutaneously. All the cells were injected at same density of 10,000 cells. Tumor formation was observed after two months of inoculation and the relative incidence calculated.

3.3.3 Adipocyte CM solely enhanced CSC properties in HCC cells

Upon the confirmation of adipocyte paracrine communication, we examined the effect of adipocyte CM on liver CSC properties. For this, the CM was collected by incubating adipocytes in DMEM serum-free medium for three days. Day 3 was chosen as we observed remarkable changes after three days of co-culture. The CM was then used for further experiments. We first tried to investigate whether adipocyte CM by itself has the potential to enhance the self-renewal properties of HCC cells in both cell lines and therefore conducted a sphere formation assay in which the sphere assay medium and CM was mixed in a 1:1 ratio, and the sphere forming ability was assessed. As demonstrated in Figure 3.3.3 (A), the secretomes in adipocyte CM were able to promote self-renewal properties in both HCC cell lines (PLC/PRF/5 and Bel-7402). This confirmed the presence of key secretomes capable of promoting the self-renewal properties of HCC cells.

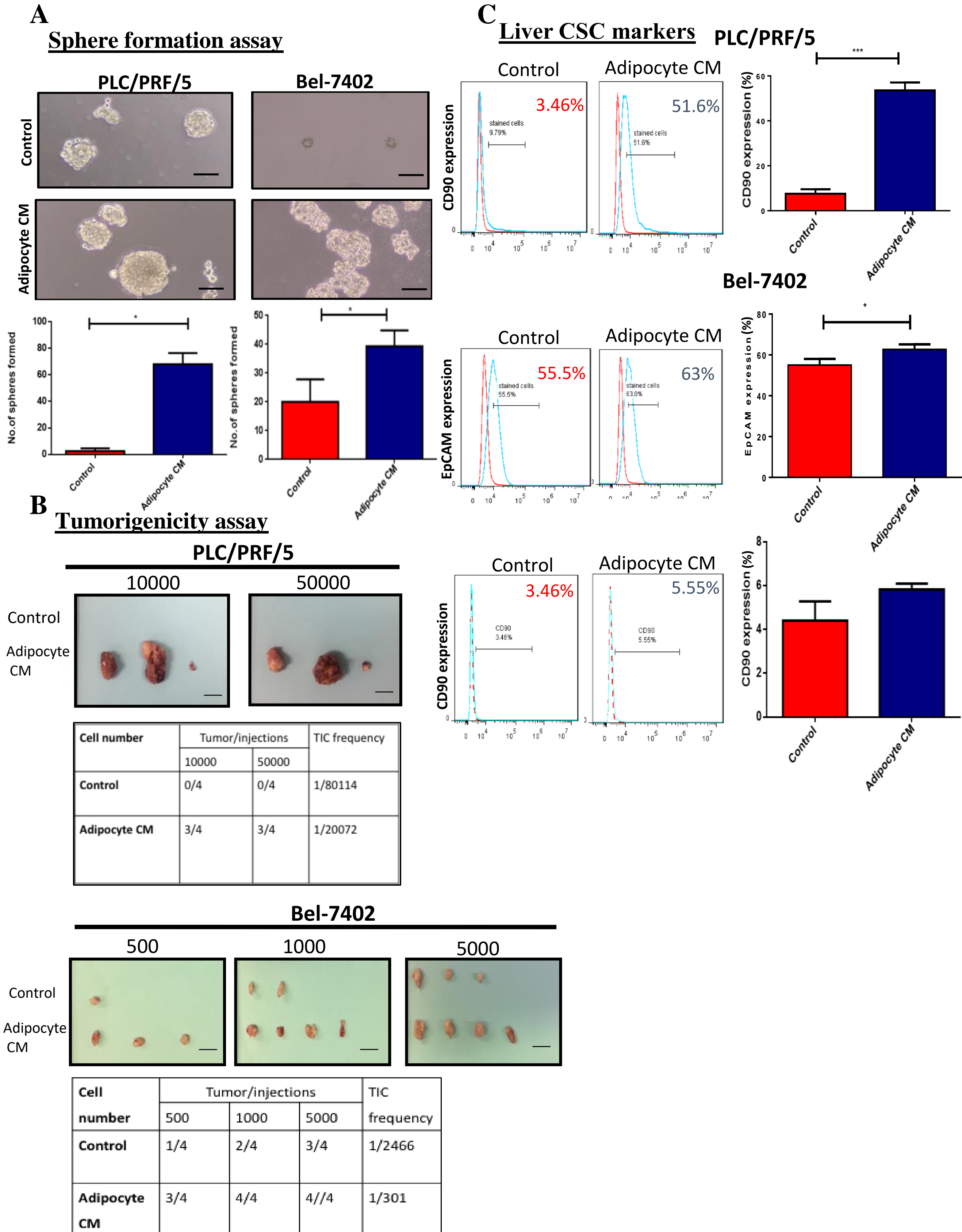
Previous assays confirmed the possible role of CM from adipocytes in promoting self-renewal and tumorigenicity in HCC cells from the co-culture setup. Next, we tried to elucidate the effect of CM in tumorigenicity. For this purpose, we subcutaneously inoculated the CM treated cancer cells into NOD/SCID mice. We performed a sequential inoculation with Bel-7402 and PLC/PRF/5 cancer cells at different densities (500, 1000 and 5000 cells for Bel-7402 and 10000 and 50000 cells with PLC/PRF/5). At all densities, CM treated cancer cells showed higher tumorigenic potential compared to their control counterparts as shown in Figure 3.3.3 (B). Although the tumor sizes after inoculation with a higher density of cancer cells were larger, compared to their control counterparts at different cell densities, both the tumor number and tumor size were found to be higher and larger, respectively, confirming the enhancement of tumorigenic potential due to exposure to the CM obtained from the adipocytes. This result also confirms significant tumor

initiating properties were attained by communicating with adipocytes cells but not with preadipocytes. Both results further verify the presence of a component in the CM from adipocytes that could play an important role in promoting the tumorigenic potential of cancer cells. Following this, we investigated other CSC properties in HCC cells upon administration of adipocyte CM.

We then investigated whether adipocyte CM enhanced the migration and invasive properties of HCC cells, which is important in metastasis since CSCs tend to evade and metastasize with self-renewal properties (Chen et al., 2013). To study this property, we incubated adipocytes in serum-free medium for 72 hours then collected the CM and introduced it into the bottom chamber of a trans-well migration chamber with an insert of 8 µm, which allows for the migration of cells from the insert chamber. CM was supplemented with 10% FBS to facilitate the migration of cells. Control-CM was obtained after incubating the cells in serum-free medium for three days. The migration of the cells as shown in Figure 3.3.3 (D) indicates that adipocytes CM drastically enhanced the migration of both Bel-7402 and PLC/PRF/5 cell lines. These data confirm the availability of a chemoattractant property available in CM in adipocyte CM that can promote cancer cell migration to other sites. Cancer cells migrating to other locations results in a poor prognosis, but cancer cells need to be able to invade through the extracellular matrix in order to do this. Therefore, to study whether adipocyte CM also promotes the invasive ability of HCC cells, we conducted an assay to study the invasive properties of the cells using a matrix gel that acts such as the extracellular matrix in the cell environment. Similar to the migration assay, adipocyte CM and control-medium supplemented with 10% FBS were used as a chemoattractant and, for both cell lines (Bel-7402 and PLC/PRF/5), showed significant differences in invasive capabilities

as shown in Figure 3.3.3 (D). These results confirm that adipocytes not only secrete components that increase the migration of the HCC cells but also boost their ability to invade. This enhancement in cancer cell properties could possibly aid in metastasis of cancer cells from their primary site to other sites. The sphere formation assays and migration and invasion assays so far have provided convincing data about a paracrine role played by adipocytes in enhancing various properties related to CSCs.

Stemness properties in HCC have been widely studied along with the discovery of liver CSC markers such as CD24, CD133, and EpCAM as described in Section 1.3.2 With accumulating evidence indicating the primary role of adipocytes in the development of CSC properties in HCC cells, we also determined if there were any changes in liver CSC markers in the presence of adipocyte CM. The expression of EpCAM and CD90 was analyzed and found to be upregulated in the presence of CM within 24 hours of incubation as demonstrated in Figure 3.3.3 (C). Interestingly, CD90 was found to be markedly increased in PLC/PRF/5. However, the basal level of EpCAM was found to be very low ~1% (data not shown) in PLC/PRF/5 making it not an appropriate cell line model to study the effects of adipocyte CM in its expression level. These results confirmed the vital role of adipocytes secretomes in enhancing key CSC properties.

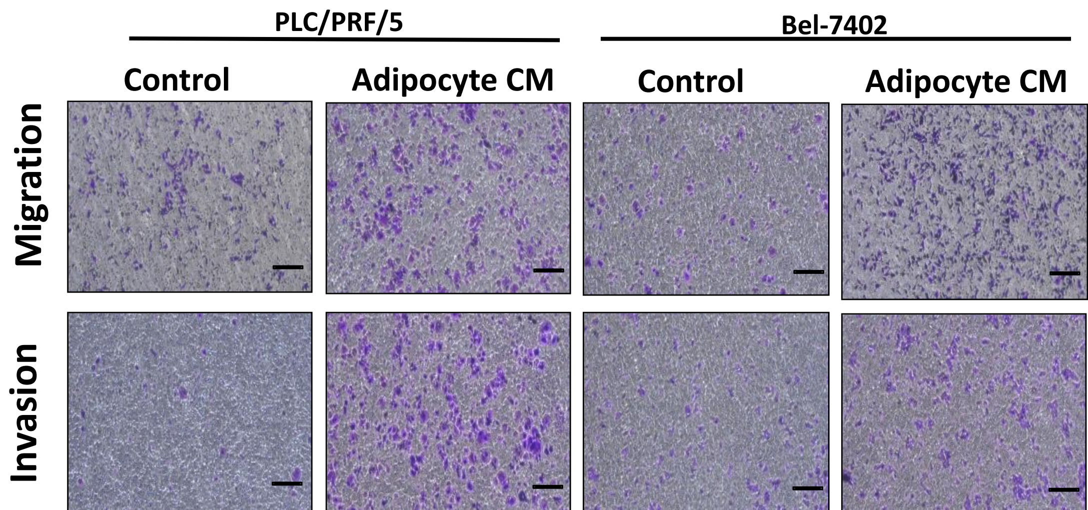


Cell number	Tumor/injections		TIC frequency
	10000	50000	
Control	0/4	0/4	1/80114
Adipocyte CM	3/4	3/4	1/20072

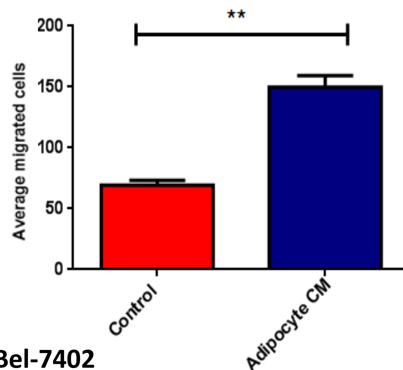
Cell number	Tumor/injections			TIC frequency
	500	1000	5000	
Control	1/4	2/4	3/4	1/2466
Adipocyte CM	3/4	4/4	4//4	1/301

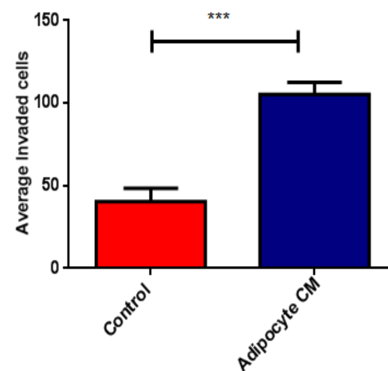
D

Migration and invasion assays

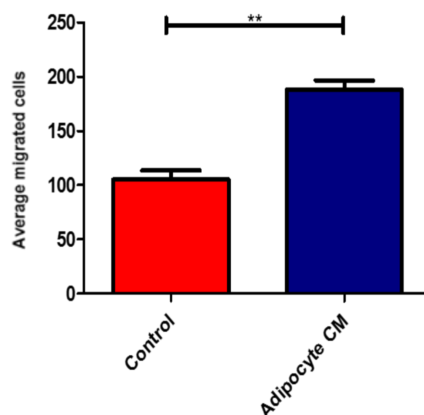


PLC/PRF/5





Bel-7402



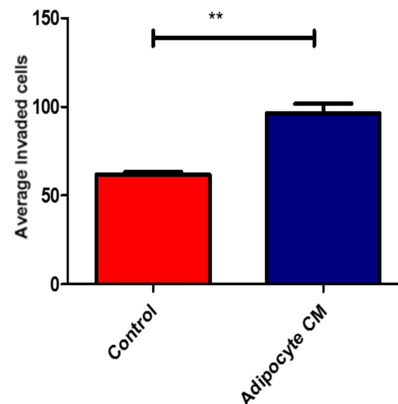


Figure 3.3.3 Adipocyte CM enhanced CSC properties in HCC. A) Adipocyte CM was able to enhance self-renewal properties in HCC cells. The scale bar is 100 µm. B) Serial dilution of adipocyte CM treated HCC cells also shows higher tumorigenicity in mice, which confirms its tumorigenic promoting ability of Adipocyte CM. C) Liver CSC markers- EpCAM and CD90 was found to be upregulated upon cultured with Adipocyte CM. D) Adipocyte CM was also found to significantly enhance both the migration and invasion abilities of HCC cells (PLC/PRF/5 and Bel-7402). The scale bar is 200µM. (* p<0.05, ** p<0.01, *** p<0.001, t-test).

3.3.4 Adipocyte CM alleviated drug sensitivity in HCC cells

One of the biggest hurdles in cancer research is the development of resistance by cancer cells against traditional therapies. Therefore, it is important we assess the effects of adipocyte CM on drug sensitivity too. It is well reported that there is a strong correlation between obesity and cancer mortality, it can be hypothesized that CSCs can play a role in this because one of their key characteristics is their ability to evade and resist traditional therapy treatment. With the establishment of adipocyte CM playing a role in self-renewal properties, tumorigenicity, expression of liver CSC markers, and promoting migration and invasive properties, we investigated whether the CM confers any resistance against traditional therapy with doxorubicin and the FDA-approved drug sorafenib. We treated the HCC cell lines (PLC/PRF/5 and Bel-7402) with 1.5μg/ml of doxorubicin and 6μM of sorafenib in the presence of CM. As demonstrated in Figure 3.3.4, in the presence of CM the cells were found to be less sensitive to both drugs since fewer cell deaths were observed in the apoptosis assay. Doxorubicin is a chemotherapeutic drug that acts via intercalation with DNA while sorafenib is a kinase inhibitor, and substantial resistance to the administration of these two types of drugs that function in completely different ways indicates that adipocytes secreted secretomes could not be drug specific but could confer resistance by simply promoting cancer cells' stemness properties. It is further interesting to note that in the presence of CM, resistance against a kinase inhibitor (sorafenib) was found to be more prominent in both cell lines, indicating the possible mechanism of adipocytes involves kinase pathways that need to be further elucidated. However, PLC/PRF/5 was treated with CM prior to drug administration, which indicates the mechanism could also vary based on different cell lines, but it does confirm adipocyte CM conferred resistance

to drugs in HCC cells, further strengthening our hypothesis that adipocyte CM aids in the development of CSC properties in HCC cells.

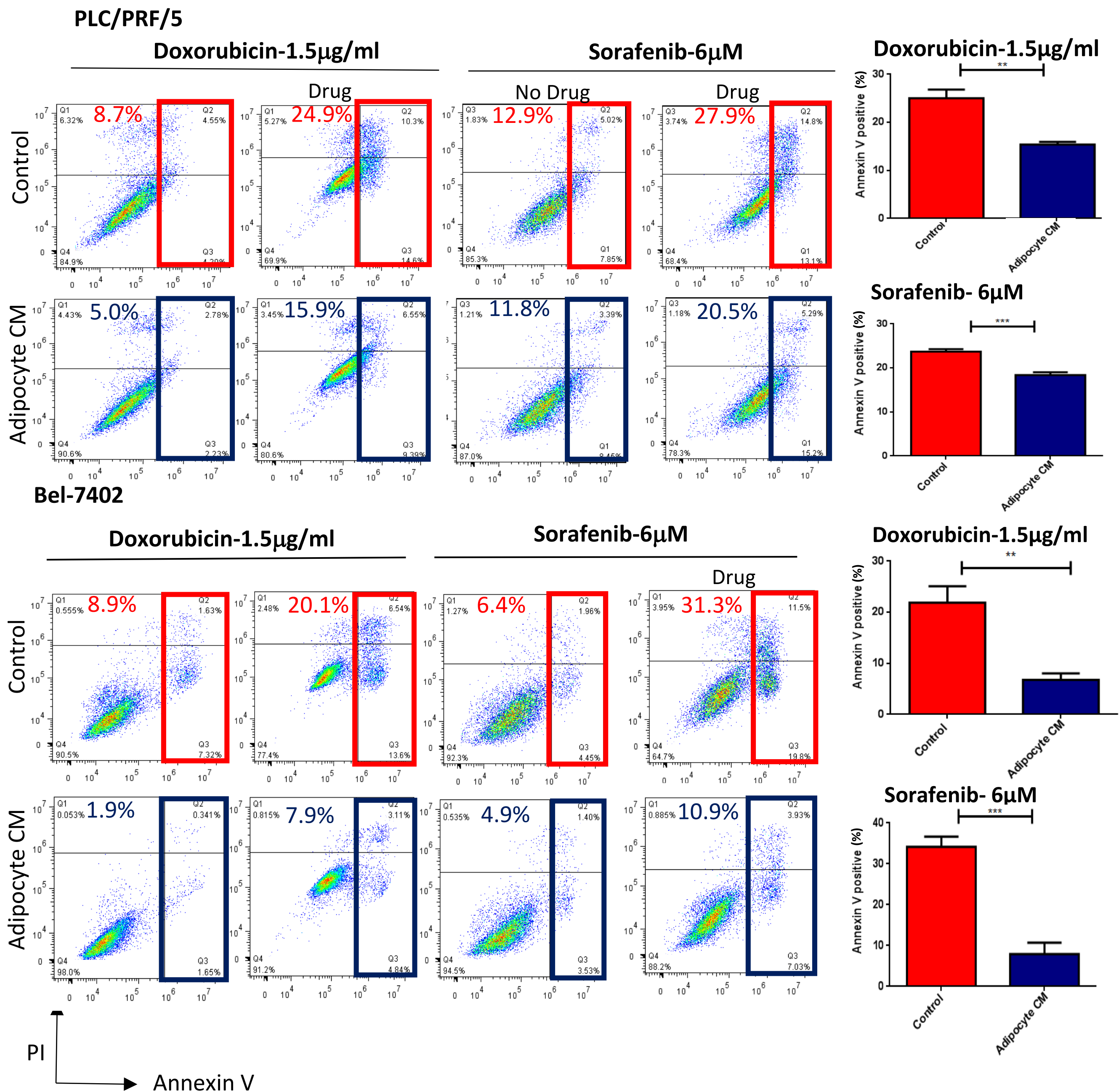


Figure 3.3.4 Adipocyte CM alleviated drug sensitivity in HCC. Adipocyte CM conferred resistance to both doxorubicin and sorafenib in both HCC cell lines as demonstrated by Annexin V assay (* p<0.05, ** p<0.01, *** p<0.001 *t* test). Cancer cells in the presence of adipocyte CM were found to be less sensitive to both drugs. A no-drug control was included to confirm that adipocyte CM alone did not affect cell survival.

3.3.5 Adipocyte CM ameliorated CSC properties in PDTX and organoids

In addition to HCC cell lines, we studied the effects of adipocyte CM on PDTX, which are obtained from patients and grown in mice. We investigated whether adipocyte CM enhanced self-renewal properties in PDTX and if it promoted their migration and invasion abilities. As demonstrated in Figure 3.3.5 (A), PDTX also showed enhanced self-renewal properties. PDTX was labelled with GFP therefore the identity of the spheres so formed was confirmed to be PDTX cells based on their GFP signal. Spheres so formed in PDTX were all GFP labelled as shown in Figure 3.3.5 (B). Additionally, both migration and invasive properties of PDTX was also enhanced in the presence of adipocyte CM as demonstrated in Figure 3.3.5 (C). The tumors propagated in mice were minced and dissociated and then centrifuged at 600 rpm to get rid of debris and other cells and then the cells were re-suspended using a syringe to dissociate them into single cells. This provided us with critical evidence that adipocyte CM indeed enhances self-renewal properties in both HCC cell lines as well as in PDTX. PDTX was also found to have enhanced properties of both migration and invasion.

Since PDTX are propagated using mice, we investigated if the same result of a high invasive nature could be reproduced in a pure patient-derived xenograft without any interference of other animals' microenvironment. Therefore, we studied the invasive nature of PDTX in the presence of adipocyte CM by making use of PDTX organoids to study this effect and as illustrated in Figure 3.3.5 (D), organoids treated with adipocyte CM was found to have enhanced invasive nature. As we hypothesized we confirmed by our previous experiments in both PDTX and HCC cell lines, adipocyte CM conferred invasiveness upon the organoid too. This result

further supports our hypothesis of a crucial role of adipocytes in regulating the CSC properties of HCC cells.

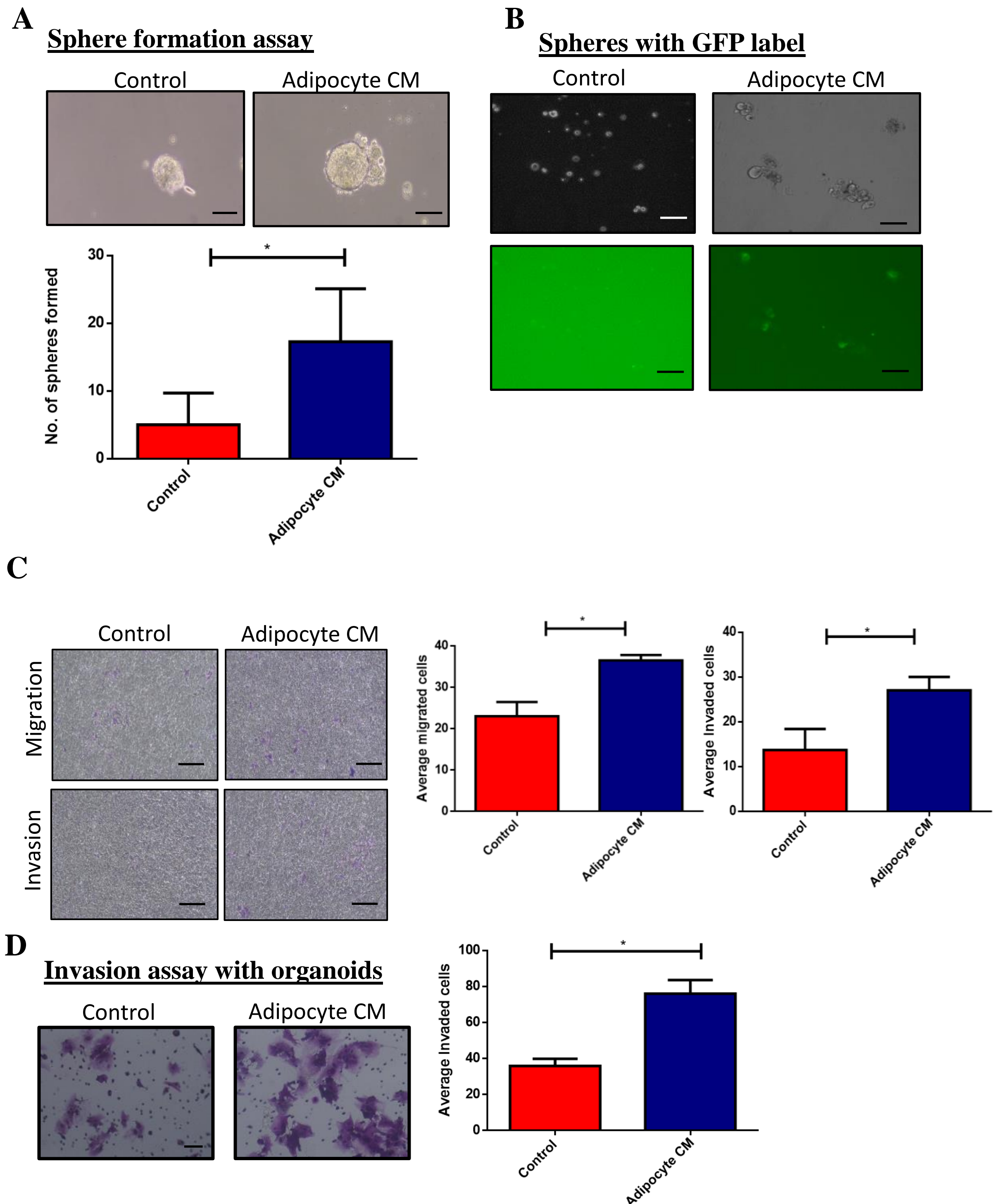


Figure 3.3.5 Adipocyte CM enhanced CSC properties- self-renewal, migration, invasion of PDTX and invasion of PDTX organoids. A) Similar to HCC cell lines, PDTX cells exhibited enhanced self-renewal properties in the presence of adipocyte CM. B) GFP labelled PDTX formed sphere in presence of adipocyte CM. The scale bar is 100 µM. C) PDTX also exhibited enhanced invasion and migration in the presence of adipocyte CM. D) Similar trend of enhancement in invasive properties was observed in PDTX organoids. Graphical representation of migrated and invasive cells is presented in graph. The scale bar for migration and invasion pictures is 200µM. (* $p<0.05$, *t* test)

3.3.6 Stimulated adipocyte CM further enhanced self-renewal properties in HCC cells

The existence of tumors in a microenvironment hints the possible manipulation of cellular factors providing a niche advantage to tumor survival. In line with this theory, we believe a possible existence of cross-talk between adipocytes and HCC cells that would further help in cancer progression. With this aim, we tried to investigate the possible effect of stimulated adipocyte CM on CSCs property of self-renewal. We hypothesized the possible reciprocal communication between adipocytes and HCC cells in which HCC cells could educate adipocytes to supply more of the key secretomes to further ameliorate its CSCs properties. Stimulated adipocyte CM (CAA-CM) was collected by incubating adipocytes with adipocyte CM previously incubated with HCC cells. Schematic drawing of the process is demonstrated in Figure 3.3.6 (A).

We conducted sphere formation assay to assess the effect on self-renewal properties of HCC Cell- PLC/PRF/5 with the CM. As demonstrated by both difference in size and number of spheres so formed, we demonstrate enhancement in sphere forming ability by stimulated CM. With reference to our results using co-culture setup in Figure 3.3.2 (B) compared to adipocyte CM only in Figure 3.3.3 (A), larger spheres were formed in co-culture set up indicating the possible reciprocal communication between the two cells. This result indicates the possible rise in secretion of key adipocytes secretomes in stimulated CM which will be investigated in the next chapter.

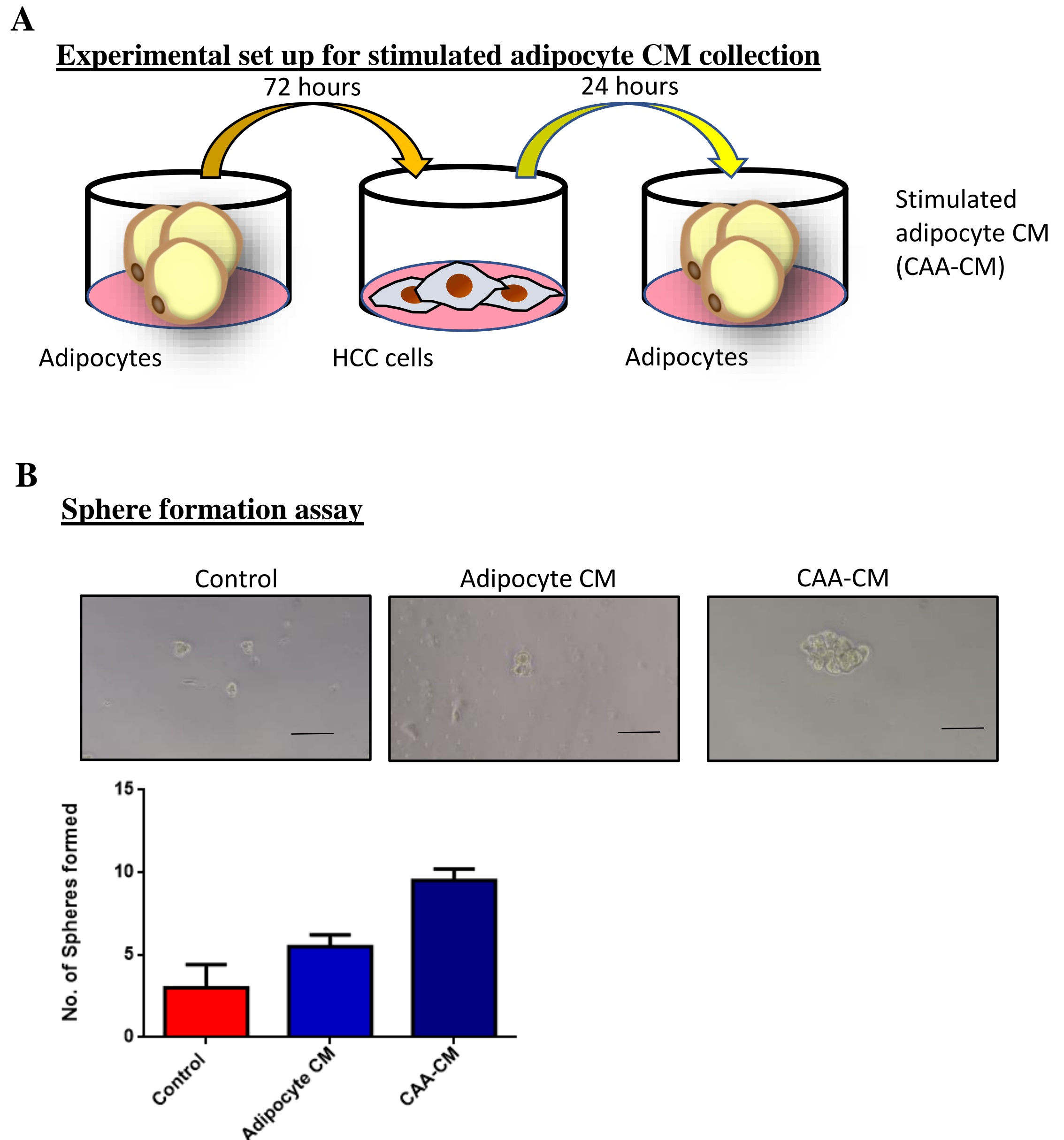


Figure 3.3.6 Stimulated adipocyte CM further enhanced self-renewal properties in HCC. A) Schematic drawing demonstrating the experimental set up for the collection of adipocyte CM. B) Sphere formation assay was performed to assess self-renewal ability in PLC/PRF/5 cells with respect to control, adipocyte CM and stimulated adipocyte CM (CAA-CM). Spheres so formed were larger in CAA-CM compared to its other counter parts. Graphical representation of total spheres so formed also demonstrate enhanced sphere forming abilities in CAA-CM. Scaling of the bar is 100µm. This result hints the possible reciprocal communication between adipocytes and HCC in tumor microenvironment.

Section 3.4 Discussion

Epidemiological studies suggest a very strong correlation between obesity and cancer mortality since it was first discovered by Calle and colleagues in 2003 (Calle et al., 2003). Since then, much research on deciphering the communication between adipocytes, one of the key cellular factors in obesity and cancer, has been conducted, such as research on how they communicate with cancer cells and aid in progression and aggressiveness. Much research on the cross communication between adipocytes and cancer has been mostly focused on adipocytes-rich microenvironment organ cancers such as breast and prostate; however, recently, studies on the adipocytes communication in leukemia (Shafat et al., 2017), gastric (Xiang et al., 2017), ovarian (Nieman et al., 2011) and melanoma (Holmes et al., 2016) have also been conducted. Most research on the communication between adipocytes and cancer has concluded there is a promoting role of adipocytes in enhancing cancer aggressiveness, which aligns with the epidemiological conclusion drawn by Calle and colleagues nearly two decades ago highlighting the important role adipocytes play in cancer progression (Calle et al., 2003).

In our research, we aimed to understand the communication between adipocytes and HCC, CSCs are a small population of cancer cells found to be resistant to traditional therapies and are capable of self-renewal and thus have the potential to metastasize and form tumors regardless of treatment with traditional therapies. Various liver CSC markers have been identified in liver CSCs as described in Section 1.4, thus helping in identification of this small population of cells under different conditions. Functional assays characterizing the CSC properties confirmed the enhancing effect of adipocytes on regulating CSC properties in HCC cells**.** Furthermore, our results indicated that HCC cells could educate adipocytes to

produce more of the soluble factors that would further enhance their CSC properties, indicating a reciprocal communication between the two types of cell.

The liver is a central system for the destruction of toxicity via the portal vein making it accessible to various soluble factors from other organs and from visceral adipocytes. Therefore, we first differentiated visceral preadipocytes into functional adipocytes. Thereafter, we established a co-culture setup that hindered physical contact between the two cell types but allowed for communication via secretomes in a paracrine manner. Sphere formation assays were conducted to study the effect of adipocytes on HCC self-renewal properties, which is a key property of CSCs. Adipocytes indeed enhanced the self-renewal property of HCC cells. The co-cultured cells were subjected to subcutaneous injection into NOD/SCID mice and their tumorigenicity was assessed. Adipocytes co-cultured cells demonstrated high tumorigenicity compared to controls and preadipocytes. These two important experiments provided us with the confidence that indeed adipocytes enhanced HCC aggressiveness by regulating its CSC properties via paracrine signaling as shown by the co-culture setup for the two experiments. Following this, we studied the enhancing effect of adipocyte CM on CSC properties in HCC. Our studies demonstrated adipocyte CM was able to enhance tumorigenicity, self-renewal properties, migration and invasion abilities and resistance to drugs in HCC. Although most of the results were obtained using HCC cell lines, we were also able to reproduce the same effect in PDTX cells and PDTX organoids, which are believed to better mimic the cell conditions *in vivo*. Moreover, we were able to postulate the existence of a possible reciprocal mechanism between the two cell types. This finding highlighted the possible modulation of the cancer microenvironment by cancer cells, which has been shown to create a favorable

niche by manipulating other noncancerous cells such as adipocytes, fibroblasts and macrophages regarding their growth and evasion of immune cells and therapies (Betof and Dewhirst., 2010). All of these results provided us with substantial evidence to identify the possible key secretomes in adipocyte CM that play a vital role in regulating CSC properties. This finding would be of great importance as of potential therapeutic use given the strong correlation between obesity and cancer mortality.

CHAPTER 4: IDENTIFICATION OF FABP4 IN ADIPOCYTE CM AS A KEY PLAYER IN REGULATING LIVER CSC PROPERTIES

Section 4.1 Introduction

In contrast to previous beliefs, adipocytes are now considered to be part of the endocrine system and play a key role in homeostasis by regulating various physiological processes through the secretion of important secretomes in response to external signals (Halberg et al., 2008). Research on secretory factors by adipocytes has revealed extracellular matrix proteins, cytokines, growth factors and hormones. With the epidemiological data demonstrating a strong correlation between obesity and cancer mortality (Calle et al., 2003), the role of these secretomes on cancer progression has been widely studied. Resistin and leptin, the first adipocytes secretomes to be reported, aid in the progression of various cancers, such as melanoma (Malvi et al., 2015), prostate (Tang et al., 2016), breast (Surmacz, 2007), colorectal (Bartucci et al., 2010), ovarian (Choi et al., 2005) and lung cancer (Tsuchiya et al., 1999). Furthermore, Tang et al. (2016) recently demonstrated the role of adipocytes-derived cathepsin B (CTSB) in promoting self-renewal properties in prostate cancer, highlighting the possible role of adipocytes in developing CSC properties. In the context of HCC, the epidemiological report suggests the detrimental role of adipocytes in HCC aggressiveness and possible liver CSC properties. Adipocytes, which act as a fat depot to release fatty acids in response to external signals, promote EMT transition in HCC cells, strongly supporting that adipocytes play a primary role in HCC development and aggressiveness; however, the key player in adipocyte CM has yet to be identified.

In the first chapter, we demonstrated that adipocytes indeed play a vital role in enhancing CSC properties in both HCC cell lines and PDTX cells. The promoting role of adipocytes on HCC cells was also replicated in PTDX, as demonstrated in Section 3.3.5 (D). Interestingly, we demonstrated that HCC cells further stimulate

adipocytes to release more secretomes, which is required to augment CSC properties. This observation provides an overview into how cancer cells build a niche for themselves to evolve against external changes and current therapies, thus developing into a highly resistant group with boosted self-renewal properties and resulting in relapse and an aggressive form of cancer. This observation led us to investigate the identity of the secretomes that play a crucial role in cancer development. Adipocytes, apart from fatty acids, secrete a pool of proteins that are involved in various physiological pathways, from the immune system, metabolism, signaling and matrix conformation (Roca-Rivada et al., 2015).

To identify the key secretomes, we conducted Orbitrap mass spectrometry analysis of the secretomes in adipocyte CM. The list of identified proteins was compared with its respective control counterpart with serum-free DMEM HG. In chapter 3, we observed further enhancement of CSC properties in adipocyte CM stimulated with HCC. We expected that the abundance of the protein in question would be further enhanced in the presence of HCC in the co-cultured medium. Therefore, we also conducted Orbitrap mass spectrometry of adipocyte CM co-cultured with HCC cells (CAA-CM). Concurrently, we conducted mass spectrometry analysis of HCC cell lines with CM (i.e., serum-free medium) in the presence of HCC cell lines to confirm that the secretomes are indeed supplied from adipocytes rather than released by HCC. The mass spectrometry data provided a list of proteins, and the proteins exclusive to adipocytes and "stimulated" adipocytes were differentiated based on their respective control counterpart. A literature review on adipocytes-specific secretomes was performed and its relative abundance level in the co-culture set up revealed six proteins and two proteins that were further secreted by adipocytes upon stimulation by HCC cells. Among them, the secretion of fatty acid

binding protein 4 (FABP4) was upregulated 8-fold in the co-culture set up and was not found released by HCC cells alone. This analysis made FABP4 our prime target for further experiments as we explored the role of FABP4 in the regulation of liver CSC properties. Recombinant FABP4 alone increased CSC properties, including self-renewal, drug resistance, invasiveness, and expression of CSC markers. The role of FABP4 in adipocyte CM was further verified by suppressing FABP4 function by using a FABP4-specific inhibitor (BMS309403) in adipocyte CM-treated HCC cells and its effects on CSC properties were examined.

Section 4.2 Experimental scheme

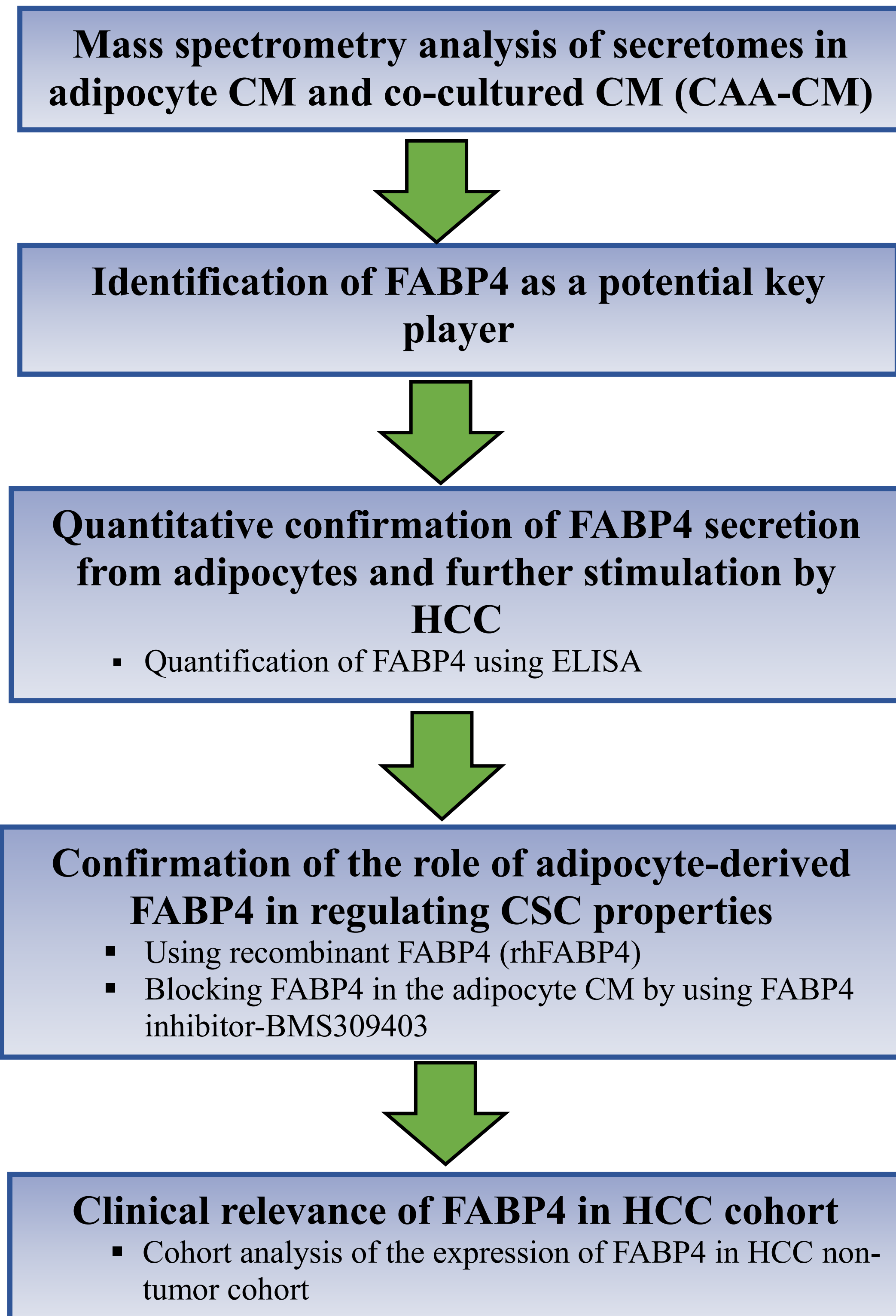


Figure 4.2 Experimental scheme of chapter 4.

Section 4.3 Results

4.3.1 Adipocytes secreted a range of proteins with various functional importance

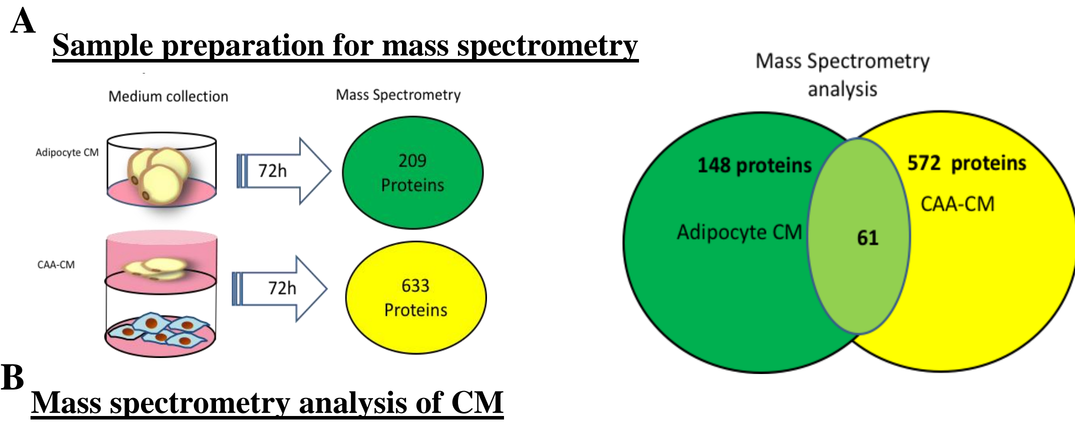
To identify the key secretomes in adipocyte CM that enhance CSC properties in HCC in chapter 3, we first conducted Orbitrap mass spectrometry analysis on the CM from adipocytes. Samples were prepared as illustrated in Figure 4.3.1 (A). For this purpose, adipocytes were incubated for 72 hours in serum-free DMEM HG medium at 37°C. Debris was removed by centrifugation at 3000 rpm for 5 minutes and the medium was subjected to subsequent mass spectrometry sample preparation, as demonstrated in Figure 4.3.1 (B). Briefly, the CM was concentrated and digested with trypsin overnight. Following digestion, the protein was desalted and run through a column. The fractionated proteins were collected and dried. The protein was re-dissolved in formic acid prior to feeding to the Orbitrap. The Orbitrap identified 209 different types of proteins in the adipocyte CM. Adipocytes-specific proteins of secretory nature were identified based on a literature review, and the top 22 proteins based on their specificity to adipocytes were selected, as listed in Table 4.3.1. The list of proteins was narrowed down to six candidates – laminin subunit gamma 1 (LAMC1), nidogen-1 (NID1), Legumain (LGMN), phospholipid transfer protein (PLTP), macrophage colony stimulating colony factor 1 (CSF1), olfactomedin-like protein 3 (OLFL3), and FABP4 – based on their novelty and their contribution to cancer progression in other types of cancer.

Since we observed enhanced CSC properties in HCC-stimulated adipocyte CM, we hypothesized that the protein abundance would be further increased when co-cultured with HCC cells. Therefore, we performed Orbitrap mass spectrometry analysis of CAA-CM. To prevent the secretion of secretomes from HCC cells, the control group consisted of HCC cells with serum-free medium. A total of 633

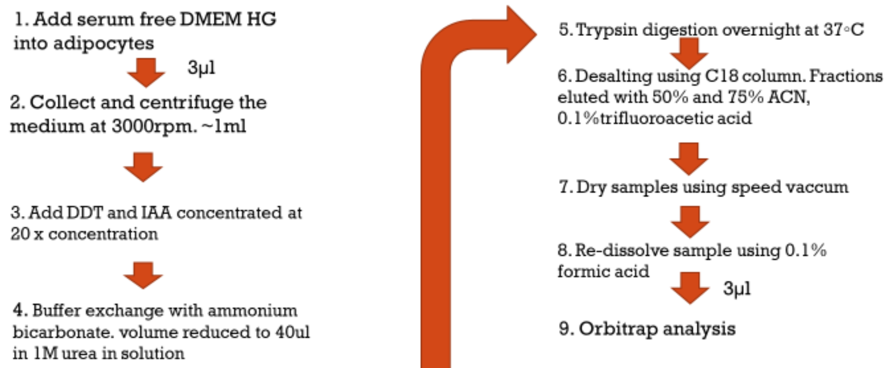
proteins were detected in CAA-CM, with 61 common proteins found between CAA-CM and adipocyte CM comprising five of the seven proteins, as shown in Figure 4.3.1 (C). The relative abundance level was based on the protein score of the mass spectrometry data to determine the relative increase in protein secretion. FABP4 secretion was increased 8-fold in the co-culture set up, followed by CSF1 (2-fold higher); however, LGMN, NID1 and LAMC1 were secreted to a lesser extent in the co-culture set up relative to adipocyte CM. PLTP1 and OLFL3 were not detected in CAA-CM. These encouraging data led us to further examine the role of FABP4 in the regulation of CSC properties.

GENE Symbol		Protein Name	Protein Score in CM	Fold change compared to CAA-CM
1	**LAMC1_HUMAN**	**Laminin subunit gamma-1**	**913**	**-0.11**
2	GELS_HUMAN	Gelsolin	547	-0.11
3	PTX3_HUMAN	Pentraxin-related protein PTX3	494	-0.53
4	LAMA4_HUMAN	Laminin subunit alpha-4	380	-0.63
5	TENA_HUMAN	Tenascin C	353	-0.93
6	LUM_HUMAN	Lumican	258	0.031
7	ANXA5_HUMAN	Annexin A5	253	0.39
8	**NID1_HUMAN**	**Nidogen-1**	221	-0.02
9	CALM_HUMAN	Calmodulin	211	-0.89
10	**LGMN_HUMAN**	**Legumain**	163	-0.29
11	FETUA_HUMAN	Alpha-2-HS-glycoprotein	126	0.69
12	POSTN_HUMAN	Periostin	114	-1.0
13	TYB10_HUMAN	Thymosin beta-10	95	0.03
14	**PLTP_HUMAN**	**Phospholipid transfer protein**	65	-1
15	AEBP1_HUMAN	Adipocyte enhancer-binding protein 1	65	-1
16	**CSF1_HUMAN**	**Macrophage colony-stimulating factor 1**	59	0.17
17	SRGN_HUMAN	Serglycin	59	-1
18	GOLM1_HUMAN	Golgi membrane protein 1	50	9.46
19	**OLFL3_HUMAN**	**Olfactomedin-like protein 3**	50	-1
20	DKK3_HUMAN	Dickkopf-related protein 3	40	-1
21	**FABP4_HUMAN**	**Fatty acid-binding protein**	33	8.8
22	PEPD_HUMAN	Xaa-Pro dipeptidase	27	1.8

Table 4.3.1 Protein secretion profile of human visceral adipocytes determined by mass spectrometry. Proteins are shortlisted from 209 original protein list and tabulated in descending order to their protein score. The proteins in red are proteins chosen for further analysis



B
Mass spectrometry analysis of CM



C
Secretomes levels in CAA-CM relative to adipocyte CM

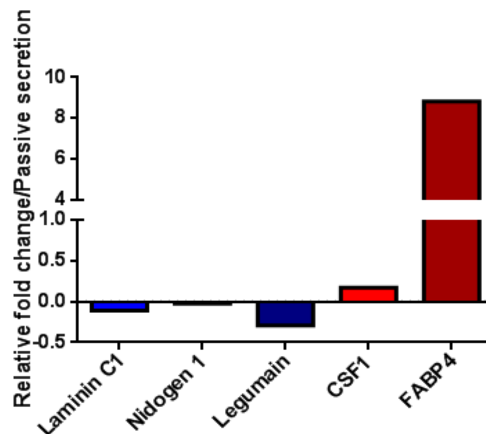


Figure 4.3.1 Identification of FABP4 as a potential player in adipocyte CM conferring CSC properties in HCC cells. A) Schematic drawing demonstrating sample preparation for mass spectrometry analysis of CM and the common proteins found in both CM and co-cultured medium illustrated in a Venn diagram. B) Stepwise protocol used for Orbitrap analysis of CM. C) A bar graph demonstrating the relative abundance of the proteins in CAA-CM with reference to their abundance in adipocyte CM based on protein score.

4.3.2 Adipocytes, but not HCC cells, preferentially secreted FABP4 into the tumor microenvironment

The mass spectrometry analysis described in section 4.3.1 on secretomes from HCC cell lines alone and adipocytes alone and from the co-culture set up indicated that FABP4 is secreted from adipocytes rather than HCC. However, to determine whether adipocytes are the dominant source of FABP4 in the tumor microenvironment and are not produced by HCC, we quantified the FABP4 concentration in CM collected from HCC cell lines - PLC/PRF/5 and Bel-7402, the two cells lines used for the study. The tumor microenvironment is comprised of various types of non-cancerous cells that co-exist to provide a suitable niche for tumor growth and development. The fatty liver tumor microenvironment, apart from adipocytes, comprises adipocytes predecessors-preadipocytes and HSC-hTERT. HSC-hTERT were recently reported to be a source of FABP4 and are highly correlated to metabolic risk factors (Chiyonobu et al., 2018). Therefore, we examined whether adipocytes are the major source of FABP4 compared to HCC and HSC-hTERT. We first studied the relative mRNA expression of FABP4 in these cell lines to have a general idea on the expression. qPCR analysis on the relative FABP4 expression level among HCC cells- Bel-7402, PLC/PRF/5, Huh 7, Hep3B and MHCC-97L and stromal cells–preadipocytes and HSC-hTERT and adipocytes. As represented in Figure 4.3.2 (A), the relative expression of FABP4 is found to be significantly upregulated followed by HSC-hTERT. The mRNA level of FABP4 in preadipocytes was found to be relatively negligible compared with other cell lines.

We further tried to quantify the concentration of FABP4 released by cells in stroma- preadipocytes, HSC-hTERT and adipocytes along with HCC cells- Bel-7402 and PLC/PRF/5. All of the CM was collected after incubation for 72 hours.

The relative amount of FABP4 secreted by all the cellular factors is demonstrated in Figure 4.3.2 (B). The ELISA confirmed the secretion of FABP4 and adipocytes as the major source of FABP4 among the stromal cellular factors. The ELISA also confirmed that FABP4 is primarily secreted by adipocytes rather than HCC cells in the co-culture set up, further confirming the stimulation of adipocytes by HCC cells to release more FABP4 into the medium. An average of ~20 ng/ml of FABP4 was passively secreted, which is similar to that in a healthy population (23.40 ng/ml) (Cabré et al., 2007).

Since we demonstrated the stimulation of release of secretomes from adipocytes by HCC cells resulting in enhancement of self-renewal properties, we tried to study by quantification of the stimulated CM. The CM was collected as illustrated in the set up in Figure 4.3.2 (C). Briefly, the adipocyte CM was incubated in HCC cells-PLC/PRF/5 and after 3 days of incubation was re-incubated with adipocytes for 24 hours. Upon stimulation, a nearly 5-fold increase in FABP4 was detected, with the release of 100 ng/ml of FABP4 upon stimulation with HCC cell lines as demonstrated in Figure 4.3.2 (D). The upregulation of FABP4 in serum (approximately 100 ng/ml) was reported in an obese population and is a significant indicator of metabolic syndrome. Apart from adipocytes, HSCs also secrete FABP4; however, adipocytes secrete twice the amount of FABP4 secreted by HSCs, making them a major source of FABP4 in the liver tumor microenvironment. Neither preadipocytes nor HCC cell lines secreted FABP4. The relative FABP4 gene expression level was also correlated to our ELISA finding in which the expression level of adipocytes was significantly upregulated compared to HSCs, preadipocytes and HCC cell lines as shown in Figure 4.3.2 (B). Both qPCR results and ELISA assays confirmed that adipocytes specifically secrete FABP4 in the tumor

microenvironment. This result suggests that a high serum level of FABP4 is found in NAFLD-induced HCC patients (Thompson et al., 2018).

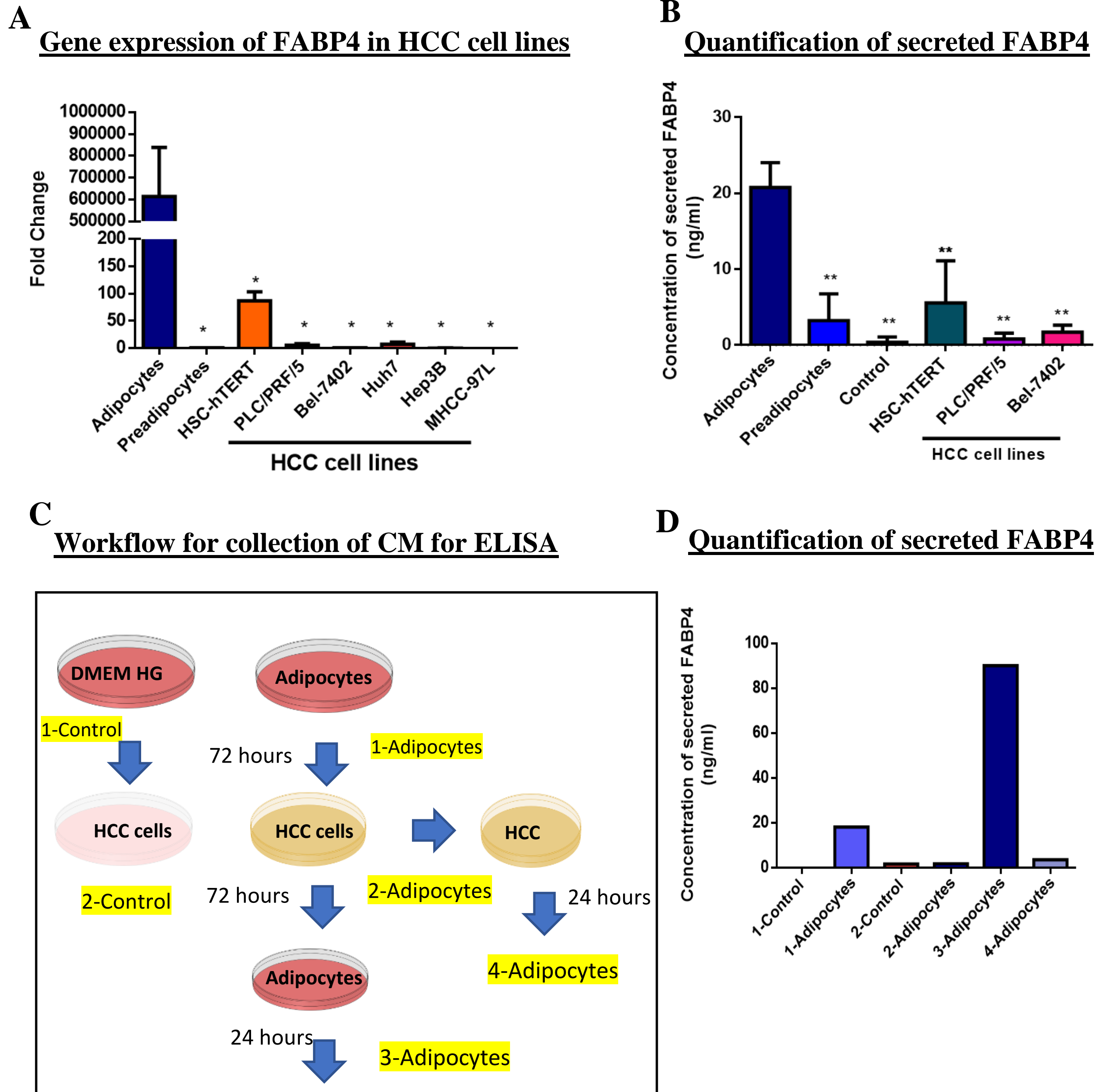


Figure 4.3.2 FABP4 was predominantly secreted by adipocytes in the tumor microenvironment. qPCR analysis normalized with GAPDH (A) of relative gene expression in HCC cell lines compared to stromal cells adipocytes, HSC-hTERT and preadipocytes. These results revealed weak expression of FABP4 in HCC cells. (B) ELISA on the secreted FABP4 by HCC cells, adipocytes, preadipocytes and HSC-hTERT revealed adipocytes as the primary source of FABP4 secretion. Control is serum free DMEM medium. (C) A schematic diagram illustrating the workflow of the collection of medium for the ELISA for stimulated medium. PLC/PRF/5 was used as the HCC cell line. (*$p<0.05$, **$p<0.01$, ***$p<0.001$, t-test). (D) ELISA of HCC-stimulated medium represented as 3-Adipocytes from workflow in (C). Compared to adipocytes only (1-Adipocytes), more FABP4 is secreted out upon stimulation with HCC medium.

4.3.3 rhFABP4 promoted CSC properties in HCC cells

To investigate the functional role of adipocytes-derived FABP4 in the regulation of CSC properties, we administered concentrations of rhFABP4 that were similar to those secreted by adipocytes (20 ng/ml) and stimulated with HCC cells (100 ng/ml) to determine whether rhFABP4 would have an analogous effect to that of adipocyte CM. First, HCC cells were subjected to a spheroid formation assay following FABP4 treatment to evaluate its effect on self-renewal ability *in vitro.* As shown in Figure 4.3.3 (A), upon administration of rhFABP4, more and larger spheres were formed compared to the control, similar to what we observed in the presence of CM and in the co-culture set up. This result confirmed that the presence of FABP4 in adipocyte CM promoted the self-renewal properties of HCC cells.

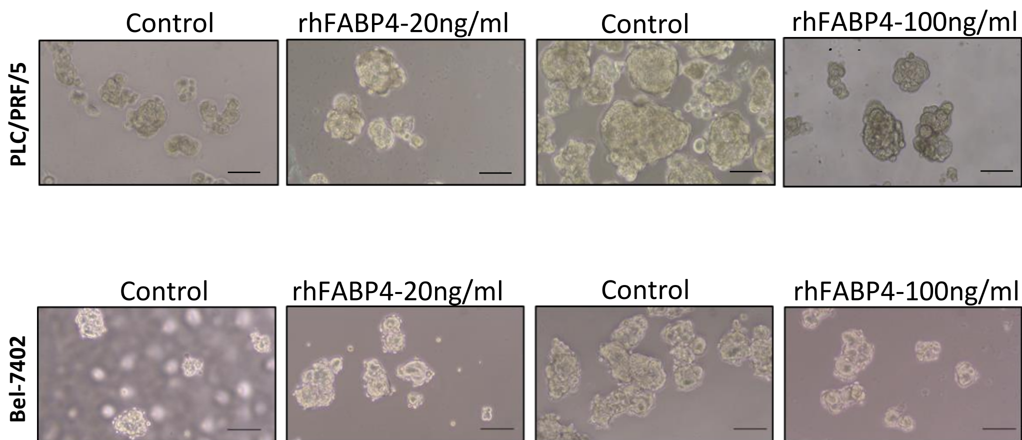
Upon confirming the promoting role of rhFABP4 in the self-renewal ability of HCC, we aimed to elucidate whether a similar effect on other CSC properties could be recapitulated. We administered rhFABP4 to assess its effect on HCC cell migration and invasion. As shown in Figure 4.3.3 (B), the invasion and migration abilities of HCC cells were enhanced in the presence of 20 ng/ml and 100 ng/ml rhFABP4. As reported in section 3.3.4, adipocyte CM provided resistance to traditional therapies of both doxorubicin and sorafenib. To confirm the involvement of FABP4 in attaining this property in HCC cells, we administered 20 ng/ml and 100 ng/ml rhFABP4 with sorafenib to deduce whether rhFABP4 alone was capable of conferring resistance against sorafenib in HCC cells. rhFABP4, at both concentrations, was able to confer resistance against sorafenib as illustrated by annexin V assay graph in Figure 4.3.3 (C), further confirming the crucial role of FABP4 in adipocyte CM.

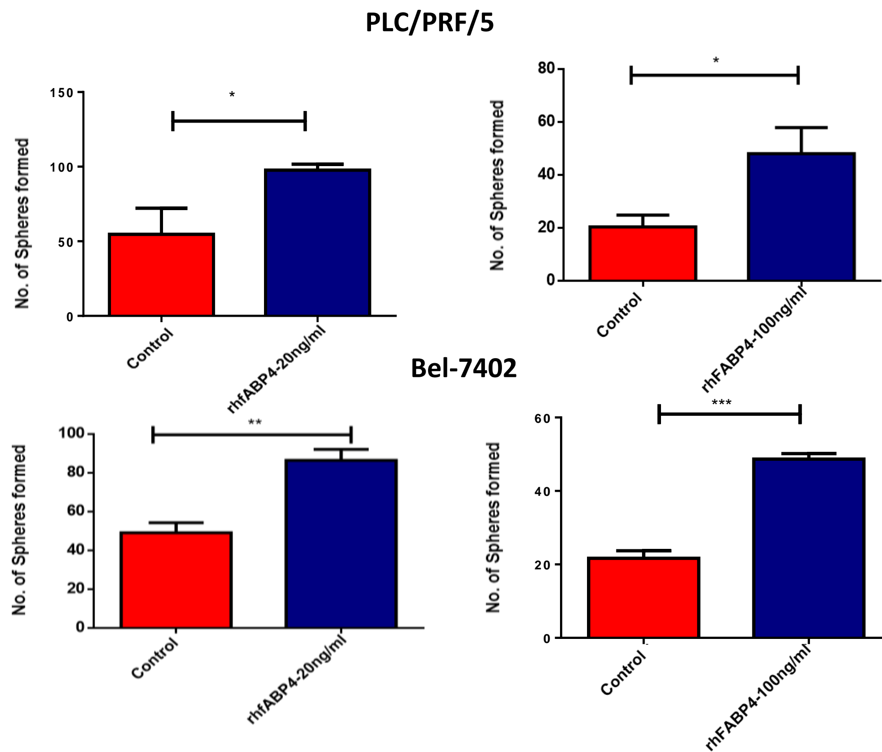
Liver CSC properties can be identified by their distinct CSC markers, as reviewed in Section 1.3.2. As reported in section 3.3.5 (C), liver CSC markers were enhanced

in the presence of adipocyte CM. To confirm whether this enhancement was attributed to FABP4, we administered rhFABP4 to HCC cells and subjected the treated cells to CSC marker analysis. The expression of CSC markers, including CD90 and EpCAM, was confirmed in rhFABP4-treated HCC cells as demonstrated in Figure 4.3.3 (D). Since treatment included rhFABP4 alone in serum-free medium, it confirmed the important role of FABP4 in adipocyte CM in regulating CSC properties in HCC cells. Furthermore, effect of FABP4 at both concentration 20ng/ml and 100ng/ml was observed in all CSC assays. This observation justifies the effect of FABP4 in an obese population, as a high level of serum FABP4 and its correlation to cancer mortality have been reported, which could be due to the development of CSC properties via FABP4.

A

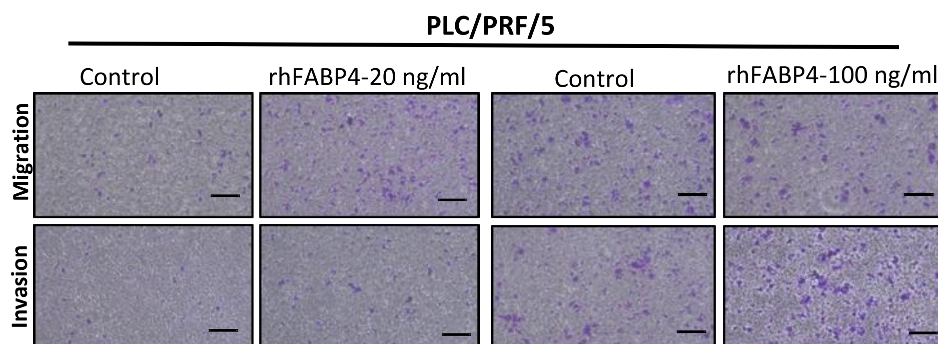
Sphere formation assay



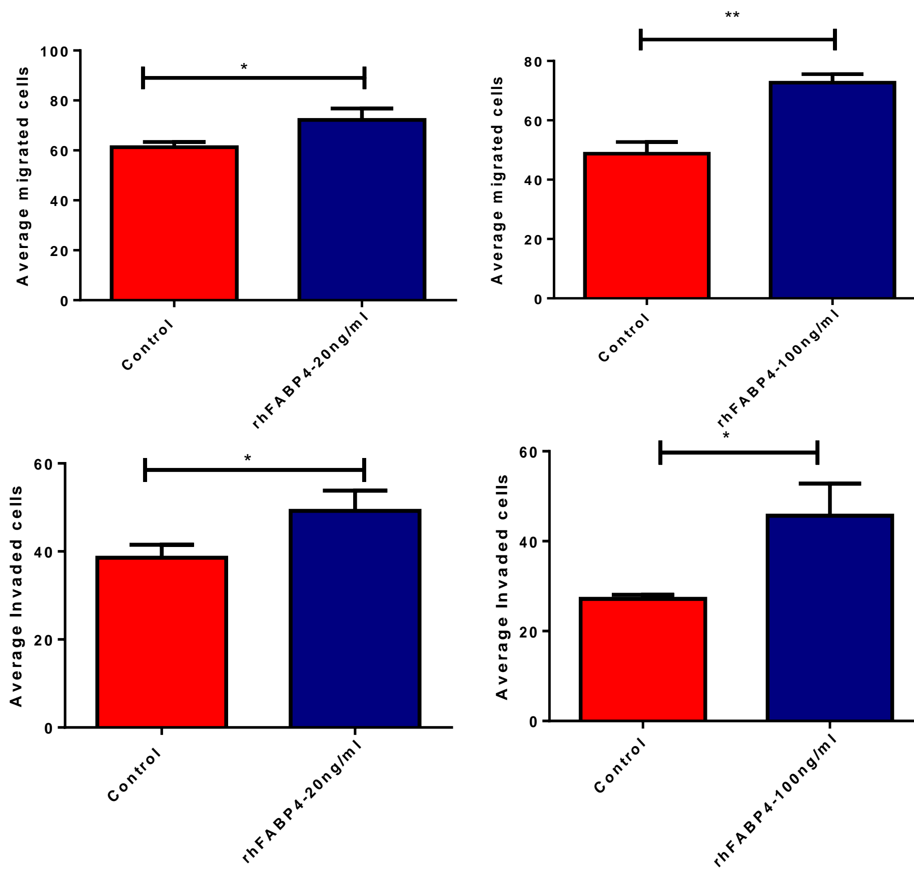


B

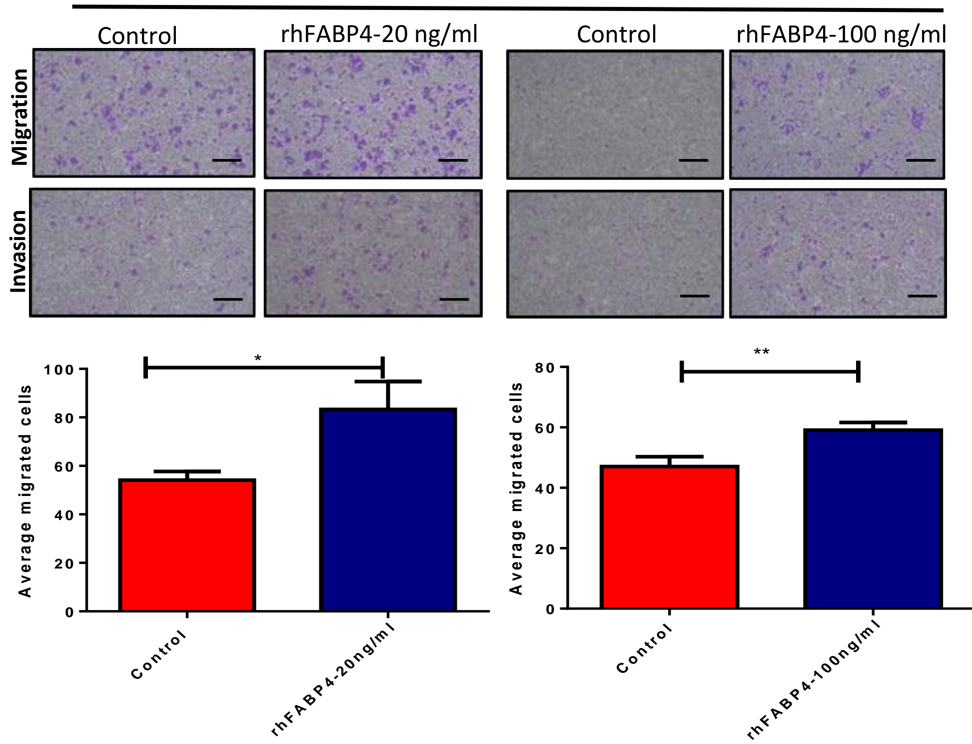
Migration and Invasion assays

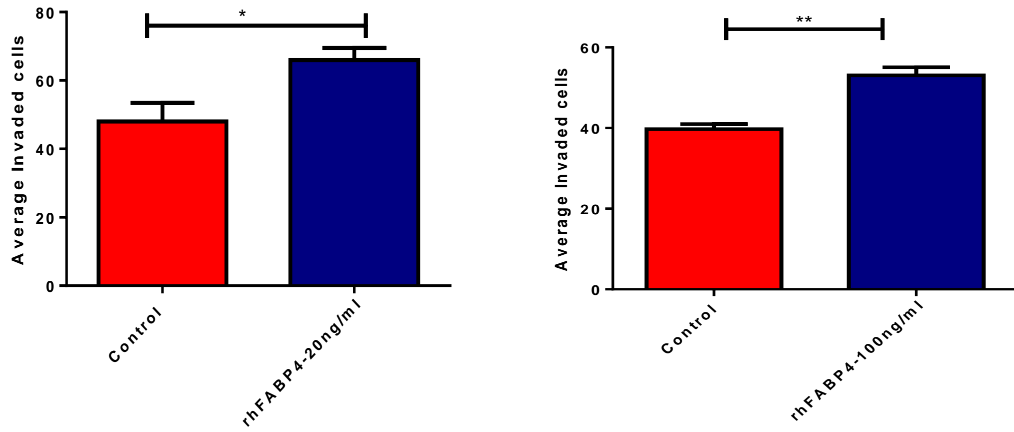


PLC/PRF/5



Bel-7402





C

Drug resistance

Control | Sorafenib-6μM | Sorafenib + rhFABP4

rhFABP4-20ng/ml

PLC/PRF/5: 12.9% | 38.5% | 17.6%

Bel-7402: 6.0% | 24.1% | 12.7%

Control | Sorafenib-6μM | Sorafenib + rhFABP4

rhFABP4-100ng/ml

PLC/PRF/5: 11.1% | 25.0% | 16.4%

Bel-7402: 3.9% | 30.7% | 19.0%

PI

Annexin V

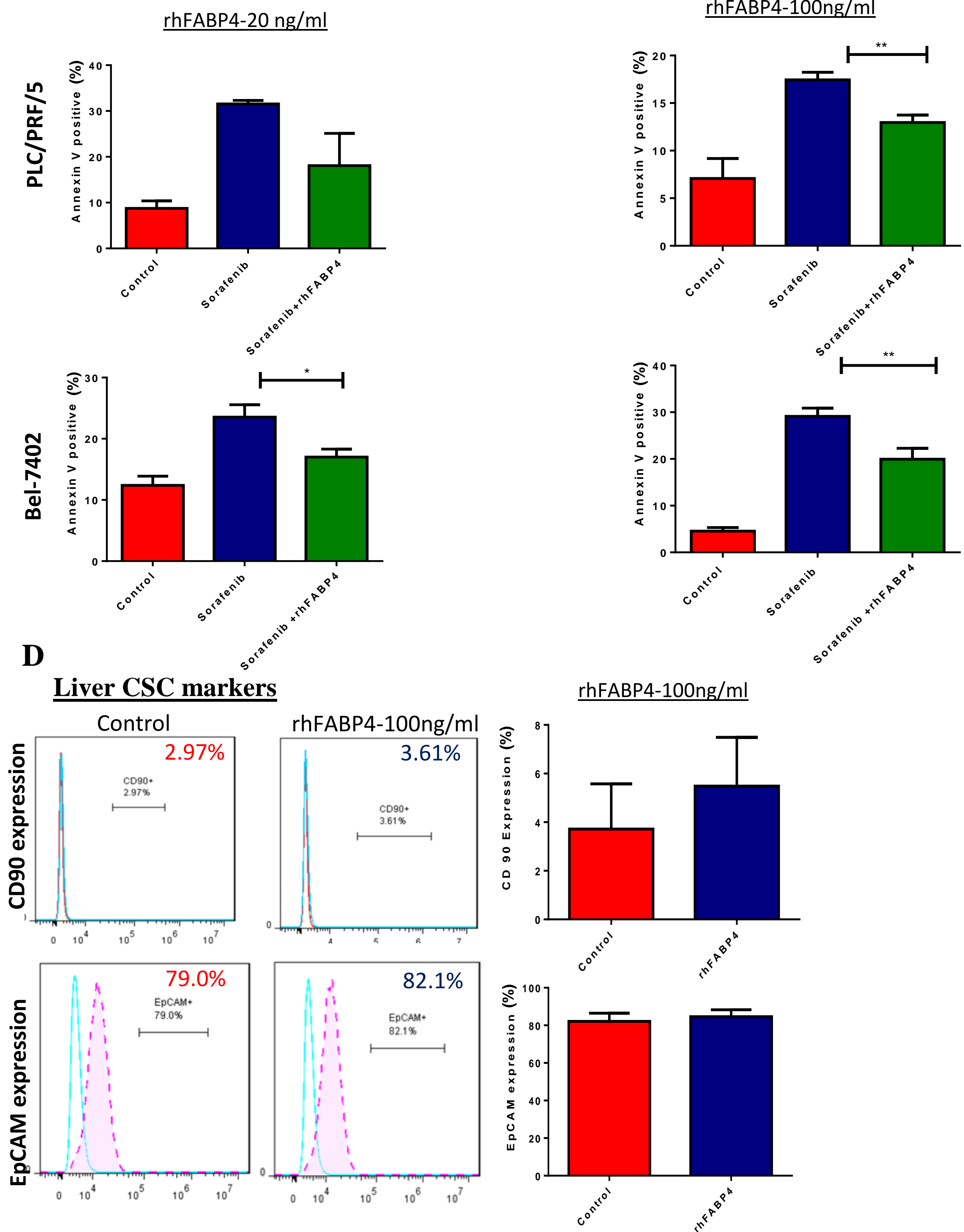


Figure 4.3.3 rhFABP4 promoted CSC properties in HCC cells. rhFABP4 was administered to PLC/PRF/5 and Bel-7402 cells at different concentrations (20 ng/ml and 100 ng/ml) and its effects on CSC properties– (A) self-renewal, (B) migration and invasion (C), drug resistance and (D) expression of liver CSC markers were examined. The scale bar for (A) sphere formation assay is 100µm and for (B) migration and invasion is 200µm. (*p<0.05, **p<0.01, ***p<0.001, t-test).

4.3.4 FABP4 inhibitor-BMS309403 (BMS) inhibited adipocyte CM-derived augmentation in CSC properties in HCC

In addition to using rhFABP4 to study the effects of FABP4 on CSC properties, it was also important to determine whether the presence of FABP4 in the medium and the inhibition of FABP4 with the use of specific inhibitors could dampen the effects of FABP4 on the regulation of CSC properties in HCC cells. For this purpose, we made use of BMS309403-a synthetically manufactured FABP4 inhibitor that inhibits FABP4 activity specifically, as demonstrated by Furuhashi and colleagues in 2007 (Furuhashi et al., 2007). We aimed to determine whether inhibition by BMS309403 attenuates the development of CSC properties in HCC. We primarily focused on the effects of the inhibitor on sphere-forming ability in the presence of adipocyte CM and its effect on drug sensitivity. Prior to administration, the IC50 value of BMS309403 was evaluated using the MTT assay. BMS309403, at a concentration of 20μM, inhibited FABP4 in adipocyte CM governed drug insensitivity in Bel-7402. For sphere formation assay which requires incubation for over six days, lower dosage of 500nM for Bel-7402 and 1μM for PLC/PRF/5 of the inhibitor was administered with subsequent addition of supplementation on consecutive days. As demonstrated in Figure 4.3.4 (A), less spheres of smaller sizes were formed in BMS309403-treated adipocyte CM. DMSO (the dissolving agent for BMS309403) was administered to assess the toxicity or protective role of the solvent. DMSO did not have any protection or toxicity role. Both HCC cell lines (Bel-7402 and PLC/PRF/5) exhibited reduced self-renewal properties in the presence of BMS309403 in adipocyte CM, confirming the role of FABP4 in enhancing self-renewal properties in HCC cells.

Following this discovery, we assessed the role of FABP4 in drug sensitivity, particularly sorafenib because of its specificity to HCC. Blockade of FABP4 in

adipocyte CM by BMS309403 showed increased drug sensitivity, as shown in Figure 4.3.4 (B). This observation was made with Bel-7402, confirming the role of FABP4 in inhibiting drug sensitivity in HCC cells. These results provide substantial evidence for the role of FABP4 in CSC properties, particularly by enhancing its self-renewal properties and relieving drug sensitivity.

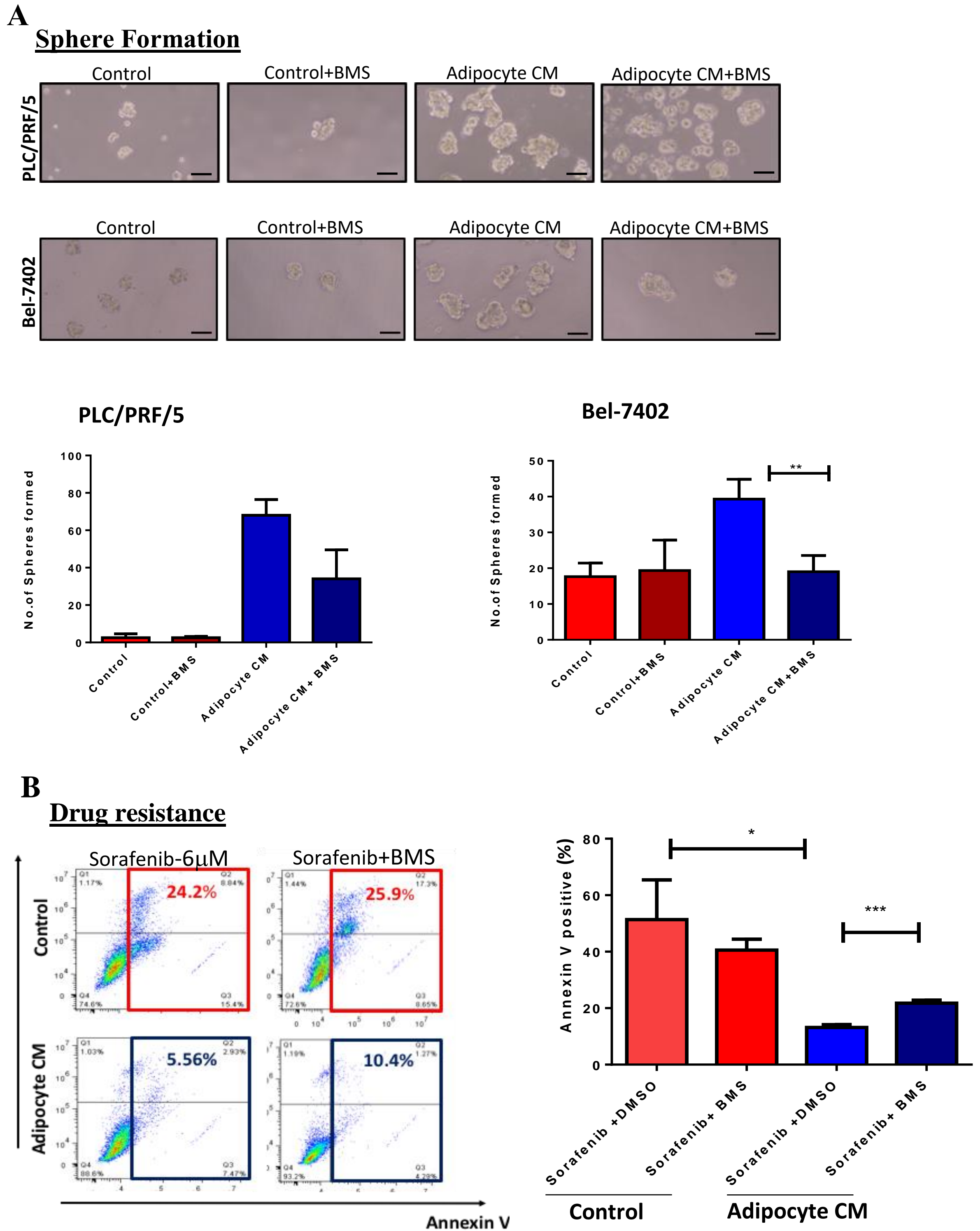


Figure 4.3.4 Inhibition of FABP4 attenuated the effects of adipocyte CM on CSC properties in HCC. PLC/PRF/5 and Bel-7402 cells were subjected to a (A) spheroid formation assay and (B) drug resistance in the presence of DMEM HG only (Control), DMEM HG with the FABP4 inhibitor-(BMS), adipocyte CM and adipocyte CM with BMS with Bel-7402 only. The effect of adipocyte CM on sphere-forming ability and drug resistance was attenuated in the presence of BMS, suggesting that FABP4 is important for both self-renewal and drug resistance mediated by adipocyte CM. The scale bar is 100 μm. (*p<0.05, **p<0.01, ***p<0.001, t-test).

4.3.5 Overexpression of FABP4 in non-tumor samples correlated to poor patient survival

Our *in vitro* assay using both recombinant FABP4 and an inhibitor demonstrated the crucial role of FABP4 in promoting CSC properties in HCC. However, its clinical relevance has yet to be determined. The serum FABP4 level is high in the obese population, with links to metabolic syndrome in diabetes (Li et al., 2016) and insulin resistance (Nakamura et al., 2017), thus indicating the possible role of FABP4 in bridging the gap between obesity and cancer mortality in HCC. To confirm our hypothesis (i.e., FABP4 is supplied from the tumor microenvironment comprised of adipocytes under obese and NAFLD conditions and eventually results in a poor prognosis), we focused on the relative expression of FABP4 in a non-tumor cohort in the publicly available TCGA dataset. A total of 50 sets were identified, and Kaplan Meir's analysis was conducted to study the correlation between FABP4 expression and survival in HCC patients. As shown in graph Figure 4.3.5 (A), in TCGA, we were able to demonstrate that high expression of FABP4 in non-tumor samples resulted in poorer survival.

Similarly, the relative expression of FABP4 in the tumor microenvironment and HCC overall survival and recurrence was assessed in GSE14520, as shown in Figure 4.3.5 (B) showed a trend similar to that of TCGA in which high expression of FABP4 correlated to low disease-free survival and recurrence. The correlation did not reach significance, which could be because in GSE14520, the primary cause of HCC is viral infection rather than fatty liver disease, as the data are collected from China, which has a higher incidence of HCC by virus. However, this result suggests the possible role of FABP4 in other causes of HCC. Additional experiments are required to study its clinical relevance.

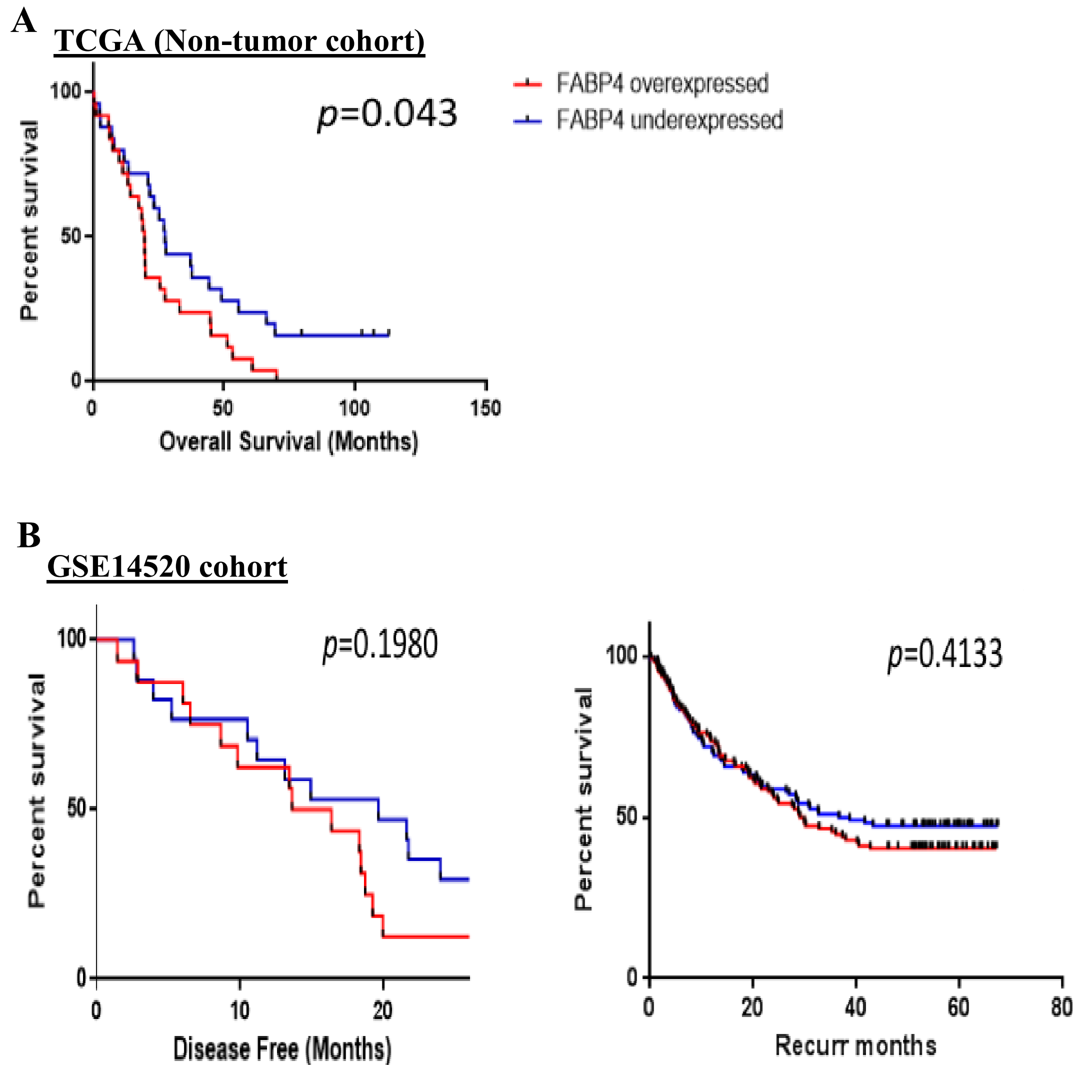


Figure 4.3.5 Expression of FABP4 in non-tumor samples correlated to poor patient survival in different HCC cohorts. Overall survival of HCC patients in publicly available TCGA data (n=50) showed poorer overall survival with high expression of FABP4 in a non-tumor cohort (A). A similar trend was observed in both disease-free survival and recurrence (B) in the GSE14520 cohort, with high expression of FABP4 associated with shorter disease-free survival and higher recurrence. Analysis of the publicly available patient cohort emphasizes the importance of FABP4 in the tumor microenvironment and its detrimental effect on patient survival.

Section 4.4 Discussion

The epidemiological report by Calle (2003) on the correlation between obesity and cancer was a key player in bringing obesity-related cellular factors into the limelight (Calle et al., 2003). Since the report, adipocytes, the key cellular factor resulting in high body fat, have been revealed as a source of various hormones, peptides and cytokines that regulate the homeostasis of various metabolic processes (Fasshauer and Blüher, 2015). Apart from metabolic disease, adipocytes have been reported to aid in cancer progression by secreting essential proteins and cytokines required for cancer progression (Booth et al., 2015).

In chapter 3, we successfully confirmed the positive role of adipocytes in HCC progression by modulating CSC properties, as evidenced by improved self-renewal, migration and invasion abilities, tumorigenic potential and enhanced drug resistance. Next, we wanted to identify the key factor released by adipocytes that resulted in the observed phenomenon. For this purpose, we conducted mass spectrometry analysis of adipocyte CM and identified 208 proteins. It is well-known that cancer cells tend to modulate the microenvironment to better develop their niche for growth and development, which could cause neighboring cells, such as adipocytes, to secrete more of the factors required for the development of more aggressive forms of cancer. With this understanding, we hypothesized that the communication between adipocytes and cancer cells in the co-culture set up would further stimulate adipocytes to release more of the essential factors that would result in their upregulation in the co-culture set up. Therefore, we conducted mass spectrometry analysis of CM from the co-culture set up. The analysis highlighted an 8-fold increase in FABP4. This surge in FABP4 production in the co-culture set up intrigued our interest in its role in the communication between adipocytes and HCC cells.

Adipocyte CM comprises a mixture of various factors, including hormones, cytokines, fats and peptides. Our mass spectrometry analysis revealed the detrimental role of FABP4 in adipocyte CM. To confirm this theory, we first quantified the amount of FABP4 present in adipocyte CM and in the co-culture set up and administered rhFABP4 to HCC cells and conducted CSCs functional assays. Upon the administration of rhFABP4, the self-renewal, migration and invasion properties were enhanced, while drug sensitivity was weakened. We also analyzed CSC properties in the presence of adipocyte CM by blocking FABP4 with the FABP4-specific inhibitor-BMS309403 and demonstrated that by simply blocking FABP4, the subsequent effects on CSC properties were attenuated. Our findings confirm the prominent role of FABP4 in adipocyte CM in regulating CSC properties in HCC.

Fatty acid binding proteins (FABPs) are lipid chaperones that transport lipids intracellularly and regulate lipid transportation. Since their discovery in 1972 (Ockner et al., 1972), 12 different isoforms have been identified (Gurung et al., 2018), with crucial roles in different types of cancer by both regulating the cancer phenotype or by modulating the cancer microenvironment. In human genome, nine FABP genes have been identified and labelled based on the relative abundance of the protein found namely- FABP1 (Liver FABP), FABP2 (Intestinal FABP), FABP3 (Heart FABP), FABP4 (Adipocyte FABP), FABP5 (Epidermal FABP), FABP6 (Ileal FABP) and FABP7 (Brain FABP) (Gurung et al., 2018). FABP8 and 9 are reported in testis and FABP10 is reported to be found in liver (Cheng et al., 2013). FABP11 is restricted to fish species (Laprairie et al., 2017). All the different isoforms of FABP consist of a basic motif of β-barrel and a single ligand (either fatty acid, cholesterol or retinoids) which is bound to the internal water filled cavity

providing an efficient design for transport for lipids (Chmurzyήska, 2006). FABP4 is of interest in cancer, as its serum level has been linked to various metabolic diseases and pathologies. FABP4 was recently found to be highly expressed in glioblastoma (Cataltepe et al., 2012) and oral squamous cancer (Lee et al., 2014) and serves as a prognostic factor in ovarian cancer (Nieman et al., 2011) and non-small cell lung cancer (Tang et al., 2016). Moreover, exogenous FABP4 enhances the proliferation and migration of prostate (Uehara et al., 2014) and breast (Guaita-Esterurelas et al., 2017) cancers, indicating its possible role in the regulation of cancer homeostasis. However, the FABP4-regulated signaling pathway has yet to be identified. Recent research on FABP4 in cancer has highlighted the activation of Akt and MAPK by exogenous supply of FABP4, resulting in cancer proliferation and metastasis (Uehara et al., 2014; Guaita-Esterurelas et al., 2017). Akt and MAPK are two important pathways that are altered in cancer cells, and previous studies on the modulation of these two pathways by FABP4 have provided a glimpse into the mechanism of FABP4 in cancer progression. In terms of CSC properties, the activation of Akt pathways has been reported to enhance tumorigenicity in colon cancer (Wang et al., 2010) and glioma (Mantwill et al., 2013), confirming the role of the Akt signaling pathway in enhancing CSC properties. In the context of HCC, the activation of Akt has been reported to cause sorafenib resistance (Chen et al., 2014). Our results with both rhFABP4 and BMS309403 highlight the vital role of FABP4 in acquiring resistance against sorafenib, indicating the possible involvement of the Akt pathway in HCC.

In summary, in this chapter, **we identified FABP4 as the prominent adipocyte-derived factor that plays a detrimental role in regulating CSC properties in HCC**. Using qPCR, ELISA and mass spectrometry analyses, we confirmed that

FABP4 is indeed secreted out from adipocytes and is further stimulated by HCC cells to be abundantly secreted by adipocytes, emphasizing the role of FABP4 in the communication between these two cell types. *In vitro* functional studies using rhFABP4 and the FABP4 inhibitor, BMS309403, enriched and attenuated CSC properties, respectively, confirming the role of FABP4 in adipocyte CM in regulating CSC properties in HCC. Our hypothesis is that FABP4 is supplied from the tumor microenvironment, particularly in cases of NAFLD-HCC and obesity. We also studied the clinical relevance of our findings by analyzing the expression of FABP4 in the tumor microenvironment and its correlation with overall survival. TCGA cohort analysis demonstrated that high expression of FABP4 in the tumor microenvironment correlated with low overall survival. These data further signify the role of FABP4 in the tumor microenvironment in HCC prognosis.

CHAPTER 5: FABP4 REGULATED CSC PROPERTIES IN HCC VIA AKT MEDIATED β-CATENIN SIGNALING PATHWAY

Section 5.1 Introduction

Our results in chapter 3 showed the pivotal role of adipocytes on regulation of liver CSC properties as both CM and co-culture set up with adipocytes promoted self-renewal, migration, invasion abilities, drug resistance and tumorigenicity in HCC cell lines. The possibility of reciprocal communication between the two cells, illustrated by further enhancement of liver CSC properties under stimulated condition, was also hinted at in the chapter. In Chapter 4, we have identified FABP4 to be the key factor in adipocyte CM, which played the major role in communication between HCC cells and adipocytes. The functional role FABP4 in liver CSC properties were well characterized with the aid of recombinant protein (rhFABP4) and FABP4 inhibitor (BMS309403). Additionally, we also found FABP4 to be further released upon stimulation by HCC cells, which suggests the possible reciprocal communication between HCC cells and adipocytes.

FABP4, a lipid chaperone, has been widely known to participate in the transport of lipids both intracellularly and extracellularly; therefore, was not considered to be relevant in major physiological homeostasis (Furuhashi et al., 2014). However, in 2010, Hsu and colleagues demonstrated the strong correlation between serum level FABP4 to metabolic syndrome that changed the perception of FABP4 (Hsu et al., 2010). High expression of FABP4 was also reported to modulate the other cellular factors, such as macrophages (Xu et al., 2016) and endothelial cells (Aragonès et al., 2012) which are key cellular factors governing the tumor microenvironment. These findings prove the existence of a diverse role of FABP4 in physiological homeostasis and, most importantly, possible role in building a suitable microenvironment.

Metabolic syndrome i.e. a predisposition factor of cancer, is widely known to have strong correlation with cancer incidence and prognosis (Braun et al., 2011). The

close relationship between metabolic syndrome and FABP4 has prompted cancer researchers to investigate the role of FABP4 in cancer progression. The first report of FABP4 in ovarian cancer was reported by Nieman and colleagues, which showed the metastatic role of adipocytes derived FABP4 in ovarian cancer (Nieman et al., 2011). FABP4 was also found to be biomarker for bladder cancer (Boiteux et al., 2009) and non-small cell lung cancer (Tang et al., 2015). However, the mechanism of FABP4 in regulating cancer has not yet been firmly established. Previous studies on role of FABP4 in cancer progression has been limited to cancer cells however FABP4 is found to be dominantly expressed in adipocytes and macrophages (Furuhashi et al, 2015). In line with this theory, the effects of exogenous supply of recombinant FABP4 have been studied in prostate and breast cancer where the tumor microenvironment comprises high proportion of adipocytes. Exogenous FABP4 was found to induce tumor progression in cancers of prostate (Uehara et al., 2014), and breast via PI3K/Akt pathway (Guiata-Esteruelas et al., 2017). Neither paper, however, did fully delineate the mechanism of exogenous FABP4 to activate PI3K/Akt. Recently, mechanistic studies concerned with FABP4 on acute myeloid leukemia (AML) cancer cell progression have demonstrated the possible role of FABP4 as regulator of transcription factor, DNMT1, found to be methylate tumor suppressor genes (Yan et al., 2017). Yan and colleagues illustrated the mechanism based on the upregulation of FABP4 internally in AML cells; nevertheless, the mechanism behind the external supply of FABP4 to regulation of liver CSCs is not yet discovered.

In this chapter, we aimed to understand how FABP4 supplied by adipocytes regulated important changes in HCC cells that resulted in the enhancement of liver CSC properties. For this purpose, we employed the next generation RNA

sequencing to compare the genetic profiles between rhFABP4 treated HCC cells and their control counterparts, followed by pathway analysis by DAVID. Upon analysis, the key components in identified pathway was further confirmed by molecular approaches.

Section 5.2 Experimental scheme

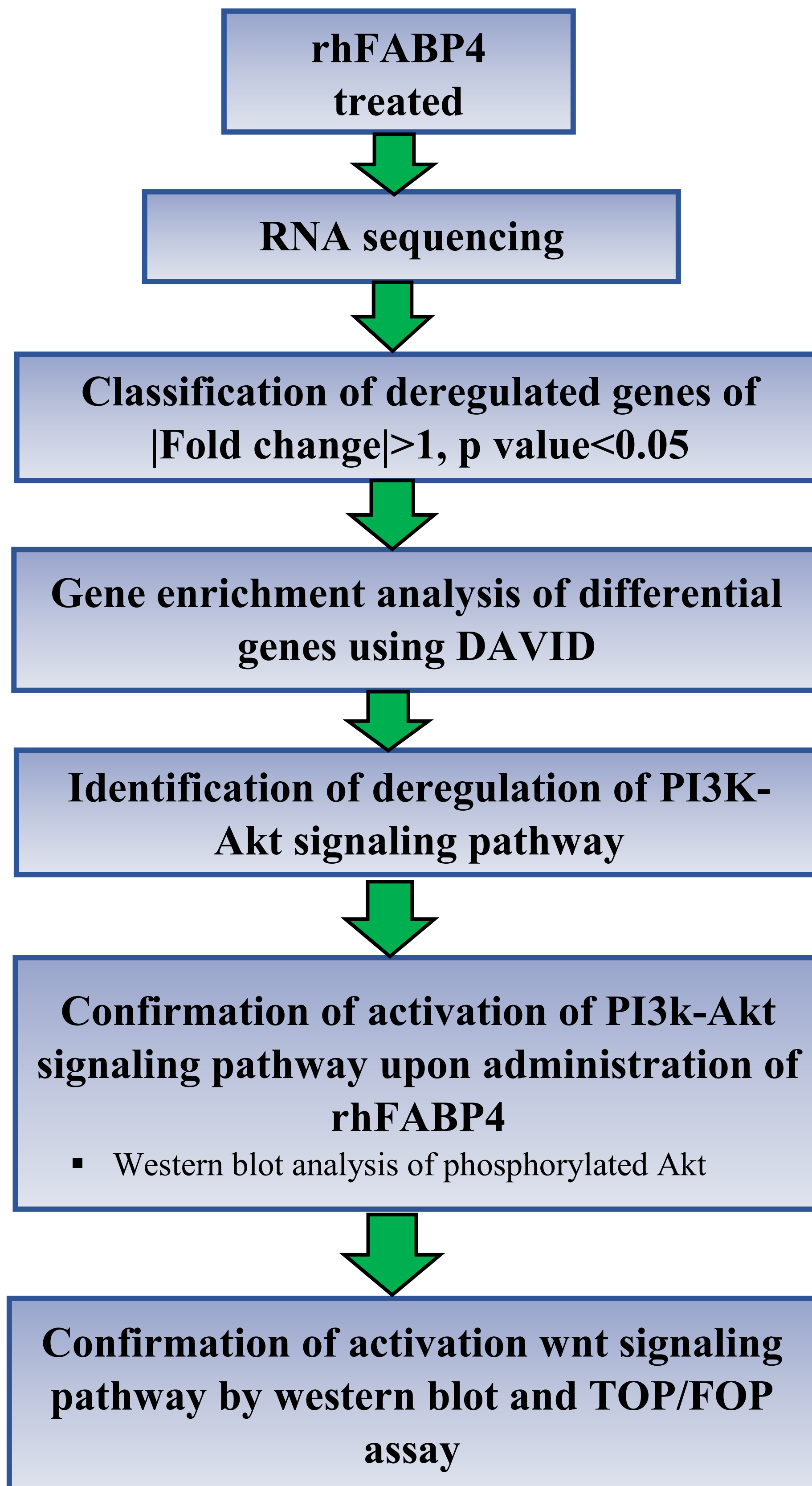


Figure 5.2 Experimental scheme of chapter 5.

Section 5.3 Results

5.3.1 Identification of PI3K-Akt as the potential signaling pathway mediated by rhFABP4

To delineate the pathways governed by rhFABP4 on HCC cells regulating liver CSC properties, we conducted RNA sequencing on rhFABP4 administered PLC/PRF/5 cells. Cells were incubated with rhFABP4 at 100ng/ml for 24 hours. RNA was extracted and subjected to RNA sequencing. The RNA sequencing detected a total 9021 genes deregulated in PLC/PRF/5 when treated with rhFABP4. Differentially regulated genes were then further narrowed down to 451 differential genes in total based on gene fold change of $|\text{Fold change}|>1$ and p value <0.05. Among 451 genes enlisted, 351 genes found to be upregulated and 100 genes found to be downregulated. Gene list was subjected to publicly available pathways analysis tool-DAVID which identified most of the genes in the list to be related to deregulated pathways in various physiological irregularities as shown in Table 5.3.1 (A). Based on Fisher exact test, a selection of critically overrepresented pathways was enlisted. The list indicated most of the overexpressed genes in the list found to be correlated to specific PI3K-Akt pathway as shown in Table 5.3.1 with significant p value of 6.6×10^{-4}. Genes related to PI3K-Akt signaling pathway were found to be significantly upregulated in the RNA sequencing data compared to its control counterpart as tabulated in Table 5.3.1 (B) that connote activation of PI3K-Akt signaling pathway.

A

Deregulated pathways

	Pathways	Count	P-Value
1	Pathways in cancer	18	3.0 E-4
2	**PI3K-Akt signaling pathway**	16	6.6E-4
3	Endocytosis	13	1.3E-3
4	Focal adhesion	12	6.9E-4
5	Rap1 signaling pathway	12	8.1E-4
6	Transcriptional mis- regulation in cancer	11	5.2E-4
7	Ras signaling pathway	11	4.8E-3
8	Cytokine-cytokine receptor interaction	11	5.4E-3
9	MAPK signaling pathway	9	6.7E-2
10	Neuroactive ligand-receptor interaction	9	9.7E-2

B

Gene hits in PI3K-Akt signaling pathway

	Name of genes (symbol)	Fold Change
1	G protein subunit gamma 10 (GNG10)	-2.99
2	**KIT proto-oncogene receptor tyrosine kinase (KIT)**	**9.06**
3	**TEK receptor tyrosine kinase (TEK)**	**7.11**
4	**Cartilage Oligomeric matrix protein (COMP)**	**3.31**
5	**Collagen type I alpha 1 chain (COL1A1)**	**1.14**
6	**Collagen type I alpha 2 chain (COL1A2)**	**8.30**
7	**Collagen type IV alpha 1 chain (COL4A1)**	**2.38**
8	**Colony stimulating factor 1 receptor (CSF1R)**	**1.67**
9	**Cyclin D2 (CCND2)**	**3.04**
10	**Fibroblast growth factor receptor 2 (FGFR2)**	**5.81**
11	**Fms related tyrosine kinase 1 (FLT1)**	**6.83**
12	**Insulin like growth factor 1 (IGF1)**	**2.41**
13	**Interleukin 7 receptor (IL7R)**	**3.86**
14	**Platelet derived growth factor receptor alpha (PDGFRA)**	**8.47**
15	**Kinase insert domain receptor (KDR)**	**2.75**
16	**Thrombospondin 2 (THBS2)**	**3.67**
17	**Von Willebrand factor (VWF)**	**4.44**

Table 5.3.1 rhFABP4 regulated various gene expression linked to carcinogenesis. rhFABP4 treated PLC/PRF/5 were subjected to RNA sequencing. Genes with fold change of |Fold change|>1 and p value <0.05 was subjected to pathway analysis using DAVID. A) Gene enrichment analysis identified PI3K-Akt signaling pathway to be overrepresented in the gene list (B) with most genes found to be upregulated.

5.3.2 rhFABP4 upregulated PI3K-Akt signaling pathway

We next sought to confirm whether PI3K-Akt signaling pathway was altered in response to rhFABP4 treatment. Exogenous supply of rhFABP4 has been previously reported to induce PI3K/Akt pathway upon phosphorylation of Akt in breast and prostate cancer (Uehara et al., 2014; Guaita-Esteruelas et al., 2017). Given our initial results on RNA sequencing analysis and previous reports on phosphorylation of Akt verified by exogenous supply of rhFABP4 in breast and prostate cancer, we examined whether PI3K-Akt signaling pathway is activated by FABP4 in HCC as concluded upon analysis of RNA sequencing data in Table 5.3.1 (A). PLC/PRF/5 were treated with rhFABP4 at 100ng/ml and proteins collected at different time points to study time dependent expression of pAkt. As shown in the Figure 5.3.2, Akt was found to be phosphorylated at Ser473 after administration of rhFABP4 for 60 minutes with continuous over phosphorylation until 24 hours of administration. The result confirmed the activation of PI3K-Akt signaling pathway complying with our results obtained from gene enrichment analysis. No notable changes in total Akt and actin was observed. This confirmed the activation of PI3K-Akt signaling pathway by rhFABP4 as demonstrated by DAVID analysis of RNA. Aberrant activation of PI3K-Akt signaling pathway has been reported in HCC development and carcinogenesis and further in-depth molecular mechanism involving FABP4/PI3K-Akt and liver CSC properties was explored in the next part of the study.

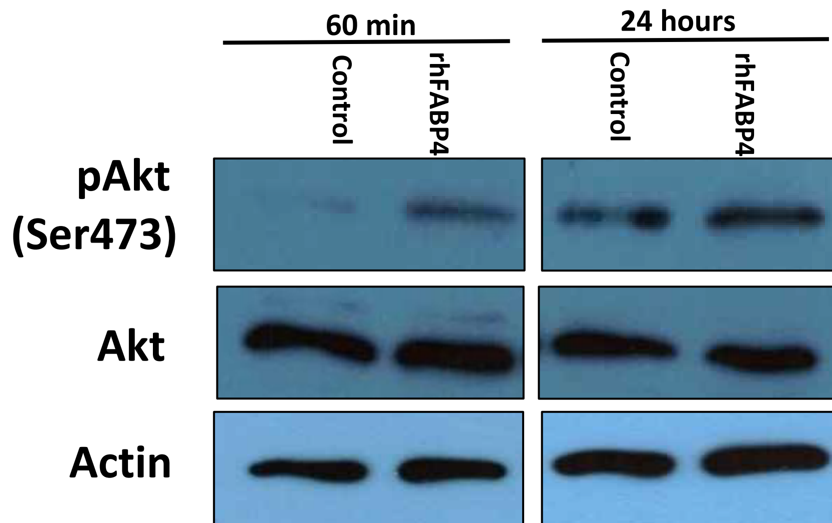


Figure 5.3.2 rhFABP4 activated PI3K/Akt pathway via phosphorylation of Akt. PLC/PRF/5 were administered with rhFABP4 at 100ng/ml at different time points and their proteins collected to analyze the phosphorylation pattern of Akt. Phosphorylation of Akt was observed within an hour of rhFABP4 administration however no changes in Akt was observed. Phosphorylation was observed until 24 hours of administration of rhFABP4.

5.3.3 rhFABP4 activated wnt/β-catenin signaling pathway mediated by phosphorylation of Akt

Our RNA sequencing data provided strong evidence of dysregulation of PI3K-Akt signaling pathway and western blot result confirmed the activation of PI3K-Akt pathway when treated with rhFABP4 alone. Activation of PI3K-Akt pathway is widely known to activate wnt/β-catenin signaling pathway which is found to regulate CSC pathway. Through membrane receptors, exogenous factors such as HGF has been reported in colon cancer to phosphorylate Akt through c-met receptor activation. Phosphorylation of Akt is demonstrated to disrupt axin-APC-GSK3β complex known to maintain β-catenin homeostasis. Disruption of the complex disables the complex to prime β-catenin for proteasomal degradation hence allows translocation of β-catenin resulting in transcription of wnt/β-catenin target genes that is found to enhance CSC properties. The report also reported an alternative route phosphorylated Akt could take to activate wnt/β-catenin signaling i.e. by auto phosphorylating β-catenin resulting in translocation of β-catenin to nucleus for further transcription.

Concurring with our previous results, we tried to deduce the status of wnt/β-catenin signaling pathway upon phosphorylation of Akt. For this purpose, we conducted western blot probing of GSK3β (Ser 9) phosphorylation and β-catenin. Upon activation of the signaling pathway, we hypothesized phosphorylation of Akt resulted in phosphorylation of GSK3β and subsequent accumulation of β-catenin. As demonstrated in Figure 5.3.3 (A) western blot results, subsequent phosphorylation of GSK3β is observed at 60 min (the same time period where phosphorylation of Akt was observed) and subsequent accumulation of β-catenin too. This western blot thus provides us a substantial evidence of the possible mode of communication of rhFABP4 via phosphorylation of Akt-GSK3β-β-catenin

eventually resulting in activation of wnt signaling pathway. To further strengthen our hypothesis and confirm the activation of wnt signaling pathway. We conducted a standard TOP/FOP assay of rhFABP4 treated cells. TOP/FOP assay is a dual luciferase reporter assay used to detect the activation of canonical wnt/β-catenin signaling pathway. Upon activation of wnt/β-catenin signaling pathway, β-catenin translocates to the nucleus and associate with TCF/LEF transcription factor to activate transcription of wnt target genes. TOP/FOP assay comprises of mutant plasmid and wild type luciferase plasmid with TCF/LEF binding region thus upon activation of wnt/β-catenin pathway, β-catenin initiates the transcription of luciferase signal and thus the signal provides an estimation of wnt/β-catenin signaling activation. We transfected PLC/PRF/5 cells with TOP/FOP plasmid in presence of rhFABP4 for six hours and changed the medium to new rhFABP4. The experiment was carried out at two different concentration of rhFABP4 20ng/ml and 100ng/ml to verify dose dependent activation of wnt/β-catenin signaling as reflected in functional assay.

Our result as illustrated in Figure 5.3.3 (B) showed dose dependent activation of wnt/β-catenin signaling upon administration of rhFABP4. This result confirmed the activation of wnt/β-catenin signaling by rhFABP4 through phosphorylation of Akt resulting in regulation of liver CSC properties in HCC. These two results thus provide us with strong evidence of involvement of Akt-GSK3β-β-catenin resulting in activation of wnt signaling pathway by rhFABP4 in HCC.

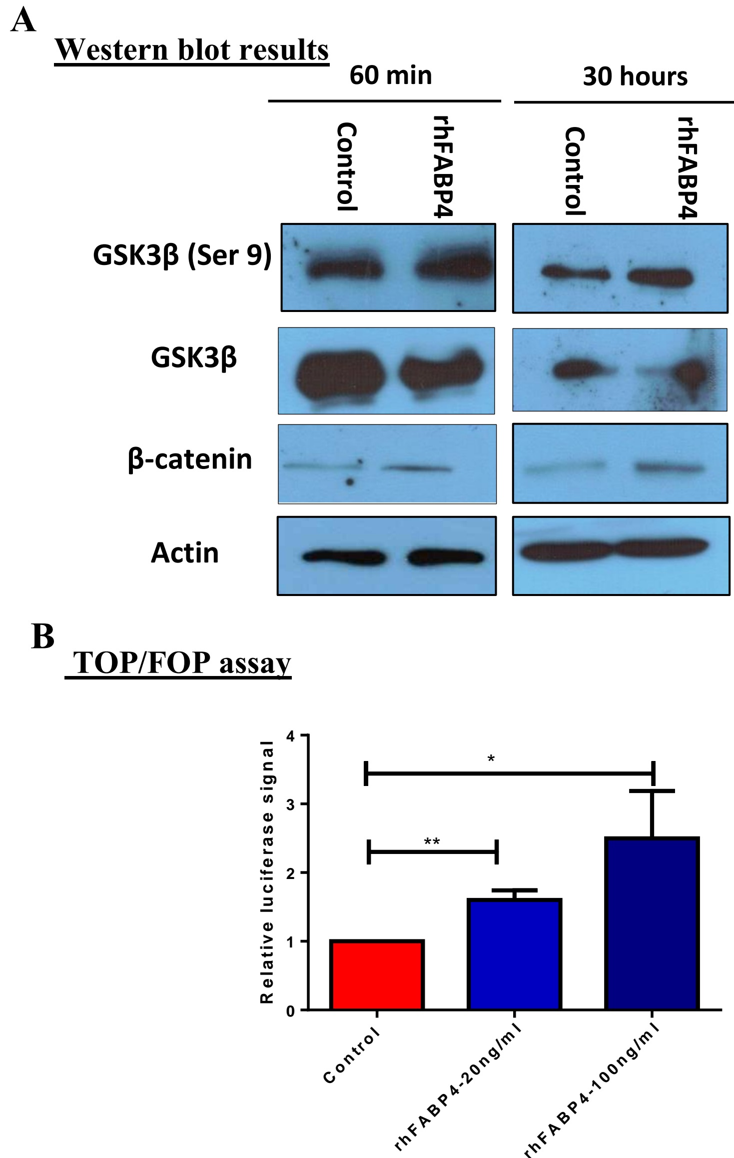


Figure 5.3.3 rhFABP4 activated wnt/β-catenin signaling pathway to regulate CSC properties in HCC. A) Western blot assay indicating the phosphorylation of GSK3β and accumulation of β-catenin upon phosphorylation of Akt by FABP4. B) TOP/FOP assay was conducted on PLC/PRF/5 to study the activation state of wnt/β-catenin signaling pathway as wnt/β-catenin signaling pathway was found to be commonly deregulated in both adipocyte CM and rhFABP4 administered HCC cell lines. TOP/FOP confirmed dose wise activation of wnt/β-catenin signaling pathways as illustrated in the graph. rhFABP4 at 20ng/ml and 100ng/ml was administered to PLC/PRF/5 and the activation of wnt/β-catenin signaling pathway measured based on the luciferase signal. (*$p<0.05$, **$p<0.01$, t-test).

Section 5.4 Discussion

Our results in chapter 3 and 4 showed the enhancing effect of adipocytes in regulation of CSC properties in HCC cells via secretion of FABP4. Although we have compelling results supporting the promoting effects of FABP4 on regulation of CSC properties in HCC, therapeutic application of the finding requires detailed mechanism of FABP4 and regulation of CSC properties. Regardless of much research on HCC, HCC still remains one of the deadliest cancers in the world with a very low 5-year survival (less than 10%). Recent approval of sorafenib by FDA as one of the few HCC targeted molecular inhibitor; this approval has provided new direction for HCC therapies which was previously dominated by resection and embolization. Molecular targets for therapeutic usage is designed upon understanding the full mechanism at the cellular level. Therefore, although we confirmed FABP4 played a crucial role in regulation of CSC properties in HCC. For possible therapeutic implication of our study, we had to elucidate the underlying mechanism to better understand as to how FABP4 regulated CSC properties which could be intervened for therapeutic purposes.

For this purpose, we subjected rhFABP4 treated PLC/PRF/5 for RNA sequencing. RNA sequencing confirmed deregulation of various genes compared to its control counterpart. The deregulated genes were further classified based on their |fold change| >1 and p value <0.05. To study the link between the expression of genes and cellular pathways involved, we carried out gene enrichment analysis using publicly available analysis software, DAVID. Gene enrichment analysis in DAVID confirmed most of the deregulated gene to be involved in cancer pathways with most genes found to be involved to PI3K-Akt signaling pathway with high significance.

PI3K-Akt signaling pathway is an important pathway found to activate wnt/β-catenin signaling pathway in cancer. Wnt/β-catenin signaling pathway is of great interest to us as it is widely known for its role in regulation of CSC properties and activation of wnt/β-catenin signaling pathway is reported to enhance self-renewal, migration, invasion, drug resistance and tumorigenicity in HCC cells (Vilchez et al., 2016). Wnt/β-catenin signaling pathway is a complex stepwise pathway involving wnt ligands, membrane receptors, axin-APC-GSK3β and β-catenin in which axin-APC-GSK3β phosphorylates free β-catenin and prime it for proteasomal degradation regulating the wnt/β-catenin signaling pathway in homeostasis (Komiya and Habas, 2008). Based on the RNA sequencing data, we hypothesized possible activation of wnt/β-catenin signaling via activation of PI3K-Akt signaling pathway by FABP4. FABP4 has been recently reported to activate PI3K/Akt/MAPK pathway in breast and prostate cancer (Uehara et al., 2014; Guaita-Esterelas et al., 2017). Exogenous supply of FABP4 has been reported to activate the pathway by phosphorylation of Akt. We therefore, examined the phosphorylation status of Akt by FABP4 at different time points. Western blot analysis of the protein phosphorylation confirmed the phosphorylation of Akt in rhFABP4 treated group. Phosphorylation could be observed within 60 minutes of administration of rhFABP4. This result provided us compelling evidence of possible activation of wnt/β-catenin signaling via Akt by FABP4 to regulate CSC properties in HCC. Following this affirmation, we conducted TOP/FOP assay to study the status of wnt/β-catenin signaling pathway in rhFABP4 treated cells which confirmed the dose dependent activation of wnt/β-catenin signaling pathway upon rhFABP4 administration. The results provided us with compelling evidence that

FABP4 regulated CSC properties in HCC via wnt/β-catenin signaling pathway and is facilitated through PI3K-Akt signaling pathway.

RNA sequencing data showed rhFABP4 treated PLC/PRF/5 showcased deregulation in more than one pathway involved in cancer pathways such as Rap 1 signaling, Ras signaling and MAPK signaling pathway. This finding indicates the possible regulation of more than one signaling pathway by FABP4 in HCC cells. Furthermore, DAVID analysis on rhFABP4 PLC/PRF/5 demonstrated upregulation of class of tyrosine kinases indicating activation of tyrosine kinase pathway which could modulate various signaling pathway involved in carcinogenesis (Paul and Mukhopadhyay, 2004). Although we confirmed the phosphorylation of Akt by rhFABP4 resulting in activation of wnt/β-catenin signaling pathway to be the underlying mechanism, since wnt/β-catenin signaling is a receptor bound signaling pathway, we were however not able to identify the receptor for FABP4 at this stage which would of great interest for therapeutic purpose.

Interestingly, we could not detect FABP4 in protein lysate of HCC administered with rhFABP4. Since the cells were treated with rhFABP4 only in serum free medium, we believe FABP4 is responsible for phosphorylation of Akt however unlike in prostate (Uehara et al., 2014) and breast cancer (Guiata-Esterelas et al., 2017) was not internalized but degraded earlier than the time point we assessed or is not internalized at all instead theorized to work as a ligand to an unknown kinase receptor and initiate the cascade of signaling through the kinase receptor. This mode of mechanism is postulated based on the ELISA result in section 4.3.2 that demonstrated decrease in concentration of FABP4 upon administration of adipocytes CM in PLC/PRF/5. This area of research is yet to be explored to better understand the fate of exogenously supplied FABP4.

In summary, our findings in chapter 5 confirmed the underlying mechanism of FABP4 in regulating liver CSC properties. FABP4 supplied by adipocytes interacts with a receptor initiating the PI3K/Akt cascade of signaling and possibly disrupting the axin-APC-GSK3β complex resulting in availability of β-catenin for transcription of wnt related genes.

CHAPTER 6: CONCLUSION AND FINAL REMARKS

Section 6.1 Conclusion

HCC remains one of the deadliest cancers in the world because of its asymptomatic nature and high aggressiveness upon diagnosis. In previous years, HCC development was predominantly caused by viral infection and exposure to aflatoxin however the development of anti-vaccine and measures to reduce exposure to aflatoxin has greatly reduced the viral and aflatoxin related HCC relatively (Sell, 2012). Regardless of preventive measures like reduction in aflatoxin and immunization against viral infection, the prevalence of HCC has not yet been managed and the prognosis of HCC diagnosed still remains very poor. This could be due to complexity of the disease itself and also because of various etiologies of HCC development and progression and the subsequent rise in other causes of HCC that requires to be addressed urgently. NAFLD, previously thought to be a benign condition of disposition of fats in liver has been reported to increase in incidence recently and surprisingly the incidence of fatty liver to HCC development has been found to increase rapidly (Baffy et al., 2012). NAFLD, the hepatic manifestation of obesity, is found to increase in incidence both in developing and developed countries (Chowdhury and Younoddi, 2016). With the rising trend in obesity and its strong correlation to development of NAFLD (Fabbrini et al., 2010; Fan et al., 2017) and to cancer mortality including HCC (Calle et al., 2003), yet the study on the influence of obesity on HCC development and progression has not been mechanistically studied.

With the discovery of correlation between obesity and cancer mortality by Calle and colleagues in 2003, scientific world took great interest in understanding the relationship and communication between one of the key cellular factors of obese microenvironment, adipocytes and that of cancer cells. Most primarily, the study

has been conducted in adipocyte rich environment cancer organs such as breast, prostate and ovarian cancers in which studies further confirmed a detrimental role of adipocytes and adipocyte secretomes in progression and aggressiveness of cancers. With the high prevalence of NAFLD induced HCC and also the rise research evidences confirming the promoting effects of adipocytes and its secretomes in cancer progression, we understood the necessity in this direction of research and aimed to decipher the role of adipocytes in HCC progression with this project. In addition to predisposition factor of NAFLD, the communication between adipocytes and its secretomes on HCC development is urgently required primarily because of the role of liver in maintaining physiological homeostasis. The liver is the center of detoxification and as per the "portal hypothesis theory" liver is reported to the directly exposed to free fatty acids and cytokines released from visceral adipocytes making its more prone to adipocyte secretomes (Rytka et al., 2011). Therefore, it is very urgent we understand the mechanistic communication between adipocytes and HCC in order to provide effective therapeutic intervention to prolong the prognosis. One of the hypothesized mechanistic progression of metastasis and relapse of cancer is governed by CSC theory as described in section 1.3. The development and existence of CSCs in HCC has been widely studied with the identification of various markers as summarized in section 1.3.2. These liver CSCs are reported to be more resilient to traditional therapies and is capable of self-renewal ability resulting in relapse of cancer and poor prognosis overall. With regards to this theory, in this research, we tried to understand the role of adipocytes in regulation of liver CSCs.

To examine our hypothesis, we first studied the communication between adipocytes and HCC cells as to whether adipocytes enhanced liver CSC properties such as self-

renewal, tumorigenicity, migration, invasion and drug resistance. For this purpose, we purchased human visceral preadipocytes and differentiated it into visceral adipocytes for the experiments. Anatomically, visceral adipocytes are found to be in close proximity to liver and based on "portal theory" is the source of free fatty acids and adipocyte secretomes delivered to liver. Our results confirmed our hypothesized as we demonstrated HCC cells in presence of adipocytes was found to develop enhanced self-renewal properties. The experiment was conducted in a co-culture setup where the two cells were physically barred with the help of an insert of 0.4µm which allowing transfer of small molecules mimicking paracrine signaling. The result confirmed the existence of paracrine signaling communication between the cells. This was an important discovery on the basis of "portal hypothesis" which indicates the accessibility of liver to adipocyte secretomes found to enhance its liver CSC properties. Furthermore, we also demonstrated a reciprocal communication between HCC cells and adipocytes and it was found HCC cells further stimulated adipocytes to produce more of the secretomes required hinting the modulation of adipocytes in tumor microenvironment similar to the microenvironment in NAFLD. In addition to self-renewal, we also demonstrated adipocyte secretomes were able to enhance other liver CSC properties such as migration, invasion, drug resistance, and expression of liver CSC markers. *In vivo* studies on tumorigenicity further confirmed adipocyte CM treated cells to have higher tumorigenicity. This is the first report demonstrating the role of adipocytes in regulating liver CSC properties via paracrine signaling pathway.

Upon this discovery, we tried to further understand the communication in depth by deducing the identification of the secretomes playing such a crucial role in regulating liver CSC properties. This discovery is of great importance in both

understanding the communication between the two cells but also pose a potential therapeutic possibility as the communication can be abrogated by designing antibodies or with the help of molecular inhibitors. For this purpose, we made use of Orbitrap mass spectrometry to analyze the protein components in adipocyte CM. Mass spectrometry on adipocyte CM provided us with a total of 209 proteins which were narrowed down to 20 proteins based on its relevance to adipocyte microenvironment, secretion and reports in cancer biology. We hypothesized increase in the secretion of the protein while in communication with HCC cells since it is important for adaptability of HCC to traditional therapies therefore with this hypothesis, we conducted mass spectrometry analysis of CM collected using co-culture set up between adipocytes and HCC cells. The result identified FABP4 as the possible candidate as the protein secretion was found to be markedly surged (~8 fold) in presence of HCC hinting the possible crucial role of FABP4 in the communication. This result was further quantified using ELISA assays which in accordance to mass spectrometry data confirmed increase in secretion of FABP4 upon stimulated by HCC. In order to confirm the functional role of FABP4, we then carried out functional assays to assess the role of FABP4 in self-renewal, migration, invasion, drug resistance, and expression of liver CSC markers by administering commercially available recombinant proteins. Concurrently, functional assays by abrogating function of FABP4 in adipocyte CM was studied with the help of FABP4 inhibitor (BMS309403). rhFABP4 enhanced liver CSCs properties while BMS309403 abolished enhancement in properties experienced in presence of adipocyte CM. These experiments confirmed the role of FABP4 present in adipocyte CM to play the major role in regulating liver CSC properties. With this

data, we are the first one to identify adipocytes derived FABP4 to play a crucial role in promoting liver CSC properties in HCC.

Following the discovery of FABP4 to be the vital secretomes in the communication between HCC and adipocytes, we next, sought to identify the mode of mechanism of FABP4 in regulation of liver CSC properties. For this purpose, we conducted RNA sequencing of rhFABP4 treated PLC/PRF/5. RNA sequencing gene enrichment analysis by DAVID revealed most of the deregulated genes were found to be involved in regulation of cancer related pathways. PI3K-Akt signaling pathway was found to be significantly deregulated followed by generic pathways related to cancer. PI3K-Akt signaling is of high relevance to our study as activation of PI3K-Akt is reported to activate wnt/β-catenin signaling pathway. Wnt/β-catenin signaling pathway is found to play a critical role in regulation of liver CSC in HCC (Vilchez et al., 2016). Onto deciphering this link, we first demonstrated the activation of PI3K-Akt signaling pathway by western blot in which we observed phosphorylation of Akt at Ser-473 upon 60 minutes of administration of rhFABP4. Following this confirmation, we studied the activation state of wnt/β-catenin signaling pathway, one of the downstream pathways of Akt phosphorylation linking to CSC properties. With the help and western blot assay of expression level of wnt signaling pathways-GSK3β and β-catenin upon phosphorylation of Akt was found to be upregulated indicating the activation of wnt signaling pathway. TOP/FOP assay further confirmed dose dependent activation of wnt/β-catenin signaling pathway upon administration of rhFABP4.

Our results in this thesis, for the first time demonstrated adipocyte derived FABP4 enhanced CSC properties in HCC via Akt mediated wnt/β-catenin signaling pathway (Figure 6.1). Our result not only demonstrated the adipocytes

play a major role in HCC aggressiveness via paracrine signaling; we also deciphered it does so by regulating CSC properties in HCC. We also identified FABP4 to be the key player in this regulation. We further delineated the mechanistic role of FABP4 in the regulation. We demonstrated FABP4 was able to activate wnt/β-catenin signaling pathway governing CSC properties via PI3K-Akt activation and subsequent phosphorylation of GSK3β and accumulation of β-catenin. This is the first time FABP4 has been found to link to regulate CSC properties of cancer and wnt signaling pathway. Furthermore, we also conducted preliminary correlation between expressions of FABP4 in the non-tumor cohort to prognosis of HCC. Overall survival analysis using TCGA non-tumor cohort established poor prognosis in HCC patients with high expression of FABP4. This correlation was found to be of high significance and similar trend in other datasets. All in all, the results in this thesis provides a new outlook to fatty acid binding role providing substantial evidence of its role in governing liver CSC properties.

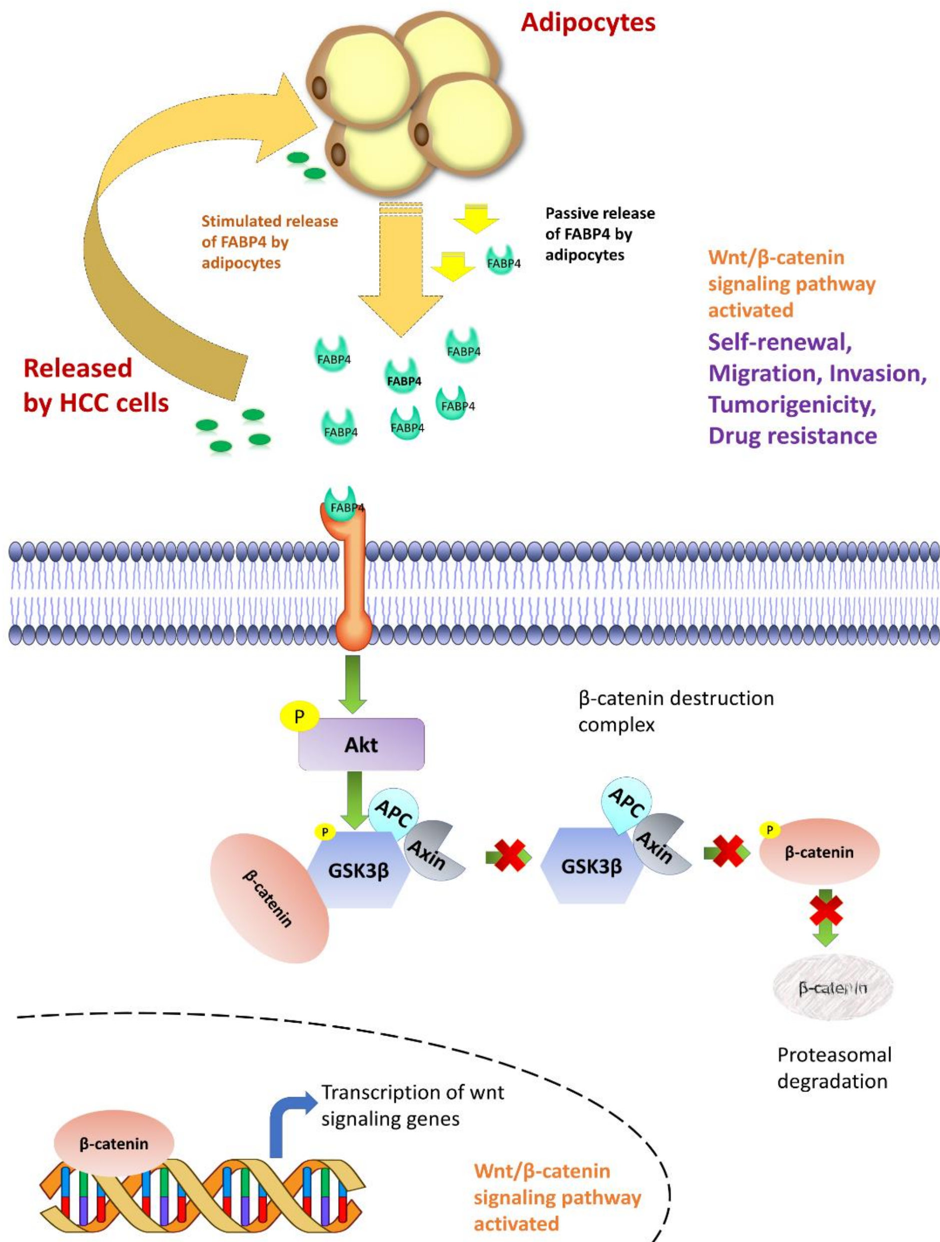


Figure 6.1 Proposed mechanism of regulation of liver CSC properties by FABP4 via wnt/β-catenin signaling pathway via Akt phosphorylation. We proposed activation of adipocytes enhanced liver CSC properties in HCC by secreting out FABP4. FABP4 upon interaction with a receptor phosphorylated Akt intracellularly activating the PI3k-Akt signaling pathway subsequently phosphorylating GSK3β and disrupting the β-catenin destruction complex. This prevents priming of β-catenin to degradation hence results in transcription of wnt/β-catenin signaling genes involved in CSC properties- self-renewal, tumorigenicity, drug resistance, migration and invasion abilities. Alternatively, β-catenin could also be phosphorylated by activated Akt resulting in its translocation to nucleus resulting in transcription of wnt/β-catenin signaling genes.

Section 6.2 Final remarks

Our results in the thesis provide compelling evidence of the role of adipocytes, particularly adipocyte secretomes playing a crucial role in regulation of liver CSC properties. The work presented in this is novel in different aspects as we for the first time demonstrate the enhancing effect of adipocytes in HCC cancer cells via regulation of CSC properties through FABP4/Akt/β-catenin signaling pathway which is novel as no one has yet reported FABP4 playing a role in Akt/β-catenin signaling pathway and hence its role in CSC properties. Although there has been reports on the role of FABP4 in cancer progression, much of the research did not address the mechanistic role of FABP4 in this progression neither its role in regulation of CSC properties (Gurung and Chung et al., 2018). Recently, Zhong and colleagues (2018) reported the tumor suppressive role of FABP4 in HCC upon over expression of FABP4 in HCC cells (Zhong et al., 2018). This result contradicts our conclusion however it is important to note that the result could be attributed to the source of FABP4 as in case of Zhong and colleagues, FABP4 is internally expressed however exogenous supply of FABP4 interacts with membrane receptors. Since we could not detect FABP4 in the protein lysate in HCC cells upon administration of FABP4, we postulate possible existence of a membrane kinase which upon interaction with FABP4 would result in phosphorylation of Akt intracellularly and hence activate wnt/β-catenin signaling pathway. This interaction is not possible upon over expression of FABP4 internally. Contradictory role of FABP4 internalized in contrast to internally expressed hint the diverse role of FABP4.These findings indicate the possible double-edged sword role of FABP4 in cancer progression in HCC which requires further exploration.

Although we have compelling *in vitro* data demonstrating the role of FABP4 in enhancing liver CSC properties, further experiments could help us to solidify our claim. To further provide supporting materials to further confirm our hypothesis, *in vivo* studies studying the effects of rhFABP4 on tumorigenicity and of FABP4 inhibitor in cancer progression requires to be studied. Detrimental role of FABP4 in obesity related HCC should also be studied by assessing tumorigenicity in FABP4$^{-/-}$ mice upon injection of DEN, followed by feeding with high fat diet. This part of the experiment is undergoing in collaboration with Dr. Ruby Hoo (HKU). The findings from this experiment will help us to evaluate the crucial role of FABP4 in HCC development. Due to limitation in cohort subjected to NAFLD induced HCC and obesity related HCC, we were unable to conclusively study the correlation between FABP4 expression level to overall and disease-free survival. To better illustrate the supply of FABP4 from tumor microenvironment to HCC instead of internal expression of FABP4 in HCC, data on expression level of FABP4 in HCC and respective FABP4 positive staining in IHC to further validate our communication mechanism however we were not able to do yet and is in the process of collaboration. In addition, although serum FABP4 is found to be higher in obese population (Karakas et al., 2009) and NAFLD (Koh et al., 2009), the correlation between NAFLD induced HCC stages and relative FABP4 level has not yet been elucidated. Based on our results, we expect to observe rise in serum level FABP4 in accordance to HCC stage and result in poor survival. Samples for this experiment is also underway in collaboration with Dr. Zhu Mo-Lin (Sun Yat Sen University, China). One of the peculiar observations we made is further secretion of FABP4 upon stimulation by HCC. Mass spectrometry observed ~ 8-fold increase in FABP4 secretion in presence of HCC and the same observation was made by ELISA when

adipocyte CM was incubated with HCC cells and re-incubated with adipocytes (CAA-CM). Based on this observation, we hypothesized possible secretion of stimulant from HCC cells that would further stimulate adipocytes to secrete more FABP4 required for regulation of CSC properties. In further experiments, we aim to deduce the stimulating agent to better understand the reciprocal communication that exists between these two cells.

Our RNA sequencing data on rhFABP4 treated cells demonstrated deregulation of various pathways involving in interaction with external environment such as immune cells (B cell activation, Toll receptor), endothelial cells (VEGF signaling), tumor cells (integrin signaling) and cytokine inflammation signaling hinting the possible role of FABP4 and adipocytes secretomes in modulating cancer microenvironment in addition to enhancing liver CSC properties. Among the secretomes we discovered, adipocyte CM comprises of mixture of important secretomes such as cathepsin B, periostin, Laminin gamma 1 along with release of fatty acids which are previously reported to play a role in cancer progression (Gondi and Rao, 2013; Lee, 2016; Zhang et al., 2017). Fatty acids alone have been reported to facilitate epithelial-mesenchymal transition in HCC cells (Nath et al., 2015). Apart from directly affecting cancer cell properties, we also identified upregulation in release of protein colony stimulating factor 1 (CSF1) in co-culture set up which is reported to regulate macrophage differentiation and affect tumor associated macrophage infiltration resulting in progression of cancer (Ding et al., 2016). Blockade of CSF1R in HCC has been reported to suppress tumor growth in HCC by shifting the polarization of macrophage to M1 type from M2 type (Ao et al., 2017). This finding indicates prominent role of adipocytes in cancer progression

either by directly characterizing properties of cancer cells and also by modulating cancer microenvironment to provide a suitable niche for cancer growth and evasion. Taken together, our result provides for the first time the role of adipocytes in HCC progression and the mechanistic mode of communication of adipocytes derived FABP4 in regulation of CSC properties via Akt mediated wnt/β-catenin signaling pathway. Furthermore, our analysis on adipocyte CM and RNA sequencing provide evidence of possible synergistic role of adipocyte secretomes in regulating cancer progression which requires to be explored further to provide novel therapeutic options in HCC.

REFERENCES

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America, 100*(7), 3983-3988.

Ao, J. Y., Zhu, X. D., Chai, Z. T., Cai, H., Zhang, Y. Y., Zhang, K. Z., et al. (2017). Colony-stimulating factor 1 receptor blockade inhibits tumor growth by altering the polarization of tumor-associated macrophages in hepatocellular carcinoma. *Molecular Cancer Therapeutics, 16*(8), 1544-1554.

Aragonès, G., Saavedra, P., Heras, M., Cabré, A., Girona, J., & Masana, L. (2012). Fatty acid-binding protein 4 impairs the insulin-dependent nitric oxide pathway in vascular endothelial cells. *Cardiovascular Diabetology*, *11*(1), 1-8.

Baffy, G., Brunt, E. M., & Caldwell, S. H. (2012). Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *Journal of Hepatology, 56*(6), 1384-1391.

Balkwill, F. R., Capasso, M., & Hagemann, T. (2012). The tumor microenvironment at a glance. *Journal of Cell Science, 125*(23), 5591-5596.

Balogh, J., Victor, D., 3rd, Asham, E. H., Burroughs, S. G., Boktour, M., Saharia, A., et al. (2016). Hepatocellular carcinoma: a review. *Journal of Hepatocellular Carcinoma, 3*, 41-53.

Bao, S., Wu, Q., Sathornsumetee, S., Hao, Y., Li, Z., Hjelmeland, A, B., et al. (2006). Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer research, 66*(16), 7843-7848.

Bartucci, M., Svensson, S., Ricci-Vitiani, L., Dattilo, R., Biffoni, M., Signore, M., et al. (2010). Obesity hormone leptin induces growth and interferes with the cytotoxic effects of 5-fluorouracil in colorectal tumor stem cells. *Endocrine-Related Cancer, 17*(3), 823-833.

Bayat Mokhtari, R., Homayouni, T. S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., et al. (2017). Combination therapy in combating cancer. *Oncotarget, 8*(23), 38022-38043.

Behan, J. W., Yun, J. P., Proektor, M. P., Ehsanipour, E. A., Arutyunyan, A., Moses, A. S., et al. (2009). Adipocytes impair leukemia treatment in mice. *Cancer Research, 69*(19), 7867-7874.

Betof, A. S. & Dewhirst, M. W. (2010). Establishing the Tumor Microenvironment. D. W. *In Tumor Microenvironment,* Siemann (Ed.).7-33.

Bhangoo, M. S., Karnani, D. R., Hein, P. N., Giap, H., Knowles, H., Issa, C., et al. (2015). Radioembolization with Yttrium-90 microspheres for patients with unresectable hepatocellular carcinoma. *Journal of Gastrointestinal Oncology, 6*(5), 469-478.

Blair, A., Hogge, D. E., Ailles, L. E., Lansdorp, P. M., & Sutherland, H. J. (1997). Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood, 89*(9), 3104-3112.

Blair, A., Hogge, D. E., & Sutherland, H. J. (1998). Most acute myeloid leukemia progenitor cells with long-term proliferative ability in vitro and in vivo have the phenotype CD34 (+)/CD71 (-)/HLA-DR-. *Blood, 92*(11), 4325-4335.

Blair, A., & Sutherland, H. J. (2000). Primitive acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo lack surface expression of c-kit (CD117). *Experimental Hematology, 28*(6), 660-671.

Blanpain, C., Mohrin, M., Sotiropoulou, P. A., & Passegue, E. (2011). DNA-damage response in tissue-specific and cancer stem cells. *Cell Stem Cell, 8*(1), 16-29.

Boiteux, G., Lascombe, I., Roche, E., Plissonnier, M.-L., Clairotte, A., Bittard, H., & Fauconnet, S. (2009). A-FABP, a candidate progression marker of human transitional cell carcinoma of the bladder, is differentially regulated by PPAR in urothelial cancer cells. *International Journal of Cancer, 124*(8), 1820-1828.

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature medicine, 3*(7), 730-737.

Booth, A., Magnuson, A., Fouts, J., & Foster, M. (2015). Adipose tissue, obesity and adipokines: role in cancer promotion. *In Hormone Molecular Biology and Clinical Investigation*, 57 (1), 57-74.

Bosch, F. X., Ribes, J., Cleries, R., & Diaz, M. (2005). Epidemiology of hepatocellular carcinoma. *Clinics in Liver Disease, 9*(2), 191-211.

Braun, S., Bitton-Worms, K., LeRoith, D. (2011). The link between the metabolic syndrome and cancer. *International Journal of Biological Sciences*, 7(7), 1003-1015.

Breitenstein, S., Dimitroulis, D., Petrowsky, H., Puhan, M. A., Mullhaupt, B., & Clavien, P. A. (2009). Systematic review and meta-analysis of interferon after curative treatment of hepatocellular carcinoma in patients with viral hepatitis. *British Journal of Surgery, 96*(9), 975-981.

Brogi, E., Wu, T., Namiki, A., & Isner, J. M. (1994). Indirect angiogenic cytokines upregulate VEGF and bFGF gene expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation, 90*(2), 649-652.

Bruix, J., Gores, G. J., & Mazzaferro, V. (2014). Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut, 63*(5), 844-855.

Budhu, A., & Wang, X.W. (2006). The role of cytokines in hepatocellular carcinoma. *Journal of leukocyte biology, 80*(6), 1197-1213.

Cabré, A., Lázaro, I., Girona, J., Manzanares, J. M., Marimón, F., Plana, N., et al. (2007). Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic patients. *Atherosclerosis, 195*(1), e150-e158.

Calder, P. C. (2011). Fatty acids and inflammation: The cutting edge between food and pharma. *European Journal of Pharmacology, 668(S1)*, S50-S58.

Calle, E. E., Rodriguez, C., Walker-Thurmond, K., & Thun, M. J. (2003). Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *New England Journal of Medicine, 348*(17), 1625-1638.

Cantor, H. M., & Dumont, A. E. (1967). Hepatic Suppression of Sensitization to Antigen absorbed into the Portal System. *Nature, 215*(5102), 744-745.

Carbone, C., Piro, G., Gaianigo, N., Ligorio, F., Santoro, R., Merz, V., et al. (2017). Adipocytes sustain pancreatic cancer progression through a non-canonical WNT paracrine network inducing ROR2 nuclear shuttling. *International Journal of Obesity, 42*(3), 334-343.

Castelli, G., Pelosi, E., & Testa, U. (2017). Liver cancer: molecular characterization, clonal evolution and cancer stem cells. *Cancers, 9*(9), 127.

Cataltepe, O., Arikan, M. C., Ghelfi, E., Karaaslan, C., Ozsurekci, Y., Dresser, K., et al. (2012). Fatty acid binding protein 4 is expressed in distinct endothelial and non-endothelial cell populations in glioblastoma. *Neuropathology and Applied Neurobiology, 38*(5), 400-410.

Chang, M. H., Chen, C. J., Lai, M. S., Hsu, H. M., Wu, T.C., Kong, M. S., et al. (1997). Universal hepatitis b vaccination in taiwan and the incidence of hepatocellular carcinoma in children. *New England Journal of Medicine, 336*(26), 1855-1859.

Chang, M. H., You, S. L., Chen, C. J., Liu, C. J., Lee, C. M., Lin, S. M., et al. (2009). Decreased incidence of hepatocellular carcinoma in hepatitis b vaccines: a 20-year follow-up study. *Journal of the National Cancer Institute, 101*(19), 1348-1355.

Chang, Y. S., Adnane, J., Trail, P. A., Levy, J., Henderson, A., Xue, D., et al. (2007). Sorafenib (BAY 43-9006) inhibits tumor growth and vascularization and induces tumor apoptosis and hypoxia in RCC xenograft models. *Cancer Chemotherapy and Pharmacology, 59*(5), 561-574.

Chen, L., Li, M., Li, Q., Wang, C. J., & Xie, S. Q. (2013). DKK1 promotes hepatocellular carcinoma cell migration and invasion through β-catenin/MMP7 signaling pathway. *Molecular Cancer, 12*(1), 1-14.

Chen, T., Yang, K., Yu, J., Meng, W., Yuan, D., Bi, F., et al. (2012). Identification and expansion of cancer stem cells in tumor tissues and peripheral blood derived from gastric adenocarcinoma patients. *Cell Research, 22(1)*, 248-258.

Chen, W., Wu, J., Shi, H., Wang, Z., Zhang, G., Cao, Y., et al. (2014). Hepatic stellate cell coculture enables sorafenib resistance in huh7 cells through HGF/c-Met/Akt and Jak2/Stat3 pathways. *BioMed Research International, 2014*, 1-10.

Cheng, A. L., Guan, Z., Chen, Z., Tsao, C. J., Qin, S., Kim, J. S., et al. (2012). Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma according to baseline status: Subset analyses of the phase III Sorafenib Asia–Pacific trial. *European Journal of Cancer, 48*(10), 1452-1465.

Cheng, A. L., Kang, Y. K., Chen, Z., Tsao, C. J., Qin, S., Kim, J. S., et al. (2009). Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular

carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *The Lancet Oncology, 10*(1), 25-34.

Cheng, L., Jin, X. K., Li, W.W., Li, S., Guo, X. N., Wang, J et al., (2013). Fatty acid binding proteins fabp9 and fabp10 participate in antibacterial responses in chinese mitten crab, Eriocheir sinensis. *PLoS ONE*, *8*(1), 1-11.

Cheung, T. T., Poon, R. T. P., Yuen, W. K., Chok, K. S. H., Tsang, S. H. Y., Yau, T., et al. (2013). Outcome of laparoscopic versus open hepatectomy for colorectal liver metastases. *ANZ Journal of Surgery, 83*(11), 847-852.

Chiang, C. H., Lai, J. S., Hung, S. H., Lee, L. T., Sheu, J. C., & Huang, K. C. (2013). Serum adiponectin levels are associated with hepatitis B viral load in overweight to obese hepatitis B virus carriers. *Obesity, 21*(2), 291-296.

Chiyonobu, N., Shimada, S., Akiyama, Y., Mogushi, K., Itoh, M., Akahoshi, K. et al. (2018). Fatty Acid Binding Protein 4 (FABP4) Overexpression in intratumoral hepatic stellate cells within hepatocellular carcinoma with metabolic risk factors. *The American Journal of Pathology, 188*(5), 1213-1224.

Chmurzyήska, A. (2006). The multigene family of fatty acid-binding proteins (FABPs): Function, structure and polymorphism. *Journal of Applied Genetics, 47*(1), 39-48.

Choi, J. H., Park, S. H., Leung, P. C. K., & Choi, K. C. (2005). Expression of leptin receptors and potential effects of leptin on the cell growth and activation of mitogen-activated protein kinases in ovarian cancer cells. *The Journal of Clinical Endocrinology & Metabolism, 90*(1), 207-210.

Chowdhury, A., & Younossi, Z. M. (2016). Global epidemiology and risk factors for nonalcoholic fatty liver disease. *Alcoholic and Non-Alcoholic Fatty Liver Disease: Bench to Bedside,* Chalasani N., Szabo G. (Ed), 21-40.

Clarke, M. F., & Hass, A. T. (2006). Cancer stem cell. *In Reviews in Cell Biology and Molecular Medicine,* R. A. Meyers (Ed.).

Coelho, P., Almeida, J., Prudêncio, C., Fernandes, R., & Soares, R. (2016). Effect of adipocyte secretome in melanoma progression and vasculogenic mimicry. *Journal of Cellular Biochemistry, 117*(7), 1697-1706.

Coelho, P., Silva, L., Faria, I., Vieria, M., Monteiro, A., Pinto, G., et al. (2017). Adipocyte secretome increases radioresistance of malignant melanocytes by improving cell survival and decreasing oxidative status. *Radiation Research, 187*(5), 581-588.

Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., & Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Research, 65*(23), 10946-10951.

Cox, C. V., Evely, R. S., Oakhill, A., Pamphilon, D. H., Goulden, N. J., & Blair, A. (2004). Characterization of acute lymphoblastic leukemia progenitor cells. *Blood, 104*(9), 2919-2925.

Cox, C. V., Martin, H. M., Kearns, P. R., Virgo, P., Evely, R. S., & Blair, A. (2007). Characterization of a progenitor cell population in childhood T-cell acute lymphoblastic leukemia. *Blood, 109*(2), 674-682.

Dalerba, P., Dylla, S. J., Park, I. K., Liu, R., Wang, X., Cho, R. W. et al. (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 104*(24), 10158-10163.

Dean, M. (2009). ABC transporters, drug resistance, and cancer stem cells. *Journal of Mammary Gland Biology and Neoplasia, 14*(1), 3-9.

Dhanasekaran, R., Bandoh, S., & Roberts, L. R. (2016). Molecular pathogenesis of hepatocellular carcinoma and impact of therapeutic advances. *F1000Res* (5)*, 1-15.*

Ding, J., Guo, C., Hu, P., Chen, J., Liu, Q., Wu, X., et al. (2016). CSF1 is involved in breast cancer progression through inducing monocyte differentiation and homing. *International Journal of Oncology*, *49*(5), 2064-2074.

Disis, M. L. (2014). Mechanism of action of immunotherapy. *Seminars in Oncology, 41*(S5), S3-S13.

Duong, M. N., Cleret, A., Matera, E. L., Chettab, K., Mathé, D., Valsesia-Wittmann, S., et al. (2015). Adipose cells promote resistance of breast cancer cells to trastuzumab-mediated antibody-dependent cellular cytotoxicity. *Breast Cancer Research, 17*(1), 1-14.

Duong, M. N., Geneste, A., Fallone, F., Li, X., Dumontet, C., & Muller, C. (2017). The fat and the bad: Mature adipocytes, key actors in tumor progression and resistance. *Oncotarget, 8*(34), 57622-57641.

El-Serag, H. B. (2012). Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology, 142*(6), 1264-1273.

Eramo, A., Lotti, F., Sette, G., Pilozzi, E., Biffoni, M., Di Virgilio, A., et al. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death and Differentiation, 15*(3), 504-514.

Fabbrini, E., Sullivan, S., & Klein, S. (2010). Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. *Hepatology, 51*(2), 679-689.

Fan, J. G., Kim, S. U., & Wong, V. W. (2017). New trends on obesity and NAFLD in Asia. *Journal of Hepatology, 67*(4), 862-873.

Fang, D., Nguyen, T. K., Leishear, K., Finko, R., Kulp, A. N., Hotz, S., et al. (2005). A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Research, 65*(20), 9328-9337.

Fang, J. H., Zhou, H. C., Zeng, C., Yang, J., Liu, Y., Huang, X., et al. (2011). MicroRNA-29b suppresses tumor angiogenesis, invasion, and metastasis by regulating matrix metalloproteinase 2 expression. *Hepatology, 54*(5), 1729-1740.

Fasshauer, M., & Bluher, M. (2015). Adipokines in health and disease. *Trends in Pharmacological Sciences, 36*(7), 461-470.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer, 127*(12), 2893-2917.

Finn, R. S., Chan, S. L., Zhu, A. X., Knox, J. J., Cheng, A.-L., Siegel, A. B., et al. (2017). Randomized phase III study of pembrolizumab versus best supportive care for second-line advanced hepatocellular carcinoma. *Journal of Clinical Oncology, 35*(S4), S503-S503.

Forner, A., Llovet, J. M., & Bruix, J. (2012). Hepatocellular carcinoma. *The Lancet,379*(9822), 1245-1255.

Fujita, T., Chiwaki, F., Takahashi, R. U., Aoyagi, K., Yanagihara, K., Nishimura, T., et al. (2015). Identification and characterization of CXCR4-positive gastric cancer stem cells. *PLoS One, 10*(6), 1-19.

Funaki, M., Kitabayashi, J., Shimakami, T., Nagata, N., Sakai, Y., Takegoshi, K., et al. (2017). Peretinoin, an acyclic retinoid, inhibits hepatocarcinogenesis by suppressing sphingosine kinase 1 expression in vitro and in vivo. *Scientific Reports, 7*(1), 1-13.

Fung, J., Lee, C. K., Chan, M., Seto, W. K., Lai, C. L., Yuen, M. F., et al. (2015). High prevalence of non-alcoholic fatty liver disease in the Chinese - results from the Hong Kong liver health census. *Liver International, 35*(2), 542-549.

Furth, J., Kahn, M. C., & Breedis, C. (1937). The transmission of leukemia of mice with a single cell. *The American Journal of Cancer, 31*(2), 276-282.

Furuhashi, M., Saitoh, S., Shimamoto, K., & Miura, T. (2014). Fatty acid-binding protein 4 (FABP4): pathophysiological insights and potent clinical biomarker of metabolic and cardiovascular diseases. *Clinical Medicine Insights: Cardiology, 8*(3), 23-33.

Furuhashi, M., Tuncman, G., Gorgun, C. Z., Makowski, L., Atsumi, G., Vaillancourt, E., et al. (2007). Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature, 447*(7147), 959-965.

Gao, M. Q., Choi, Y. P., Kang, S., Youn, J. H., & Cho, N. H. (2010). CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. *Oncogene, 29*(18), 2672-2680.

Gao, Q., Wang, X. Y., Qiu, S. J., Zhou, J., Shi, Y. H., Zhang, B. H., et al. (2011). Tumor stroma reaction-related gene signature predicts clinical outcome in human hepatocellular carcinoma. *Cancer Science, 102(8)*, 1522-1531.

Ghouri, Y. A., Mian, I., & Rowe, J. H. (2017). Review of hepatocellular carcinoma: Epidemiology, etiology, and carcinogenesis. *Journal of Carcinogenesis, 16(1)*, 1-10.

Gilbert, C. A., & Ross, A. H. (2009). Cancer stem cells: cell culture, markers, and targets for new therapies. *Journal of Cellular Biochemistry, 108*(5), 1031-1038.

Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., et al. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell, 1*(5), 555-567.

Global Burden of Disease Liver Cancer (2017). The burden of primary liver cancer and underlying etiologies from 1990 to 2015 at the global, regional, and national level: Results from the global burden of disease study 2015. *Journal of the American Medical Association Oncology, 3*(12), 1683-1691.

Gondi, S. C., and Rao, J.S. Cathepsin B as a cancer target (2013). *Expert opinion on therapeutic targets, 17*(3), 281-291.

Gravitz, L. (2014). Liver cancer. *Nature, 516,* (S1).

Guaita-Esteruelas, S., Bosquet, A., Saavedra, P., Guma, J., Girona, J., Lam, E. W., et al. (2017). Exogenous FABP4 increases breast cancer cell proliferation and activates the expression of fatty acid transport proteins. *Molecular Carcinogenesis, 56*(1), 208-217.

Gurung, S., Chung, K. P. S., & Lee, T. K. (2018). Emerging role of fatty acid binding proteins in cancer pathogenesis. *Histology and Histopathology*, 18014.

Halberg, N., Wernstedt-Asterholm, I., & Scherer, P. E. (2008). The adipocyte as an endocrine cell. *Endocrinology and Metabolism Clinics of North America, 37*(3), 753-768.

Hashimoto, R., Katoh, Y., Miyamoto, Y., Nakamura, K., Itoh, S., Daida, H., et al. (2012). Enhanced accumulation of adipocytes in bone marrow stromal cells in the presence of increased extracellular and intracellular (Ca^{2+}). *Biochemical and Biophysical Research Communication, 423*(4), 672-678.

Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., et al. (2003). Cancerous stem cells can arise from pediatric brain tumors. *Proceedings of the National Academy of Sciences of the United States of America, 100*(25), 15178-15183.

Hemming, A. W., Cattral, M. S., Reed, A. I., Van Der Werf, W. J., Greig, P. D., & Howard, R. J. (2001). Liver transplantation for hepatocellular carcinoma. *Annals of Surgery, 233*(5), 652-659.

Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M. et al. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell, 1*(3), 313-323.

Himbert, C., Delphan, M., Scherer, D., Bowers, L. W., Hursting, S., & Ulrich, C. M. (2017). Signals from the adipose microenvironment and the obesity–cancer link—a systematic review. *Cancer Prevention Research, 10*(9), 494-506.

Ho, M. M., Ng, A. V., Lam, S., & Hung, J. Y. (2007). Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Research,67*(10), 4827-4833.

Holczbauer, A., Factor, V. M., Andersen, J. B., Marquardt, J. U., Kleiner, D. E., Raggi, C., et al. (2013). Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. *Gastroenterology, 145*(1), 221-231.

Holmes, D. (2016). Adipose tissue: Adipocyte exosomes drive melanoma progression. *Nature Reviews- Endocrinology, 12*(8), 436.
Hospital Authority (2017). "Hong Kong Cancer Registry." Retrieved on 24th July 2018: http://www3.ha.org.hk/cancereg/topten.html

Hosen, N., Park, C. Y., Tatsumi, N., Oji, Y., Sugiyama, H., Gramatzki, M., et al. (2007). CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America, 104*(26), 11008-11013.

Hoy, A. J., Balaban, S., & Saunders, D. N. (2017). Adipocyte–tumor cell metabolic crosstalk in breast cancer. *Trends in Molecular Medicine, 23*(5), 381-392.

Hsu, B. G., Chen, Y. C., Lee, R. P., Lee, C. C., Lee, C. J., & Wang, J. H. (2010). Fasting serum level of fatty-acid-binding protein 4 positively correlates with metabolic syndrome in patients with coronary artery disease. *Circulation Journal, 74*(2), 327-331.

Huang, T. S., Shyu, Y. C., Chen, H. Y., Yuan, S. S., Shih, J. N., & Chen, P. J. (2013). A systematic review and meta-analysis of adjuvant interferon therapy after curative treatment for patients with viral hepatitis-related hepatocellular carcinoma. *Journal of Viral Hepatitis, 20*(10), 729-743.

Ikeda, M., Morizane, C., Ueno, M., Okusaka, T., Ishii, H., & Furuse, J. (2018). Chemotherapy for hepatocellular carcinoma: current status and future perspectives. *Japanese Journal of Clinical Oncology, 48*(2), 103-114.

Issa, D., & Alkhouri, N. (2017). Nonalcoholic fatty liver disease and hepatocellular carcinoma: new insights on presentation and natural history. *Hepatobiliary Surgery and Nutrition, 6*(6), 401-403.

Ivanov, A. V., Bartosch, B., Smirnova, O. A., Isaguliants, M. G., & Kochetkov, S. N. (2013). HCV and oxidative stress in the liver. *Viruses, 5*(2), 439-469.

Jaiswal, S., Jamieson, C. H., Pang, W. W., Park, C. Y., Chao, M. P., Majeti, R., et al. (2009). CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell, 138*(2), 271-285.

Jin, L., Hope, K. J., Zhai, Q., Smadja-Joffe, F., & Dick, J. E. (2006). Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature Medicine, 12*(10), 1167-1174.

Jordan, C. T., Upchurch, D., Szilvassy, S. J., Guzman, M. L., Howard, D. S., Pettigrew, A. L., et al. (2000). The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia, 14*(10), 1777-17784.

Kallini, J. R., Gabr, A., Salem, R., & Lewandowski, R. J. (2016). Transarterial radioembolization with Yttrium-90 for the treatment of hepatocellular carcinoma. *Advances in Therapy, 33*(5), 699-714.

Karakas, S. E., Almario, R. U., & Kim, K. (2009). Serum fatty acid binding protein 4, free fatty acids, and metabolic risk markers. *Metabolism, 58*(7), 1002-1007.

Khan, H., Pillarisetty, V. G., & Katz, S. C. (2014). The prognostic value of liver tumor T cell infiltrates. *Journal of Surgical Research, 191*(1), 189-195.

Kim, E. R., & Chang, D. K. (2014). Colorectal cancer in inflammatory bowel disease: the risk, pathogenesis, prevention and diagnosis. *World Journal of Gastroenterology, 20*(29), 9872-9881.

Kise, K., Kinugasa-Katayama, Y., & Takakura, N. (2016). Tumor microenvironment for cancer stem cells. *Advances in Drug Delivery Review, 99*(PtB), 197-205.

Koh, J. H., Shin, Y. G., Nam, S. M., Lee, M. Y., Chung, C. H., & Shin, J. Y. (2009). Serum adipocyte fatty acid-binding protein levels are associated with nonalcoholic fatty liver disease in type 2 diabetic patients. *Diabetes Care, 32*(1), 147-152.

Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis, 4*(2), 68-75.

Korkaya, H., Paulson, A., Iovino, F., & Wicha, M. S. (2008). HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene, 27*(47), 6120-6130.

Kraus, N. A., Ehebauer, F., Zapp, B., Rudolphi, B., Kraus, B. J., & Kraus, D. (2016). Quantitative assessment of adipocyte differentiation in cell culture. *Adipocyte, 5*(4), 351-358.

Kreso, A., & Dick, J. E. (2014). Evolution of the cancer stem cell model. *Cell Stem Cell, 14*(3), 275-291.

Krishnamurthy, S., Dong, Z., Vodopyanov, D., Imai, A., Helman, J. I., Prince, M. E., et al. (2010). Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. *Cancer Research, 70*(23), 9969-9978.

Krummel, M. F., & Allison, J. P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *The Journal of Experimental Medicine, 182*(2), 459-465.

Kubota, K., Makuuchi, M., Kusaka, K., Kobayashi, T., Miki, K., Hasegawa, K., et al. (1997). Measurement of liver volume and hepatic functional reserve as a guide to decision-making in resectional surgery for hepatic tumors. *Hepatology, 26*(5), 1176-1181.

Kudo, M. (2017). Systemic therapy for hepatocellular carcinoma: 2017 Update. *Oncology, 93(S1)*, 135-146.

Kudo, M (2015). Molecular targeted therapy for hepatocellular carcinoma: Where are we now? *Liver cancer 4*(3), I-VII.

Lai, C. L., Ratziu, V., Yuen, M. F., & Poynard, T. (2003). Viral hepatitis B. *The Lancet, 362*(9401), 2089-2094.

Lanza, E., Donadon, M., Poretti, D., Pedicini, V., Tramarin, M., Roncalli, M., et al. (2016). Transarterial therapies for hepatocellular carcinoma. *Liver Cancer, 6*(1), 27-33.

Lapraire, R. B., Denovan-Wright, E. M. and Wright, J. M. (2017). Differential regulation of the duplicated fabp7, fabp10 and fabp11 genes of zebrafish by peroxisome proliferator activated receptors. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 213*, 81-90.

Lau, E. Y. T., Lo, J., Cheng, B. Y. L., Ma, M. K. F., Lee, J. M. F., Ng, J. K. Y., et al. (2016). Cancer-associated fibroblasts regulate tumor-initiating cell plasticity in hepatocellular carcinoma through c-Met/FRA1/HEY1 signaling. *Cell Reports, 15*(6), 1175-1189.

Laurent, V., Guérard, A., Mazerolles, C., Le Gonidec, S., Toulet, A., Nieto, L., et al. (2016). Periprostatic adipocytes act as a driving force for prostate cancer progression in obesity. *Nature Communications, 7*, 1-15.

Leach, D. R., Krummel, M. F., & Allison, J. P. (1996). Enhancement of antitumor immunity by CTLA-4 blockade. *Science, 271*(5256), 1734-1736.

Lee, D., Wada, K., Taniguchi, Y., Al-Shareef, H., Masuda, T., Usami, Y., et al. (2014). Expression of fatty acid binding protein 4 is involved in the cell growth of oral squamous cell carcinoma. *Oncology Reports*, *31*(3), 1116-1120.

Lee, J. I. (2016). Role of Periostin in Hepatocellular Carcinoma: The importance of tumor microenvironment. *Gut Liver, 10*(6), 871-872.

Lee, T. K., Cheung, V. C., Lu, P., Lau, E. Y., Ma, S., Tang, K. H., et al. (2014). Blockade of CD47-mediated cathepsin S/protease-activated receptor 2 signaling provides a therapeutic target for hepatocellular carcinoma. *Hepatology, 60*(1), 179-191.

Lee, T. K., Castilho, A., Cheung, V. C., Tang, K. H., Ma, S., and Ng, I. O. (2011) CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. *Cell stem cell, 9*(1), 50-63.

Li, J. C., Wu, D. A., Hou, J. S., Subeq, Y. M., Chen, H. D., & Hsu, B. G. (2016). High Serum adipocyte fatty acid binding protein is associated with metabolic syndrome in patients with type 2 diabetes. *Journal of Diabetes Research, 2016*, 1-7.

Li, C., Lee, C. J., & Simeone, D. M. (2009). Identification of human pancreatic cancer stem cells. *Methods in molecular biology, 568*, 161-173.

Llovet, J. M., & Bruix, J. (2003). Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. *Hepatology, 37*(2), 429-442.

Llovet, J. M., & Bruix, J. (2008). Molecular targeted therapies in hepatocellular carcinoma. *Hepatology, 48*(4), 1312-1327.

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., et al. (2008). Sorafenib in advanced hepatocellular carcinoma. *New England Journal of Medicine, 359*(4), 378-390.

Ma, S., Chan, K. W., Hu, L., Lee, T. K., Wo, J. Y., Ng, I. O., et al. (2007). Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology, 132*(7), 2542-2556.

Ma, S., Chan, K. W., Lee, T. K., Tang, K. H., Wo, J. Y., Zheng, B. J., et al. (2008). Aldehyde dehydrogenase discriminates the CD133 liver cancer stem cell populations. *Molecular Cancer Research, 6*(7), 1146-1153.

Majeti, R., Chao, M. P., Alizadeh, A. A., Pang, W. W., Jaiswal, S., Gibbs, K. D., et al. (2009). CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. *Cell, 138*(2), 286-299.
Malvi, P., Chaube, B., Pandey, V., Vijayakumar, M. V., Boreddy, P. R., Mohammad, N., et al. (2015). Obesity induced rapid melanoma progression is reversed by orlistat treatment and dietary intervention: Role of adipokines. *Molecular Oncology, 9*(3), 689-703.

Mantwill, K., Naumann, U., Seznec, J., Girbinger, V., Lage, H., Surowiak, P., et al. (2013). YB-1 dependent oncolytic adenovirus efficiently inhibits tumor growth of glioma cancer stem like cells. *Journal of Translational Medicine, 11*, 216-216.

Masuzaki, R., Karp, S. J., & Omata, M. (2016). NAFLD as a risk factor for HCC: new rules of engagement? *Hepatology International, 10*(4), 533-534.

Matsui, W. H. (2016). Cancer stem cell signaling pathways. *Medicine, 95*(S1), S8-S19.

Mazzaferro, V., Regalia, E., Doci, R., Andreola, S., Pulvirenti, A., Bozzetti, F., et al. (1996). Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *New England Journal of Medicine, 334*(11), 693-699.

McGlynn, K. A., & London, W. T. (2011). The global epidemiology of hepatocellular carcinoma: present and future. *Clinics in Liver Disease, 15*(2), 223-243.

Memon, K., Lewandowski, R. J., Riaz, A., & Salem, R. (2013). Yttrium 90 microspheres for the treatment of hepatocellular carcinoma. *Recent Results in Cancer Research, 190*, 207-224.

Meyer, K. A., Neeley, C. K., Baker, N. A., Washabaugh, A. R., Flesher, C. G., Nelson, B. S., et al. (2016). Adipocytes promote pancreatic cancer cell proliferation via glutamine transfer. *Biochemistry and Biophysics Reports, 7*, 144-149.

Mohanta, S., Siddappa, G., Valiyaveedan, S. G., Dodda Thimmasandra Ramanjanappa, R., Das, D., Pandian, R., et al. (2017). Cancer stem cell markers in patterning differentiation and in prognosis of oral squamous cell carcinoma. *Tumour Biology, 39*(6), 1-14.

Nakamura, R., Okura, T., Fujioka, Y., Sumi, K., Matsuzawa, K., Izawa, S., et al. (2017). Serum fatty acid-binding protein 4 (FABP4) concentration is associated with insulin resistance in peripheral tissues, A clinical study. *PLoS One, 12*(6), 1-14.

Nath, A., Li, I., Roberts, L. R., & Chan, C. (2015). Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma. *Scientific Reports, 5*, 1-19.

Nathan, H., Schulick, R. D., Choti, M. A., & Pawlik, T. M. (2009). Predictors of survival after resection of early hepatocellular carcinoma. *Annals of Surgery, 249*(5), 799-805.

Nguyen, P. H., Giraud, J., Chambonnier, L., Dubus, P., Wittkop, L., Belleannee, G., et al. (2017). Characterization of biomarkers of tumorigenic and chemoresistant cancer stem cells in human gastric carcinoma. *Clinical Cancer Research, 23*(6), 1586-1597.

Nieman, K. M., Kenny, H. A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M. R., et al. (2011). Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature Medicine, 17*(11), 1498-1503.

Nio, K., Yamashita, T., & Kaneko, S. (2017). The evolving concept of liver cancer stem cells. *Molecular Cancer, 16*(1), 1-12.

Ntambi, J. M., & Young-Cheul, K. (2000). Adipocyte differentiation and gene expression. *The Journal of Nutrition, 130*(12), 3122S-3126S.

O'Brien, C. A., Pollett, A., Gallinger, S., & Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature, 445*(7123), 106-110.

Ockner, R. K., Manning, J. A., Poppenhausen, R. B., & Ho, W. K. (1972). A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science, 177*(4043), 56-58.

Okazaki, T., & Honjo, T. (2007). PD-1 and PD-1 ligands: from discovery to clinical application. *International Immunology, 19*(7), 813-824.

Oliveira, C. P., Stefano, J. T., & Carrilho, F. J. (2017). Clinical patterns of hepatocellular carcinoma (HCC) in non-alcoholic fatty liver disease (NAFLD): a multicenter prospective study. *Hepatobiliary Surgery and Nutrition, 6*(5), 350-352.

Pang, R., Law, W. L., Chu, A. C., Poon, J. T., Lam, C. S., Chow, A. K., et al. (2010). A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. *Cancer stem cell,* 6(6), 603-615.

Pappenheim, A. (1917). Prinzipen der neuren morphologichschen haematozytologie mach zytogenetischer grundlage. *Folia Haematol ,* 91-101.

Paul, M. K., & Mukhopadhyay, A. K. (2004). Tyrosine kinase – Role and significance in Cancer. *International Journal of Medical Sciences, 1*(2), 101-115.

Pece, S., Tosoni, D., Confalonieri, S., Mazzarol, G., Vecchi, M., Ronzoni, S., et al. (2010). Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell, 140*(1), 62-73.

Pienta, K. J., McGregor, N., Axelrod, R., & Axelrod, D. E. (2008). Ecological therapy for cancer: defining tumors using an ecosystem paradigm suggests new opportunities for novel cancer treatments. *Translational Oncology, 1*(4), 158-164.

Poon, R. T. P., Fan, S. T., Tsang, F. H. F., & Wong, J. (2002). Locoregional therapies for hepatocellular carcinoma: a critical review from the surgeon's perspective. *Annals of Surgery, 235*(4), 466-486.

Prince, M. E., Sivanandan, R., Kaczorowski, A., Wolf, G. T., Kaplan, M. J., Dalerba, P., et al. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America, 104*(3), 973-978.

Roca-Rivada, A., Bravo, S. B., Perez-Sotelo, D., Alonso, J., Castro, A. I., Baamonde, I., et al. (2015). CILAIR-Based secretome analysis of obese visceral and subcutaneous adipose tissues reveals distinctive ECM remodeling and inflammation mediators. *Scientific Reports, 5*, 1-13.

Rytka, J. M., Wueest, S., Schoenle, E. J., & Konrad, D. (2011). The portal theory supported by venous drainage-selective fat transplantation. *Diabetes, 60*(1), 56-63.

Sangro, B., Yau, T., Hsu, C., Kudo, M., Crocenzi, T. S., Choo, S. P., et al. (2017). Nivolumab in sorafenib-experienced patients with advanced hepatocellular carcinoma (HCC) with or without chronic viral hepatitis: CheckMate 040 study. *Journal of Hepatology, 66*(1), S34-S35.

Sell, S. (2012). Prevention of hepatocellular carcinoma: Vaccination and dietary changes show promise to eliminate most cases of human liver cancer. *Journal of carcinogenesis & mutagenesis, 3*(e108), 1-4.

Shafat, M. S., Oellerich, T., Mohr, S., Robinson, S. D., Edwards, D. R., Marlein, C. R., et al. (2017). Leukemic blasts program bone marrow adipocytes to generate a protumoral microenvironment. *Blood, 129*(10), 1320-1332.

Shaheen, S., Ahmed, M., Lorenzi, F., & Nateri, A. S. (2016). Spheroid-formation (colonosphere) assay for in vitro assessment and expansion of stem cells in colon cancer. *Stem Cell Review, 12*(4), 492-499.

Shen, J., Yeh, C. C., Wang, Q., Gurvich, I., Siegel, A. B., & Santella, R. M. (2016). Plasma adiponectin and hepatocellular carcinoma survival among patients without liver transplantation. *Anticancer research, 36*(10), 5307-5314.

Sheng, X., Tucci, J., Parmentier, J. H., Ji, L., Behan, J. W., Heisterkamp, N., & Mittelman, S. D. (2016). Adipocytes cause leukemia cell resistance to daunorubicin via oxidative stress response. *Oncotarget, 7*(45), 73147-73159.

Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., & Dirks, P. B. (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Research, 63*(18), 5821-5828.

Stefanou, N., Papanikolaou, V., Furukawa, Y., Nakamura, Y., & Tsezou, A. (2010). Leptin as a critical regulator of hepatocellular carcinoma development through modulation of human telomerase reverse transcriptase. *BMC Cancer, 10*, 442-442.

Surmacz, E. (2007). Obesity hormone leptin: a new target in breast cancer? *Breast Cancer Reearchs, 9*(1), 1-2.

Suzuki, T., Shinjo, S., Arai, T., Kanai, M., & Goda, N. (2014). Hypoxia and fatty liver. *World Journal of Gastroenterology, 20*(41), 15087-15097.

Takaishi, S., Okumura, T., Tu, S., Wang, S. S. W., Shibata, W., Vigneshwaran, R., et al. (2009). Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells, 27*(5), 1006-1020.

Takaki, A., & Yamamoto, K. (2015). Control of oxidative stress in hepatocellular carcinoma: Helpful or harmful? *World Journal of Hepatology, 7*(7), 968-979.

Takayasu, K., Arii, S., Ikai, I., Omata, M., Okita, K., Ichida, T., et al. (2006). Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. *Gastroenterology, 131*(2), 461-469.

Tang, K. D., Liu, J., Jovanovic, L., An, J., Hill, M. M., Vela, I., et al. (2016). Adipocytes promote prostate cancer stem cell self-renewal through amplification of the cholecystokinin autocrine loop. *Oncotarget, 7*(4), 4939-4948.

Tang, Z., Shen, Q., Xie, H., Zhou, X., Li, J., Feng, J., et al. (2016). Elevated expression of FABP3 and FABP4 cooperatively correlates with poor prognosis in non-small cell lung cancer (NSCLC). *Oncotarget, 7*(29), 46253-46262.

Tang, K. H., Ma, S., Lee, T. K., Chan, Y. P., Kwan, P. S., Tong, C. M., et al. (2012). CD133(+) liver tumor-initiating cells promote tumor angiogenesis, growth, and self-renewal through neurotensin/interleukin-8/CXCL1 signaling. *Hepatology, 55*(3), 807-820.

Thompson, K.J., Austin, R.G., Nazari, S. S., Gersin, K.S., Iannitti, D. A. & McKillop, I. H. Altered fatty acid binding protein (FABP4) expression and function in human and animal models of hepatocellular carcinoma. *Liver International, 38(*6), 1074-1083.

Tsai, J. C., Goldman, C. K., & Gillespie, G. Y. Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB and bFGF. *Journal of neurosurgery, 82*(5), 864-873.

Tsuchiya, T., Shimizu, H., Horie, T., & Mori, M. (1999). Expression of leptin *receptor* in lung: leptin as a growth factor. *European Journal of Pharmacology, 365*(2), 273-279.

Uehara, H., Takahashi, T., Oha, M., Ogawa, H., & Izumi, K. (2014). Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression. *International Journal of Cancer, 135*(11), 2558-2568.

Ullah, M., Stich, S., Häupl, T., Eucker, J., Sittinger, M., & Ringe, J. (2013). Reverse differentiation as a gene filtering tool in genome expression profiling of adipogenesis for fat marker gene selection and their analysis. *PLoS One, 8*(7), 1-14.

Vilchez, V., Turcios, L., Marti, F., & Gedaly, R. (2016). Targeting Wnt/β-catenin pathway in hepatocellular carcinoma treatment. *World Journal of Gastroenterology, 22*(2), 823-832.

Voena, C., & Chiarle, R. (2016). Advances in cancer immunology and cancer immunotherapy. *Discovery Medicine, 21*(114), 125-133.

Wang, M., Zhao, J., Zhang, L., Wei, F., Lian, Y., Wu, Y., et al. (2017). Role of tumor microenvironment in tumorigenesis. *Journal of Cancer, 8*(5), 761-773.

Wang, Y. K., Zhu, Y. L., Qiu, F. M., Zhang, T., Chen, Z. G., Zheng, S., et al. (2010). Activation of Akt and MAPK pathways enhances the tumorigenicity of CD133+ primary colon cancer cells. *Carcinogenesis, 31*(8), 1376-1380.

Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., & Ferrante, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation, 112*(12), 1796-1808.

Wen, Y. A., Xing, X., Harris, J. W., Zaytseva, Y. Y., Mitov, M. I., Napier, D. L., et al. (2017). Adipocytes activate mitochondrial fatty acid oxidation and autophagy to promote tumor growth in colon cancer. *Cell Death and Disease, 8*(2), 1-12.

Wilhelm, S. M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., et al. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Research, 64*(19), 7099-7109.

Wolfson, B., Eades, G., & Zhou, Q. (2015). Adipocyte activation of cancer stem cell signaling in breast cancer. *World Journal of Biological Chemistry, 6*(2), 39-47.

Wright, M. H., Calcagno, A. M., Salcido, C. D., Carlson, M. D., Ambudkar, S. V., & Varticovski, L. (2008). Brca1 breast tumors contain distinct CD44 (+)/CD24 (-) and CD133 (+) cells with cancer stem cell characteristics. *Breast Cancer Research, 10*(1), R10-R10.

Wroblewski, L. E., Peek, R. M., & Wilson, K. T. (2010). Helicobacter pylori and gastric cancer: factors that modulate disease risk. *Clinical Microbiology Reviews, 23*(4), 713-739.

Wu, X. Z., & Chen, D. (2006). Helicobacter pylori and hepatocellular carcinoma: correlated or uncorrelated? *Journal of Gastroenterology and Hepatology, 21*(2), 345-347.

Wu, C.Y., Chen, Y. J., Ho, H. J., Hsu, Y. C., Kuo, K. N., Wu, M. S., et al. (2012). Association Between nucleoside analogues and risk of hepatitis B virus-related hepatocellular carcinoma recurrence following liver resection. *Journal of the American Medical Association, 308*(18), 1906-1914.

Xiang, F., Wu, K., Liu, Y., Shi, L., Wang, D., Li, G., et al. (2017). Omental adipocytes enhance the invasiveness of gastric cancer cells by oleic acid-induced activation of the PI3K-

Akt signaling pathway. *The International Journal of Biochemistry and Cell Biology, 84*, 14-21.

Xu, H., Hertzel, A. V., Steen, K. A., & Bernlohr, D. A. (2016). Loss of fatty acid binding protein 4/ap2 reduces macrophage inflammation through activation of sirt3. *Molecular Endocrinology, 30*(3), 325-334.

Yamashita, T., Honda, M., Nakamoto, Y., Baba, M., Nio, K., Hara, Y., et al. (2013). Discrete nature of EpCAM+ and CD90+ cancer stem cells in human hepatocellular carcinoma. *Hepatology, 57*(4), 1484-1497.

Yamashita, T., & Wang, X. W. (2013). Cancer stem cells in the development of liver cancer. *Journal of Clinical Investigation, 123*(5), 1911-1918.

Yamashita, T., Ji, J., Budhu, A., Forgues, M., Yang, W., Wang, H. Y., et al. (2009). EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology, 136*(3), 1012-1024.
Yan, F., Shen, N., Pang, J. X., Zhang, Y. W., Rao, E. Y., Bode, A. M., et al. (2017). Fatty acid-binding protein FABP4 mechanistically links obesity with aggressive AML by enhancing aberrant DNA methylation in AML cells. *Leukemia, 31*(6), 1434-1442.

Yang, Z. F., Ho, D. W., Ng, M. N., Lau, C. K., Yu, W. C., Ngai, P., et al. (2008). Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell, 13*(2), 153-166.

Yang, Z. F., Ngai, P., Ho, D. W., Yu, W. C., Ng, M. N., Lau, C. K., et al. (2008). Identification of local and circulating cancer stem cells in human liver cancer. *Hepatology, 47*(3), 919-928.

Ye, H., Adane, B., Khan, N., Sullivan, T., Minhajuddin, M., Gasparetto, M., et al. (2016). Leukemic stem cells evade chemotherapy by metabolic adaptation to an adipose tissue niche. *Cell Stem Cell, 19*(1), 23-37.

Yeung, T. M., Gandhi, S. C., Wilding, J. L., Muschel, R., & Bodmer, W. F. (2010). Cancer stem cells from colorectal cancer-derived cell lines. *Proceedings of the National Academy of Sciences,107*(8), 3722-3727.

Younossi, Z. M., Otgonsuren, M., Henry, L., Venkatesan, C., Mishra, A., Erario, M., & Hunt, S. (2015). Association of nonalcoholic fatty liver disease (NAFLD) with hepatocellular carcinoma (HCC) in the United States from 2004 to 2009. *Hepatology, 62*(6), 1723-1730.

Zhang, S., Balch, C., Chan, M. W., Lai, H. C., Matei, D., Schilder, J. M., et al. P. (2008). Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Research, 68*(11), 4311-4320.

Zhang, S. S., Han, Z. P., Jing, Y. Y., Tao, S. F., Li, T. J., Wang, H., et al. (2012). CD133 (+) CXCR4 (+) colon cancer cells exhibit metastatic potential and predict poor prognosis of patients. *BMC Medicine, 10,* 1-14.

Zhang, Y., Xi, S., Chen, J., Zhou, D., Gao, H., Zhou, Z., et al. (2017). Overexpression of LAMC1 predicts poor prognosis and enhances tumor cell invasion and migration in hepatocellular carcinoma. *Journal of Cancer, 8*(15), 2992-3000.

Zhao, S., Chen, C., Chang, K., Karnad, A., Jagirdar, J., Kumar, A. P., & Freeman, J. W. (2016). CD44 expression level and isoform contributes to pancreatic cancer cell plasticity, invasiveness and response to therapy. *Clinical Cancer Research, 22*(22), 5592-5604.

Zhong, C. Q., Zhang, X. P., Ma, N., Zhang, E. B., Li, J. J., Jiang, Y. B., et al. (2018). FABP4 suppresses proliferation and invasion of hepatocellular carcinoma cells and predicts a poor prognosis for hepatocellular carcinoma. *Cancer Medicine, 7*(6), 2629-2640.

Zhu, R. X., Seto, W. K., Lai, C. L., & Yuen, M. F. (2016). Epidemiology of hepatocellular carcinoma in the Asia-Pacific region. *Gut Liver, 10*(3), 332-339.

Zhu, Z., Hao, X., Yan, M., Yao, M., Ge, C., Gu, J., & Li, J. (2010). Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. *International Journal of Cancer, 126*(9), 2067-2078.