THE ROLES OF CHEMOKINES AND CHEMOKINE RECEPTORS IN TUMOR METASTASIS

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Metastasis is often a deadly disease but highly inefficient as it involves several complex sequential processes such as invasion, hematogenous dissemination and distant organ colonization. Tumor suppressor E-cadherin, which is the key biomarker for epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET), plays essential roles in cancer invasion and colonization. Organ colonization has been proposed to be the rate-limiting step of metastasis as most cancer cells undergo apoptosis in these restrictive foreign microenvironments. However, some metastatic cells manage to evade apoptosis by undergoing cellular quiescence and later grow out to form deadly overt metastases.

Herein, we investigated the roles of chemokines and chemokine receptors in mediating tumor phenotypic transition during cancer invasion and tumor escape from dormancy during liver colonization. Chemokine receptor CXCR3 mediates immune cell chemotaxis but inhibits adherent cell locomotion. These opposing effects on cellular motility are accomplished via two CXCR3 isoforms. We hypothesized that the anti-migratory isoform CXCR3B promoted E-cadherin expression to allow cancer cell seeding. CXCR3B overexpression induced E-cadherin expression and CXCR3B-knockdown reduced total and membranous E-cadherin. We also found that the correlation between CXCR3B and E-cadherin expression was significantly conserved in human prostate cancer liver metastasis in mice.

Next, we utilized *in vitro* and *ex vivo* co-culture systems to uncover the soluble factors that mediate tumor emergence from dormancy. *In vitro* co-culture with hepatic stellate cells (HSCs) significantly elevated tumor growth. Protein analyses of primary liver non-parenchymal cells and immortalized HSCs revealed increased secretion of pro-inflammatory chemokines IL-8 and MCP-1. We further noted that IL-8, not MCP-1, directly augmented cancer cell growth and mediated tumor escape from dormancy, potentially through ERK activation.

In summary, we establish novel functions for CXCR3 and IL-8 during tumor metastasis. CXCR3B can induce E-cadherin re-expression that may facilitate cancer cell seeding and protect the cells from drug-induced apoptosis. HSC-derived pro-inflammatory chemokine IL-8 can drive tumor escape from dormancy in the liver. These findings may have substantial implications on the management and treatment of metastatic disease. Targeting IL-8 or inflammation in general, may prove beneficial to curb cancer outgrowth at this rate-limiting step of metastasis.

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PREFACE

Berakit-rakit ke hulu, berenang-renang ke tepian,

Bersakit-sakit dahulu, bersenang-senang kemudian

This is a short Malay proverb which literally means “no pain, no gain”. I have always known that pursuing a doctoral degree is not an easy feat and it is indeed physically and mentally exhaustive. Fortunately, I have been blessed with supportive family and friends all around me and across the globe and I am thankful to everyone that has helped me to reach this point of my life.

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LIST OF ABBREVIATIONS

AR: androgen receptor

cAMP: cyclic adenosine monophosphate

CRPC: Castrate-resistant prostate cancer

CXCL: Cysteine-X-cysteine ligand

CXCR: Cysteine-X-cysteine receptor

CYP/CYP P450: Cytochrome P450

ECM: Extracellular matrix

EGFR: Epidermal growth factor receptor

EMT: Epithelial-to-mesenchymal transition

ER: Estrogen receptor

HER2: Human epidermal growth factor receptor 2

HSC: Hepatic stellate cell

IL8: Interleukin-8

KC: Kupffer cell

LPS: Lipopolysaccharides

LSEC: Liver sinusoidal endothelial cell

MCP-1: Monocyte chemoattractant protein-1

MET: Mesenchymal-to-epithelial transition

MMP: Matrix metalloproteinase

MPS: Micro-physiologic system

PDGFR: Platelet derived growth factor receptor

PF4: Platelet factor 4

PKA: Protein kinase A

PLC: Phospholipase C

PR: Progesterone receptor

PSA: Prostate specific antigen

TNBC: Triple negative breast cancer

1.0 OVERVIEW: CANCER AND METASTASIS

Cancer is a disease of dysregulated cell division or cell death. Carcinogenesis or tumor formation occurs in almost all human cell types primarily the epithelial cells (carcinoma), blood and lymphatic cells (leukemia and lymphoma) and connective tissue cells (sarcoma). Neoplastic transformation is potentiated by both intracellular (i.e. DNA damage and genetic mutation) and extracellular factors (i.e. tumor microenvironment, inflammation and exposure to carcinogen) (Hanahan and Weinberg 2000). More than 100 driver gene mutations, both acquired and inherited mutations, have been identified. These genes, such as TP53, BRCA1/2, RAS/RAF, HER2, MYC and JAK, play important roles in cell cycle regulation, cell survival and genome maintenance (Vogelstein et al. 2013). Gene mutations such as specific nucleotides mutation, chromosomal rearrangement, gene truncation, deletion or amplification, result in loss of gene functions to prevent carcinogenesis or gain of functions to enable neoplastic transformation (Vogelstein et al. 2013). These genetic aberrations always go hand in hand with the extrinsic factors to facilitate or expedite tumor formation. For example, smoking is the number one risk factor for lung cancer and exposure to tar and other carcinogens in cigarettes causes DNA damage to the lung cells (Hecht 2012). Although DNA damage alone is sufficient to induce carcinogenesis, lung cancer development in smokers is accelerated by inflammatory reactions, invoked by the body to clear foreign bodies and to repair cellular damages, that elevate pneumocytes proliferation (Takahashi et al. 2010). Similar observations are also documented in colorectal and breast cancers where pro-inflammatory factors within the specified tumor microenvironments drive tumorigenesis (Erreni, Mantovani and Allavena 2011, Williams, Yeh

and Soloff 2016). During carcinogenesis, cancer cells progressively acquire several malignancy characteristics such as maintenance of proliferative signals, resistance to cell death, evasion or suppression of immune responses, induction of tumor angiogenesis, deregulation of cellular energetics and ultimately activation of invasion and migratory properties for dissemination (Hanahan and Weinberg 2000).

Metastasis is the main cause of cancer-related deaths (Siegel, Miller and Jemal 2017). Typically, patients succumb to this disease due to increased tumor burden in the secondary sites and acute organ failure (Mogrovejo et al. 2014). The exact mechanisms driving the acquisition of metastatic characteristics is unclear. Recent data suggest that genomic instability drive phenotypic transition for metastasis and confer resistance to drugs (Lee et al. 2011, Gao et al. 2016).

Metastasis involves several sequential steps and each step requires the metastatic tumor cells to undergo additional changes such as epithelial and mesenchymal phenotypic plasticity (Kalluri and Weinberg 2009, Gao et al. 2016), increased expression of matrix-degrading matrix metalloproteinases (MMPs) enzymes (Deryugina and Quigley 2006) and increased expression of cytokines and growth factors and their respective receptors (Hanahan and Weinberg 2000).

First, conversion to mesenchymal phenotype liberates the cancer cells from cell-cell contacts that inhibit cell motility and proliferation. In addition, cadherin junctional complexes, comprised of E-cadherin and catenin proteins, are thought to be important in sequestering catenin proteins at the cell membrane. Hence, EMT results in increased level of cytoplasmic β-catenin and p-120 catenin which may translocate into the nucleus and contribute to mesenchymal phenotype (Wells, Yates and Shepard 2008). Recent *in vivo* data show that reduction of E-cadherin leads to the accumulation and nuclear translocation of cytoplasmic β-catenin to drive

colon cancer proliferation. This observation is supported by clinical observation where E-cadherin is reduced in a subpopulation of β-catenin driven pancreatic and colon cancers (Huels et al. 2015). Thus, EMT promotes cells motility and drives tumor cells transformation and proliferation via the associated catenin proteins. Second, metastatic cancer cells also up-regulate the expression of MMPs, especially at the leading edge, to degrade extracellular matrix (ECM) components to 1) allow the cells to migrate through the ECM and 2) release growth factors that are trapped within the ECM (Deryugina and Quigley 2006). Lastly, as the carcinoma cells are just part of a larger organ system that includes parenchymal and non-parenchymal cells that signal to the carcinoma cells, metastasis has been shown to be regulated by non-genetic means. Cancer cells face countless obstacles during dissemination. Hence, during this process, metastatic cells typically upregulate the production of growth signals or stimulate stromal cells to produce these factors to stimulate invasive behavior, promote intravasation or extravasation, induce angiogenesis and facilitate metastatic colonization (Hanahan and Weinberg 2011). For instance, tumor-associated macrophages (TAMs) have been shown to secrete various growth and angiogenic factors and also facilitate matrix degradation to promote invasion and metastasis (Balkwill and Mantovani 2001). Specifically, deletion of Csf-1 gene to prevent macrophages accumulation in polyoma-middle T-driven mammary tumor did not affect primary tumor development but almost completely abrogated metastasis which could be reversed by re-expression of Csf-1 (Lin et al. 2002). An *in vivo* study revealed that disseminating mesenchymal cancer cells secreted thrombospondin-2 to activate fibroblasts in the lung which then induced the cancer cells to undergo MET to promote efficient lung colonization (Del Pozo Martin et al. 2015). In addition to TAMs and fibroblasts, mesenchymal stem cells (MSCs) have also been shown to play roles in driving tumorigenesis and metastasis. *In vitro* co-culture of breast cancer

cells with breast-derived MSCs significantly promotes cancer cells proliferation and invasion (Zhang et al. 2013) and a recent *in vivo* study demonstrated that the MSCs within tumor microenvironment up-regulated the expression of discoidin domain receptor 2 (DDR2) and the secretion collagen type I to induce breast cancer migration and lung metastasis (Gonzalez et al. 2017).

Cellular crosstalk between cancer cells and stromal cells is a truly complex event involving various different cell types and a plethora of factors including growth factors, cytokines and ECM components. In this study, we focus on the roles of chemokine receptor CXCR3 and pro-inflammatory cytokines, IL-8 and MCP-1, in facilitating tumor metastasis.

1.1 PRIMARY AND METASTATIC CANCERS: INCIDENCE, PREVALENCE AND MORTALITY RATE

Currently, there are more than 14 million Americans affected by cancer. An additional 1.6 million new cases is estimated to be diagnosed and about 600,000 cancer-related deaths are estimated to be reported in 2017 (American Cancer Society 2017). Extensive efforts to treat and cure cancers have been made in the US and worldwide but unfortunately, cancer incidence and mortality rates in the US remain on a high plateau for the past 20 years (Kochanek et al. 2016). In fact, malignant neoplasms are the second highest cause of deaths in the US since 1960s with negligible amelioration as opposed to mortality due to heart diseases which has been steadily declining (Kochanek et al. 2016). Some of the highly prevalent cancer types include the cancer of reproductive organs, colon, rectum, lung, skin, bladder, kidney and blood cells (American Cancer Society 2017). Among these cancer types, breast and prostate cancers are the most

common cancers diagnosed in women and men, respectively, while lung cancer is the number one cause of cancer-related deaths in both sexes followed by colorectal, breast and prostate cancers (American Cancer Society 2017). These reports highlight metastatic cancer as one of the main health concerns and serve as the prime motivation for this study. This work utilizes breast and prostate cancer cells as models to study some aspects of cancer metastasis.

1.2 BREAST CANCER

Breast cancer is the most frequent cancer type diagnosed in women. Breast cancer is estimated to be diagnosed in about 250,000 patients and causes approximately 40,000 deaths in 2017 alone (American Cancer Society 2017). More than 60% of the diagnosed cases are primary breast cancer, followed by regional breast cancer and about 6% of the patients are estimated to be diagnosed with metastatic breast cancers (American Cancer Society 2017). The 5-year survival rate for patients with primary breast cancer is close to 99% but this rate precipitously regresses to only 25% for metastatic breast cancer patients (American Cancer Society 2017).

1.2.1 Breast Cancer Pathogenesis

Molecular pathogenesis of breast cancer is quite complex. Breast cancer typically develops from abnormal proliferation of the epithelium of the ducts and can be divided into estrogen receptor (ER)-dependent and ER-independent pathogenesis. ER-driven tumorigenesis is due to genetic alterations including 16q deletion and 1q gains which include tumor suppressor ADAMTS18 on 16q23.1. ER-independent tumorigenesis is caused by other genomic instability including

dysregulated/loss of TP53, BRCA1/2 or amplified HER2 gene expression (Nordgard et al. 2008, Allison 2012)

Improved understandings of breast cancer biology and technological advancements permit physicians and scientists to analyze the cancer cells at molecular levels. Several cancer specific markers have been identified and employed to group patients based on the expression of these markers including estrogen receptor (ER), progesterone receptor (PR) and HER2 (Kittaneh, Montero and Gluck 2013). Genome-wide cancer cell profiling further divides breast cancers into at least six distinct molecular subgroups: basal-like, HER2+, normal-breast-like, luminal-A subtype, luminal-B subtype and claudin low (Perou et al. 2000). About 15% of breast cancer patients have the basal-like or triple negative breast cancer (TNBC) subtype which mainly characterized by the lack of ER, PR and HER2 expression. 40% of breast cancer patients are luminal-A subtype and 20% of the patients are luminal-B subtype. Luminal A subtype typically expresses ER and PR but is negative for HER2. Similarly, luminal-B subtype also expresses ER or PR but may also express HER2 and typically shows poorer prognosis than luminal A subtype. Lastly, 20% breast cancer patients are in the HER2 subtype due HER2 gene amplification. Normal-like and claudin-low subtypes are less common (American Cancer Society 2013). Molecular clustering based on the expression of these markers are crucial to determine the prognosis of the disease, suitable treatment modalities and facilitate the evaluation of treatment efficacy. In addition, this clustering method allows for a more specific disease modeling. Currently, there are several most commonly used breast cancer cell lines such as MCF-7, MDA-MB-231, T47D, BT747 and SKBR3. MCF7 and T47D cell lines represent the luminal A subtype, BT747 cell line is luminal B subtype, and MDA-MB231 cell line is basal-like or TNBC subtype (Holliday and Speirs 2011). Molecular and phenotypic heterogeneity of breast cancers

are important parameters and current studies should encompass broad selections of cell lines so that experimental data can be correctly extrapolated into clinical setting.

In this study, we probed the growth of the less aggressive MCF7 breast tumor cell line and the more aggressive MDA-MB-231 tumor cells in response to growth factors secreted by hepatic stellate cells. The MDA-MB231 cells were also utilized in 3D *ex vivo* liver model to study tumor escape from dormancy in the liver.

1.2.2 Breast Cancer Recurrence and Metastasis

Metastatic disease is the main cause of cancer-related mortality (Chaffer and Weinberg 2011). Although most of the newly diagnosed breast cancers are primary tumor, up to 50% of the patients can suffer recurrence at various time points post primary treatment and the majority of the late recurrences are distant metastases (Lee 1985, Karrison, Ferguson and Meier 1999). Metastasis is more frequent in patients with large or advanced primaries (with nodal involvement) but still affects patients with small and node negative primary tumor following surgery, indicating that tumor metastasis is an early event during cancer development (Heimann and Hellman 2000).

Bone and lung are the main sites of first distant recurrence following surgical intervention whereas liver (~5%) and brain metastases are rarely diagnosed (Lee 1985). However, at autopsy, the leading sites of breast cancer metastasis are lung/pleura (65%) followed by bone (65%), liver (60%) and brain (22%) (Tabariès and Siegel 2011). Patients with advanced disease at diagnosis also show similar pattern of distribution. This suggests that liver and brain metastases are underdiagnosed or missed until they form macrometastases that disrupt the functions of the host organs. Analysis of metastatic distribution of breast cancer subtypes reveals

distinct patterns among ER/PR, HER2 and TNBC cancer subtypes. Aggressive tumors such as TNBC and HER2 breast tumors metastasize at higher frequency to the lung, liver and brain as opposed to luminal type breast cancers that primarily disseminate to the bone (Kennecke et al. 2010, Yuan et al. 2014). Additionally, young patients, typically diagnosed with aggressive breast cancers, are more frequently diagnosed with liver metastases (Cummings et al. 2014). Breast cancer patients with liver metastases have poorer prognosis when compared to lung and bone metastases with a 5-year survival percentage of merely 5.5-8.5% and a median of 14-16 months of survival times (Tabariès and Siegel 2011).

In this study, we focus on studying breast cancer dormancy and metastasis in the liver as this aspect, in addition to other reasons that will be discussed later, is clinically relevant but relatively understudied when compared to bone and lung metastases.

1.2.3 Breast Cancer Treatment

Several treatment options are available for breast cancer patients depending on the stages and the types of the cancers.

Breast cancer cells develop from the epithelium lining of the breast ducts (more than 80% of the cases) or the lobules (about 10%). Abnormal cells typically undergo hyperplasia or neoplasia to form solid mass *in situ*. Lobular neoplasia/lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS) are sometimes considered as stage 0 breast cancers. LCIS refers to the abnormal cells of the breast lobule but are not pre-cancer cells. Thus, LCIS may or may not be treated following initial biopsy but some guidelines recommend surgical excision to rule out any malignant cells. Since LCIS increases the risk of developing breast cancer, chemoprevention or bilateral prophylactic mastectomy are among the suggested alternative

options (Oppong and King 2011). DCIS, on the other hand, are pre-cancer cells. Thus, these cells are typically resected either through the removal of the entire breast (total/simple mastectomy) including axillary lymph nodes (modified radical mastectomy) or the removal of tumor nodules and surrounding tissues only to conserve the breast (lumpectomy) with or without adjuvant therapies (American Cancer Society 2013).

Primary breast cancers (stage I to III) are often treated with mastectomy or lumpectomy with or without radiation. Mastectomy may be the preferred option in cases with multicentric tumor nodules and in patients that are diagnosed with aggressive breast cancers (PDQ Adult Treatment Editorial Board 2002, Cao, Olson and Tyldesley 2013). Several clinical trials reported that mastectomy reduced local recurrence when compared to lumpectomy but did not significantly improve long-term survival (Cao, Olson and Tyldesley 2013). In addition to the local therapies (surgery and radiation therapy), adjuvant therapies such as hormonal therapy, chemotherapy and targeted therapy may also be performed depending on the tumor stage and molecular subtypes.

The distinction of breast cancers into several molecular subtypes has led to more efficacious therapies. The ER/PR positive breast cancer cells require estrogen/progesterone hormones to survive and proliferate. Currently, there are several adjuvant or neoadjuvant therapies to reduce or block estrogen activity in cancer cells. Aromatase inhibitors such as anastrozole block aromatase enzymatic activity that prevents the conversion of androgen into estrogen, thus decreasing estrogen level in the body. Another method of hormonal intervention is to specifically bind estrogen receptor with selective estrogen receptor degrader drugs (SERD) such as fulvestrant that will alter the hydrophobicity of ER, rendering it unstable and leads to ER degradation. The third hormonal therapy option is selective estrogen receptor modulator (SERM)

drugs such as tamoxifen that can act as estrogen antagonists to competitively bind to the ER but inhibit the transcription of estrogen target genes in the cancer cells (American Cancer Society 2013).

Adjuvant therapies with radiation, chemotherapy or hormone therapy (tamoxifen) can improve disease-free survival and/or overall survival of breast cancer patients treated with either mastectomy or lumpectomy (Allred et al. 2012, American Cancer Society 2013). In ER positive breast cancer patients, continuing adjuvant tamoxifen for five years significantly reduced the recurrence percentage from 40% to approximately 25% and reduced breast cancer mortality from 33% to 23% within the first decade post-surgery (Davies et al. 2011). The prolonged tamoxifen treatment, however, slightly increased thromboembolic and uterine cancer-related mortality. Extending adjuvant tamoxifen to ten years further reduced the risk of recurrence after 10 years (Davies et al. 2013). Additionally, tamoxifen is also an effective preventive tool for women with high risk for breast cancer as it can substantially reduce the cumulative rate of invasive breast cancer (Fisher et al. 2005).

HER2 amplified breast cancers can be specifically targeted with HER2 monoclonal antibody trastuzumab and small molecule tyrosine kinase inhibitor lapatinib. The exact mechanism(s) of action for trastuzumab is/are unclear but it has been proposed to limit cancer cell proliferation and survival by inducing HER2 internalization and degradation that disrupt downstream signaling pathways (Vu and Claret 2012). Trastuzumab may also promote cancer cell killing through antibody-dependent cellular cytotoxicity (Vu and Claret 2012). Four large multicenter clinical trials revealed an improvement in disease-free survival and substantial reduction in risk of death after two years of adjuvant trastuzumab therapy (Krishnamurti and Silverman 2014). Unfortunately, resistance against trastuzumab has been documented within just

one year of treatment through a variety of proposed mechanisms such as increased Mucin-4 glycoprotein expression that masks the binding site to prevent trastuzumab binding to HER2 and truncation of HER2 extracellular domain that prevents drug binding to the receptors (Vu and Claret 2012). Resistant cancer cells also overexpress other tyrosine kinase receptors and alter the intracellular signaling cascades to compensate or overcome HER2 inhibition by trastuzumab (Vu and Claret 2012).

Chemotherapeutic drugs are currently used as adjuvant, neoadjuvant and metastatic cancer therapies. There are several types of chemotherapy drugs with different efficacy, toxicity and modes of action. Some of the commonly used drugs are anthracyclines (i.e. doxorubicin), taxanes (i.e. paclitaxel and docetaxel), alkylating agents (i.e. carboplatin) and pyrimidine antagonists (i.e. 5-fluorouracil). These drugs are also used in combination such as the cyclophosphamide, methotrexate and 5-fluorouracil (CMF) regimen. These drugs target actively proliferating cancer cells by disrupting tumor cell cycling at specific phases or interfering DNA duplication. Cancer cells, however, acquire resistance against chemotherapeutic drugs through several mechanisms such as overexpression of transmembrane efflux pump, P-glycoprotein to pump out the drugs (Hassan et al. 2010).

One of the current challenges in breast cancer treatment and management is detecting and treating dormant tumor cells. These cells are insensitive to chemotherapy drugs due to their limited cell cycling and usually re-awaken years or decades post-surgery. The concept of tumor dormancy provides an alternative therapeutic strategy that is by keeping the tumor in dormant state which would prevent metastatic tumor burden and shift this incurable disease to a chronic disease. Dormancy also offers a long therapeutic

window before the tumors manifest. Thus, understanding the factors for emergence is of utmost importance and will be discussed in Chapter 3.

1.3 PROSTATE CANCER

Prostate cancer is the most commonly diagnosed cancer among men in the US and the third leading cause of cancer-related death in men. In 2017, approximately 160,000 new cases are estimated to be diagnosed and about 26,000 deaths are projected to be reported (American Cancer Society 2017). Similar to most other cancer types, the majority of the newly diagnosed cases are primary cancers with a 5-year survival of almost 100%. A small percentage of the diagnosed cases (4%) are metastatic prostate cancer with a 5-year survival of merely 30% (American Cancer Society 2017). Prostate cancer incidence demonstrated an exponential growth in the early 1990s following the introduction of prostate-specific antigen (PSA) screening. However, the specificity and sensitivity of PSA screening was not clearly established and led to overtreatment that caused harmful side effects and complications (PDQ Screening and Prevention Editorial Board 2016). The US Preventive Services Task Force (USPSTF) and European Society for Medical Oncology (ESMO) recommended against PSA screening as the sole diagnostic procedure (Moyer 2012, Parker et al. 2015). Nowadays, several diagnostic methods have been utilized such as PSA testing, serial digital rectal examination (DRE), biopsy and ultrasonography or magnetic resonance imaging to accurately distinguish prostate cancer from other conditions and are recommended on high-risk individuals instead of mass population screening (Parker et al. 2015). These approaches help in early primary cancer detection that can be effectively treated with prostatectomies or external-beam radiation therapy. However, cancer

relapse is still a major problem affecting 20-50% of the patients within 10 years post primary interventions (Paller and Antonarakis 2013). Recurrent and high grade prostate cancers are often treated with androgen deprivation therapy (ADT) but can develop resistance through various mechanisms within 12- 18 months (Bubley and Balk 1996).

1.3.1 Prostate Cancer Pathogenesis

The prostate gland is lined with columnar epithelium and is separated from the underlying stroma by a layer of basal cells. Testosterone and 5α-dihydrotestosterone (DHT) are required for normal prostate development as well as prostate carcinogenesis. Binding of these hormones to androgen receptor (AR) triggers AR conformational changes and induces AR dimerization and translocation into the nucleus to induce the expression of AR target genes such as KLK3 gene for PSA production and other genes for cell survival and proliferation (Feldman and Feldman 2001). One of the most common prostatic pathologies is benign prostate hyperplasia (BPH). BPH, highly prevalent in the elderly, is caused by an abnormal growth of the columnar epithelium due to high level of circulating DHT but is not considered as a pre-malignant tissue or a precursor of prostate cancer (Bostwick et al. 2004). Prostate epithelium undergoes several transformations prior to prostate malignancy starting with proliferative inflammatory atrophy (PIA) lesions. PIA lesions develop from dysregulated regenerative proliferation of the epithelium in response to injury and oxidative stress caused by chronic inflammation marked by high levels of glutathione S-transferase Pi 1 (GSTP1) and cyclooxygenase-2 (COX-2) (Nelson, De Marzo and Isaacs 2003). Loss of GSTP1 expression, due to hypermethylation, could indicate PIA transitions into precancerous prostatic intraepithelial neoplasia (PIN) lesions (Nelson, De Marzo and Isaacs 2003). NKX3.1, an essential prostate-specific homeobox gene for normal prostate development,

has been proposed to be the gatekeeper gene for prostate cancer development from PIN as progressive loss of NKX3.1 has been documented in 20% of PIN lesions, 6% of low-grade prostate cancer, 22% of high-grade prostate cancers, 34% of castrate-resistance prostate cancer (CRPC) and 78% of metastatic prostate cancers (Nelson, De Marzo and Isaacs 2003). Other genetic modifications that contribute to prostate carcinogenesis include loss of tumor suppressor PTEN and reduced level of cyclin-dependent kinase inhibitor p27 (Nelson, De Marzo and Isaacs 2003). High-grade and metastatic cancers eventually acquire another genetic modification to become CRPC through several mechanisms such as amplified AR expression or increased DHT secretion, AR activation by various non-androgen ligands, ligand-independent AR or AR-independent mechanisms through other oncogenes or loss of tumor suppressor genes (Feldman and Feldman 2001).

Prostate cancers are graded differently than breast and some other cancer types. Gleason score is used to grade prostate cancer based on the morphology of the tissues or cells and will be given a score from 1-5 with 1 being close to normal-like appearance and 5 being most aberrant/abnormal dysplastic appearance. The tissues will be graded twice with one score being the worst grade and the other being the most common grade and the individual scores will be added. Essentially, Gleason scores range from 2-10. Gleason score below 6 are usually relegated to ‘watchful waiting’ of close follow-up without immediate intervention, while scores of 9 or 10 result in the recommendation for surgery or radiation. The intermediary scores of 6-8 are therapeutic conundrums.

1.3.2 Prostate Cancer Recurrence and Metastasis

PSA level in prostate cancer patients is usually monitored for biochemical recurrence (BCR) following prostatectomies or radiation therapy. Shorter PSA doubling times (PSADT) have been reported to be significantly associated with metastasis and prostate-cancer specific mortality (Freedland et al. 2005). However, BCR is not an independent prognostic marker for prostate cancer recurrence as many patients experiencing BCR can remain metastasis-free. Thus, in addition to PSADT, several parameters namely Gleason score and initial timing of PSA elevation are included as important risk factors for metastasis (Pound et al. 1999).

Prostate cancers primarily recur in the bone followed by bladder, lung, liver and other organs (Bubendorf et al. 2000). Prostate cancer bone metastases are mainly osteoblastic through the secretion of bone morphogenetic proteins (BMPs) and various growth factors leading to painful, immature and excessive bone formation (Logothetis and Lin 2005). **In this study, we are interested in prostate cancer liver metastases. Prostate cancer liver metastasis is rarely diagnosed, accounting for less than 10% of the cases (Lamothe et al. 1986, Wang et al. 2013) due to technical limitations as ultrasonography, computed tomography and other imaging modalities are incapable of detecting micrometastases in the liver (Pouessel et al. 2007). However, at autopsy, up to 48% of prostate cancer patients had liver involvement (de la Monte, Moore and Hutchins 1986, Bubendorf et al. 2000), suggesting underdiagnosis due to minimal impact on health. Additionally, 66% of CRPC patients had liver involvement, second to bone metastases (Shah et al. 2004). It was also noted that most of prostate cancer liver metastases were hormone-refractory cancers that typically developed during the late stage of disease progression (Pouessel et al. 2007, Wang et al. 2013).**

Most cancer cell lines were derived from the metastatic sites. LNCaP is a commonly used androgen receptor positive cell line that expresses epithelial markers E-cadherin, cytokeratin-8 and cytokeratin-18 (Sobel and Sadar 2005). These cells, though derived from a lymph node, did not undergo phenotypic switching as observed in other metastatic cells and have a very limited metastatic potential *in vivo* (Sato et al. 1997, Thalmann et al. 2000). Other commonly used prostate cancer cell lines include PC3 and DU145 cells, derived from prostate cancer bone and brain metastases, respectively. **Both of these cell lines, used in our study in Chapter 2, do not express androgen receptor and lack membrane E-cadherin expression indicating high metastatic potential (Logothetis and Lin 2005).**

1.3.3 Prostate Cancer Treatment

Several treatment options are available for prostate cancer patients. American Society of Clinical Oncology (ASCO) recommends active surveillance (AS) for low-risk localized prostate cancer patients with the lowest Gleason score (≤ 6) that should include 2-3 PSA tests/year, annual DRE and serial biopsies and disease management should also include age, ethnicity and tumor volume into account (Chen et al. 2016). Similarly, ESMO clinical guidelines recommend watchful surveillance for low-risk patients but patients may undergo brachytherapy, radical prostatectomy (RP) or radiotherapy (RT). Comparison between watchful surveillance and RP on a Scandinavian cohort showed a slight difference in cancer-related deaths after 23 years of follow-up (247 vs 200 deaths for surveillance vs RP) with significant side effects such as erectile dysfunction and urinary leakage in the RP. Additionally, PIVOT trial involving 731 North American men showed no significant difference in overall mortality rate and prostate cancer specific mortality rate between watchful waiting and surgery (Parker et al. 2015).

For intermediate-risk patients (Gleason score of 4+3), ASCO recommends active therapy of either RP or RT but recommends AS for patients with Gleason score 3+4 (Chen et al. 2016). For high-grade (Gleason score ≥ 8) tumors, adjuvant RT following RP reduces local recurrence and clinical progression (Freedland et al. 2014). Other options for these groups include neoadjuvant ADT with RP and RP plus pelvic lymphadenectomy. Patients with hormone-naïve metastatic prostate cancers can temporarily benefit from ADT treatment (Parker et al. 2015) but there are no specific targeted therapies for CRPC other than chemotherapies (usually docetaxel plus prednisone) that provide modest survival benefits (Tannock et al. 2004). Although these cells are termed "castrate-resistant", the cells still rely on androgen for growth but are no longer responsive to primary hormone treatment. Second-line chemotherapy or hormone therapy post-docetaxel such as abiraterone, enzalutamide, cabazitaxel and radium-223 are more potent and have been clinically tested to improve overall survival (Parker et al. 2015).

An alternative option to circumvent chemoresistance is to prevent tumor metastasis altogether by restoring epithelial markers such as E-cadherin in the tumor cells. The role of E-cadherin in tumor metastasis and current efforts to target this adhesion molecule will be discussed in subsequent sections and chapters.

1.4 CANCER METASTASIS

1.4.1 Metastatic Sequence

Metastatic, the main cause of cancer-related mortality, is a complex disease as the cells, in addition to neoplastic transformations, need to acquire additional changes to enable

dissemination and survival in foreign microenvironments. Complete understanding of this disease, however, is still lacking leading to paucity in effective treatments.

Several key steps are required for successful dissemination: 1) primary tumors undergo genetic and phenotypic modifications to enable tumor cell invasion through the surrounding normal tissues and across the blood vessel barrier 2) the tumor cells intravasate into the blood circulation, resist anoikis while in the circulation and extravasate into secondary organs 3) the tumor cells undergo phenotypic reversion to successfully seed into secondary organs 4) the tumor cells survive and proliferate in the new microenvironments (Figure 1).

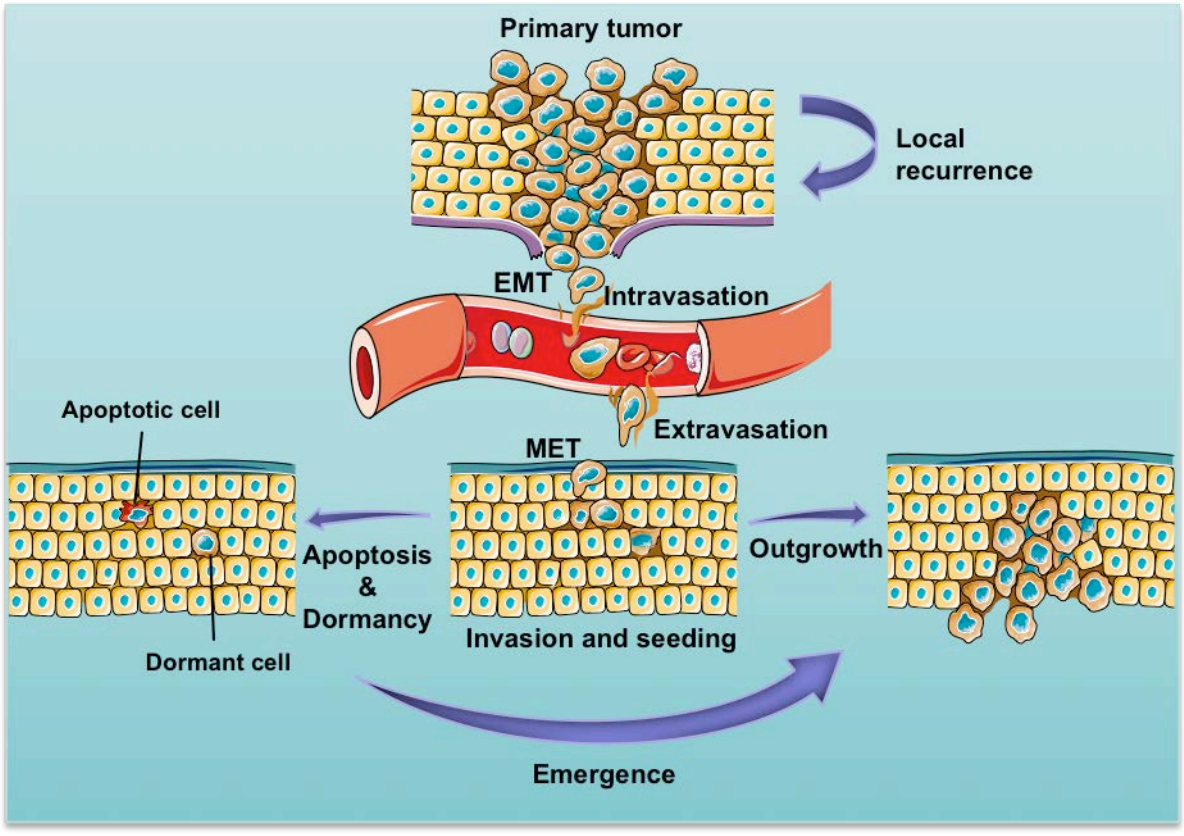


Figure 1: Schematic of tumor progression.

Cancer cells undergo several processes to successfully metastasize to distant organ. EMT is an important first step that enables the cancer cells to invade and intravasate into the blood vessels. Surviving cancer cells are typically

trapped within the capillary beds and extravasate from the lumen to seed into secondary organs. The parenchyma of the secondary organs such as the hepatocytes nurtures the cancer cells to revert to epithelial phenotype to form cellular connections. Most metastatic cancer cells undergo apoptosis due to host's immunoregulation and lack of survival factors. Some of the cells become quiescent under this restrictive microenvironment and may emerge from dormancy years or decades post primary treatment. Additionally, some of the highly aggressive and proliferative metastatic cancer cells can rapidly form micro- and macro-metastases but can be effectively targeted with current chemotherapeutic drugs.

1.4.2 Cancer Invasion: MMPs Secretion and EMT

Several underlying molecular mechanisms have been proposed for each step of metastasis. First of all, cancer cells secrete matrix metalloproteinases (MMPs) and undergo epithelial-to-mesenchymal transition (EMT) to potentiate the initial invasion step. MMPs are protein-degrading enzymes that allow tumor cells to migrate and release mitogenic and angiogenic factors sequestered within the extracellular matrices (ECM). Clinically, elevated expression of MMP-1, -2, -3, -7, -9, -13 and -14 are associated with poorly differentiated tumors, invasive stage, metastasis and shorter survival. However, these associations are still ambiguous as further analyses and studies showed contradicting and inconclusive findings especially for MMP-8 and MMP-3 (Deryugina and Quigley 2006).

Epithelial cells are arranged in sheets and connected to neighboring cells via tight junctions and gap junctions. This arrangement is crucial for cellular communication, limits cell proliferation and migration and establishes cell polarity. Cell polarity is important in regulating the accessibility of factors-containing fluids from the apical side to the basolateral side (Wells, Yates and Shepard 2008). Epithelial cells may undergo phenotypic transition under certain circumstances. Epithelial to mesenchymal transition (EMT) is characterized by the loss of

epithelial cell markers such as E-cadherin, gain of mesenchymal cell markers and also loss of cells polarity which results in increased cell motility. During implantation, EMT facilitates the invasion and anchoring of cytotrophoblast into the endometrium to form placenta (Kalluri and Weinberg 2009). During embryogenesis and development, EMT is required for various processes such as neuroectoderm migration to different parts of the body (Kalluri and Weinberg 2009). EMT also occurs in cancer cells but is distinct from physiologic EMT where epithelial cancer cells do not fully revert into mesenchymal cells; cancer cell transition does not encompass complete roles of mesenchymal cells and the cancer cells typically do not completely revert to mesenchymal phenotype but they rather express differential levels of both epithelial and mesenchymal markers (Davies, Jiang and Mason 2000, Wells, Yates and Shepard 2008, Sethi et al. 2010, Araki et al. 2011).

Molecular mechanisms for EMT have been described. Most of these mechanisms stem from crosstalk between tumor cells and tumor stroma. Transforming growth factor beta (TGFβ) is one of the most potent and important EMT inducer. TGFβ primarily induces EMT through SMAD signaling. Binding of TGFβ to its receptors, TβRI and II, activates SMAD2/3 to complex with SMAD4. The SMAD complexes translocate into the nucleus to combine with SNAIL, SLUG and zinc-finger-E-box-binding (ZEB) transcription factors (TFs) to repress or activate target genes including other TFs such as TWIST (Lamouille, Xu and Derynck 2014). TGFβ can also induce EMT via SMAD-independent pathway. Phosphorylation of TβRI on a tyrosine residue induces the phosphorylation of SRC homology 2 domain-containing-transforming A (SHCA) that creates a docking site for growth factor receptor-bound 2 and son of sevenless (GRB2/SOS) to initiate signaling cascades involving PI3K, MAPK and RHO GTPases (Lamouille, Xu and Derynck 2014). Activation of Rac, RHO and CDC42 promote actin

reorganization, lamellipodia and filapodia formation while activation of RHOA results in the dissolution of tight junctions. Mammalian TOR complex 1 (mTORC1), downstream of PI3K-AKT, is required for protein synthesis, motility and invasion in TGFβ-induced EMT. Additionally, TGFβ-induced MAPK activation stabilizes SNAIL and TWIST to drive EMT (Lamouille, Xu and Derynck 2014). EMT through non-SMAD pathways can also be activated by other growth factors such as epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) (Lamouille, Xu and Derynck 2014). Some chemokines such as IL-8 can also induce EMT through Brachyury transcription factor (Fernando et al. 2011).

EMT is marked by reduced expression of epithelial markers such as epithelial-cadherin (E-cadherin), zona-occludin-1 (ZO-1), SNAIL, SLUG, TWIST and/or gain of mesenchymal markers such as neural-cadherin (N-cadherin) and Vimentin. Single-pass transmembrane protein E-cadherin, one of the most reliable EMT marker, forms trans-cadherin interaction with neighboring cells. Intracellularly, this protein interacts directly with β- and p120-catenins with β-catenin contains binding domain for α-catenin that connects the junctional complexes to actin filaments. E-cadherin has been reported to be reduced or absent in high grade primary prostate tumors, metastatic prostate tumors, metastatic breast cancers and other tumors (Umbas et al. 1992, Oka et al. 1993, Kalluri and Weinberg 2009). In these cells, E-cadherin level is regulated via several different ways. In metastatic MDA-MB-231 breast cancer cells, E-cadherin is epigenetically regulated where the promoter of E-cadherin is hypermethylated, thus preventing E-cadherin gene transcription. *In vitro* co-culture and *in vivo* liver metastases experiments showed that secondary organ parenchyma such as the hepatocytes can alleviate E-cadherin promoter methylation to promote E-cadherin re-expression due to lack of methylation

maintenance in the dividing cells (Chao, Shepard and Wells 2010). Treatment with mitomycin-C to limit cell replication prevented loss of methylation and E-cadherin re-expression. In prostate cancer cells DU145 and PC3, E-cadherin protein level is down-regulated due to reciprocal activity of EGFR signaling. Activation of EGFR triggers intracellular phosphorylation cascades including β- and γ- catenin phosphorylation leading to the dissociation of the catenins from adheren junctions that eventually results in the destabilization and degradation of the adheren junctions (Jawhari, Farthing and Pignatelli 1999).

Given the central role of E-cadherin in EMT, strategies have been developed to restore this protein mainly by regulating epigenetic events by targeting DNA methyltransferases (DNMT) and histone deacetylase (HDAC) to prevent tumor metastasis. 5-aza-2'-deoxycytidine, a DNMT inhibitor, could restore E-cadherin in MDA-MB435S cells and suppress *in vivo* tumor growth (Wu, Sarkissyan and Vadgama 2016). Oxamflatin, a HDAC inhibitor, induced E-cadherin expression and decreased the viability of MKN45 gastric cancer cells (Faghihloo et al. 2016). Similarly, MS-275, a class I HDAC inhibitor, induced strongest E-cadherin re-expression in lung cancer cells when compared to other HDAC inhibitors and impaired cancer growth and proliferation in E-cadherin dependent manner (Kakihana et al. 2009). However, the use of drugs targeting epigenetic events in solid cancers is still limited. Luteinizing-hormone-releasing hormone (LHRH) and EGFR inhibitors have also been shown to induce E-cadherin expression in prostate cancer cells (Yates, Wells and Turner 2005).

1.4.3 Cancer Circulation: Intra- and Extravasation and Evading Anoikis

The second step of metastasis is cancer cell intravasation into the blood or less commonly into the lymphatic circulation. Cancer cells intravasate into the circulation via several

mechanisms such as the induction of angiogenic sprouting, recruitment of endothelial progenitor cells, vessel co-opting and vasculature mimicry (Hillen and Griffioen 2007). These newly formed vessels are typically leaky and allow the cancer cells to enter the vasculature. In addition to inducing new vasculature, cancer cells also secrete soluble factors such as VEGFA to increase vessel porosity that will permit cancer cell entry into the circulation. Notch signaling has been shown to aid cancer cells latching onto endothelial cells during initial steps of intravasation. MMP-1, through protease-activated receptor 1 (PAR1) and a disintegrin and metalloproteinase 12 (ADAM12), remodel and disrupt vascular endothelial cadherin (VE-cadherin) to allow paracellular intravasation (Reymond, d'Agua and Ridley 2013). TGFβ, in addition to its role in EMT, also facilitates cancer intravasation and extravasation (Tsai et al. 2012). Recent publication revealed that cancer cells intravasation was initiated early during primary tumorigenesis and intratumoral intravasation can be independent and parallel to stromal invasion (Deryugina and Kiosses 2017).

Within the circulation, cancer cells face survival challenges from anoikis, flow shear stress and innate immune responses. Cancer cells are devoid of integrin-ECM crosstalk in the blood stream, thus exposing them to anoikis. EMT provides initial protection against anoikis mainly through the activation of PI3K-AKT survival pathway (Guadamillas, Cerezo and Del Pozo 2011). Circulating cancer cells also overcome anoikis by increasing autocrine secretion of c-met, PDGFR, TrkB receptor and EGFR for pro-survival signaling loop (Guadamillas, Cerezo and Del Pozo 2011). Other mechanisms to evade anoikis include autophagy to obtain energy during starvation and entosis where cancer cells invade and reside within a homotypic cell to either being degraded or remain viable (Guadamillas, Cerezo and Del Pozo 2011). Circulating cancer cells have also been found to interact with platelets through tissue factors and selectins to

form microemboli to shield themselves from both blood flow shear stress and immune responses (Valastyan and Weinberg 2011).

During extravasation, cancer cells have been shown to adopt similar strategies to leukocyte transendothelial migration that involve initial adhesion to endothelial cells via selectins and integrins interactions (Reymond, d'Agua and Ridley 2013). Cancer cell adhesion to endothelial cells is also facilitated by glycosylated CD44 and N-cadherin. Additionally, αVβ3 integrin on cancer cells interact with platelet endothelial cell adhesion molecule 1 (PECAM1) during extravasation. Cancer cells also secrete growth factors such as VEGFA and TGFβ that increase actomyosin contractility and disrupt VE-cadherin to increase the permeability of endothelial barrier (Reymond, d'Agua and Ridley 2013).

1.4.4 Cancer Colonization: MET

Following extravasation, cancer cells integrate themselves into the secondary organs by forming cell-cell contact with the organs' parenchyma. Cancer cells undergo mesenchymal to epithelial transition (MET) to form cell-cell contact and initiate intracellular signaling for survival (Wells, Yates and Shepard 2008). *Ex vivo* analyses revealed that metastatic prostate cells form heterotypic E-cadherin interaction with hepatocytes (Yates et al. 2007). Additionally, *in vitro* co-culture of metastatic breast and prostate cancer cells with liver parenchyma stimulates E-cadherin re-expression in the cancer cells (Yates et al. 2007, Chao, Shepard and Wells 2010). In doxycycline-induced Twist1 mouse model, topical doxycycline administration on the skin permitted EMT reversion to MET at the metastatic sites whereas systemic doxycycline resulted in irreversible EMT. Reversion of EMT in this model resulted in higher metastatic frequency (Tsai et al. 2012). Histopathologic analyses on human tumor tissues corroborated experimental

observations for MET where lung, liver and brain metastatic breast cancers showed higher E-cadherin staining than their primary pairs (Chao et al. 2011). Moreover, E-cadherin staining was more intense in small prostate metastatic nodules indicating reversion to epithelial phenotype and initiation of EMT for secondary metastases in the larger nodules (Chao et al. 2011).

E-cadherin re-expression also provides the cancer cells with survival and chemo-protective advantages. Increased E-cadherin in prostate cancer liver nodules were more resistant to paclitaxel which can be reversed with AKT inhibitor, implying elevated AKT-mediated pro-survival pathway in these E-cadherin expressing prostate cancer cells (Ma et al. 2016). Similar observation was also documented in breast cancer cells challenged with camptothecin and staurosporine (Chao et al. 2012).

1.4.5 Cancer Survival and Proliferation in Distant Organs

Lastly, upon extravasation, the cancer cells will face a variety of obstacles in surviving and colonizing the secondary organs. Tissue microenvironment of the secondary organ differs greatly than that of the primary sites where certain growth factors and hormones are severely lacking. In addition, the organs' resident cells including resident macrophages secrete pro-apoptotic factors or chemoattractant to recruit leukocytes to target the cancer cells. As a consequence, majority of the cells undergo apoptosis or growth arrest (Luzzi et al. 1998, Kienast et al. 2010). In addition to the restrictive microenvironment, the sequential metastasis steps contain numerous possible check points, making this process inefficient. Therefore, even though millions of cells from the primary mass are shed into the bloodstream daily (Butler and Gullino 1975), only few cells develop into micrometastases or clinically overt metastases, indicating efficient rate-limiting step(s) for metastasis (Luzzi et al. 1998, Valastyan and Weinberg 2011).

Distant organs colonization has been noted to be the rate-limiting step of cancer metastasis (Wells, Yates and Shepard 2008, Valastyan and Weinberg 2011). Metastatic colonization will be discussed in further details in Chapter 3.

One of the issues that are still unclear regarding cancer metastasis is the timing for initial tumor dissemination. The progression model is the prevailing paradigm for metastasis where a subpopulation of tumor cells progressively undergoes genetic and epigenetic alterations to eventually acquire full metastatic potentials (Hunter, Crawford and Alsarraj 2008). Based on this model, it is inferred that metastasis occur during the later stages of disease progression. However, the presence of "metastatic poor-prognosis" gene signature in primary tumor mass to predict metastatic incidence with 90% accuracy indicates that tumor cells acquire metastatic capabilities early during disease progression (van 't Veer et al. 2002). Similarly, genomic aberration pattern in primary tumor mass from M0 patients resembles M1-like genotype. Importantly, chromosomal aberrations found in disseminated M1 cells are absent in disseminated M0 cells indicating tumor dissemination prior to genetic alteration, contradicting the current paradigm (Schmidt-Kittler et al. 2003). This temporal issue is crucial as one of the pursued therapies for metastatic disease is to re-instate the expression of tumor suppressor E-cadherin to prevent primary tumor cells from escaping the primary sites but may be impractical if tumor cells already metastasized early on. On the other hand, given the essential roles of E-cadherin in metastatic seeding and survival during MET, anti-E-cadherin therapy might be immensely useful in curbing tumor metastasis at its rate-limiting step. Thus, more efforts should focus on establishing the functional roles and molecular mechanisms of MET.

1.5 LIVER AS A METASTATIC SITE

The following sections contain materials from:

(Khazali, Clark and Wells 2017). A Pathway to Personalizing Therapy for Metastases Using Liver-on-a-Chip Platforms. 2017 Jun;13(3):364-380.

Liver is one of the metastatic sites for various solid cancers. This is due to both liver physiology and blood circulation that facilitate the transportation and retention of circulating cancer cells in liver sinusoids.

Liver as a metastatic site poses significant challenges in cancer treatment as it is the main site for drug metabolism. Most current and past studies on metastasis rely on animal models, specifically mouse models that can be genetically altered to mimic human diseases (Vandamme 2014). Although these models offer a lot of advantages and have led to remarkable scientific breakthroughs, these models pose some serious limitations with the difference in drug response and metabolism between human and animal being the prime disadvantages (Cheung and Gonzalez 2008). Almost 95% of tested compounds fail clinical trials (Rubin 2008, Moreno and Pearson 2013) and this astonishingly high attrition rate, primarily due to drug toxicity often only in subsets of patients, results in cumulative expenditures of over two billion dollars for a successful drug approval by the US Food and Drug Administration (FDA) (DiMasi, Grabowski and Hansen 2016). Therefore, there is a pressing need for a more reliable and robust model to study liver metastases and drug discovery as they pertain to the human condition.

1.5.1 Liver Physiology

Liver is the largest internal organ. It is located in the right upper quadrant of the abdominal cavity and below the diaphragm. The liver receives oxygenated blood from the aorta through hepatic artery. The liver also receives nutrient-rich blood from the gastrointestinal through portal vein that drains into the central vein. The liver is divided into four main lobes that consist of hexagonal hepatic lobules as the liver functional units (Vidal-Vanaclocha 2011). Central vein, located at the center of each lobule, connects to portal triads at each corner of the hexagonal-shaped lobule through liver sinusoids (Figure 2A).

As an organ, the liver plays pivotal roles in a lot of physiological processes including production of thrombopoietin and coagulation factors for hemostasis, gluconeogenesis and glycogen storage for energy regulation, xenobiotics metabolism, lipid metabolism, storage of retinoic acid and production of bile acid.

The liver is made up of several different types of cells. The hepatocytes constitute 60-70% of liver cell number (90% of liver mass) with the rest of liver volume is made up of the cholangiocytes/biliary cells (2-3%) and the non-parenchymal cells (NPC) that include liver-specific endothelial cells (2.5%), hepatic stellate cells (2-8%), Kupffer cells (2%), oval cells and some other types of cells (Geerts 2001, Damm et al. 2013). Liver non-parenchymal cells have distinct functions and roles in supporting liver physiology. These NPCs also have specific cellular arrangements and morphologies that are crucial for their functions (Figure 2B).

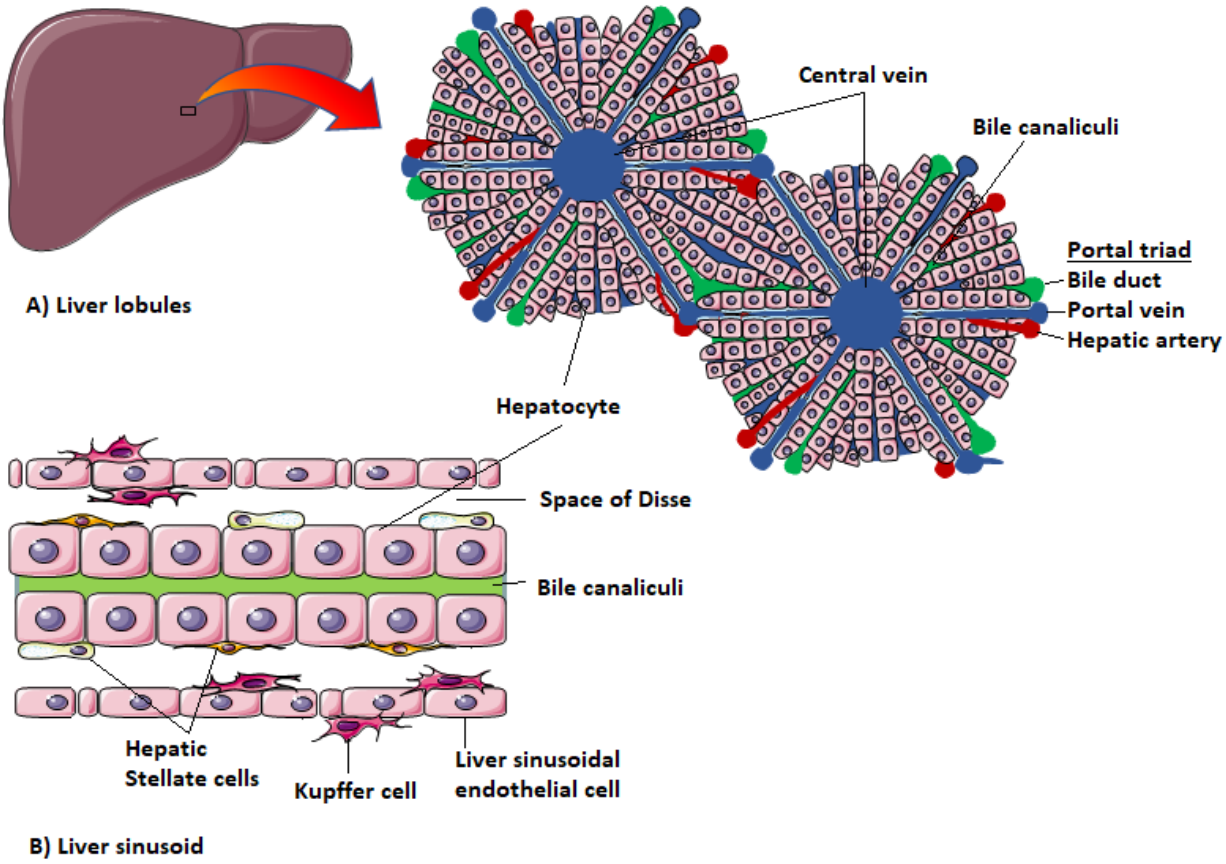


Figure 2: Schematics of liver structure and liver sinusoid.

A) Hepatocytes are arranged into hexagonal-shape liver lobules with the central veins at the center of the liver functional units. Portal triads consisting of bile ducts, portal veins, hepatic arteries are found at each corner of the hexagonal liver lobules and connect to the central vein through liver sinusoids. B) Liver sinusoids are lined with liver sinusoidal endothelial cells. Kupffer cells actively monitor the sinusoids for foreign bodies. Hepatic stellate cells reside within the space of Disse and remain dormant in normal livers. Quiescent stellate cells (yellow) stores vitamin A whereas activated stellate cells (orange) transdifferentiate into myofibroblasts and secrete ECM components during liver fibrosis. Drawn from templates provided by Servier Medical Arts (refer to supplementary information Appendix D for the permission of use).

The sinusoids are lined with liver sinusoidal endothelial cells (LSECs). These cells are the first point of contact with foreign bodies including circulating cancer cells. One of the specific features of the LSEC is fenestration which are small cytoplasmic windows or pores ranging from 20-300nm in diameter that filter fluids and particles from the blood vessels. The LSEC also has high endocytotic capacity due to high expression of mannose receptors and other scavenger receptors (Elvevold et al. 2008). This scavenger feature is coupled with efficient catabolism and degradation to clear waste products in the blood (Elvevold, Smedsrod and Martinez 2008). Since the LSEC is unique compared to most other endothelial cells, efforts have been made to immortalize these cells. Most primary, immortalized, iPS cell-derived endothelial cells and human LSE cell lines including TMNK-1 and TRP3 cells express endothelial cell markers such as CD34 and von Willebrand factor (vWF) and exhibit normal endothelial cell functions such as uptake of acetylated-low density lipoprotein or soluble materials, responsiveness to vascular endothelial growth factor (VEGF) and FGF and formation of vascular tube-like structure on basement membrane gel but lack the ability to establish true fenestration (Matsumura et al. 2004, Adams et al. 2013, Parent et al. 2014). Primary LSECs are highly fenestrated but tend to lose this feature within 1-2 days of *in vitro* culture (Elvevold, Smedsrod and Martinez 2008). Primary liver cancer and metastatic cancer cells also induce defenestration, possibly by depleting nutrients that are required for fenestrae maintenance (Braet et al. 2003).

Within the sinusoids, Kupffer cells (KCs), the liver resident macrophages, are usually found anchored to the endothelium. Similar to the LSEC, KC also expresses high levels of scavenger receptors, toll-like receptors, complement receptors and antibody receptors since the main function of these cells is to detect and phagocytose pathogens or foreign molecules (Jenne and Kubes 2013). Binding to these foreign bodies also triggers the secretion of various pro-

inflammatory cytokines such as TNFα, ROS, IFNγ, IL-6, CCL2 and other factors which can directly affect the invading pathogens or indirectly through the recruitment of neutrophils, bone marrow-derived monocytes and natural killer cells (Brodt 2016). The KC is also an important antigen-presenting cells, expressing major histocompatibility complex (MHC) class I and II and co-stimulatory molecules to activate T-cells. Although the KC was discovered in the 1800s, the ontogeny of these cells are still debatable as some studies showed that these cells consist of differentiated bone marrow-derived monocytes whereas others showed that the KC is locally derived in the liver from progenitor cells during liver development or from hematopoietic stem cells in the liver (Jenne and Kubes 2013, Zigmond et al. 2014). These different ontogenies may give rise to several subpopulations of KCs. Inherently, KCs are genetically and phenotypically different from the infiltrating monocyte-derived macrophages and involve mainly in the resolution phase following liver injury by increasing phagocytosis of apoptotic bodies and ECM remodeling (Zigmond et al. 2014). Additionally, the different subsets of Kupffer cells, at least in mice, show different functions. F4/80+/CD68+ KC showed potent phagocytic activity and elevated reactive oxygen species production following lipopolysaccharides (LPS) stimulation whereas F4/80+/CD11b+ KC displayed cytokines producing phenotype following LPS treatment (Kinoshita et al. 2010). Similar to general macrophages, KCs can also be polarized into pro-inflammatory M1 and anti-inflammatory M2 macrophages that differently affect tumor growth (Yang et al. 2016). KC has also been reported to display bimodal roles in cancer progression where during pre-metastatic stage, KC inhibits cancer growth *in vivo* but increase tumor burden at later stage through the secretion of VEGF secretion and depletion of iNOS (Wen, Ager and Christophi 2013).

Hepatic stellate cells (HSCs) are another liver resident cells that are typically found in the space of Disse. These fat-storing cells had also been previously referred as lipocytes, mesenchymal cells, perisinusoidal cells and Ito cells (Friedman 2008). In normal livers, the HSCs are in a quiescent state. The main functions of these cells are to store vitamin A lipid droplets and aid immunoregulation by secreting chemoattractants such as CCL2, RANTES and CCL21 to recruit monocytes and neutrophils (Friedman 2008).

HSCs are mostly studied for their roles in liver fibrosis. Liver injury or infection exposes the HSCs to various paracrine stimuli such as TGFβ and PDGF leading to HSC activation which is marked by vitamin A secretion, cell proliferation, increased contractility, transdifferentiation into myofibrobalsts and ECM components production (Friedman 2008).

The roles of HSC in liver and bile tumorigenesis have been extensively studied. HSCs have also been shown to increase the proliferation, migration and invasion of hepatocellular carcinoma (HCC), cholangiocarcinoma, Lewis lung carcinoma and colon carcinoma through growth factors production, ECM synthesis and tumor angiogenesis induction (Kang, Gores and Shah 2011).

There are several hepatic stellate cell lines that sufficiently replicate primary stellate cell (Herrmann, Gressner and Weiskirchen 2007). LX-1 and LX-2 stellate cells, immortalized with SV40-Tag, display stellate cells characteristics including expression of stellate cell markers such as glial fibrillary acidic protein (GFAP), alpha-smooth muscle actin (αSMA), vimentin, secretion of collagen in response to TGF-β1 stimulation and the ability to store and metabolize retinoic acid (Xu et al. 2005). Similarly, TWNT-1 and HSC-T6 cells exhibit the functions and markers of hepatic stellate cell (Vogel et al. 2000, Watanabe et al. 2003). All cell lines, however, are in the activated state which is marked by the lack of retinoic acid storage, increased level of αSMA

expression and collagen type I secretion. *In vitro* HSC activation, caused by stiff culture condition (Friedman et al. 1989) and the isolation process that simulates liver injury, could be reversed or prevented by culturing the cells on laminin-coated surface (Stone et al. 2015) or on soft surface/gel (Caliari et al. 2016). Thus, proper cultivation and maintenance of these cells are essential to limit and prevent hepatic stellate activation that can artificially affect the cancer cells in the liver model for liver metastasis study.

1.5.2 Primary Liver Malignancy

Liver plays a lot of vital roles in the body and has been implicated in numerous pathological conditions such as cirrhosis, hepatitis, hereditary diseases, benign liver lesions, alcohol liver disease, fatty liver disease, liver malignancy and various other diseases. Sexual and racial disparities in the incidence of liver diseases have been documented. Women are more susceptible to cirrhosis, benign liver lesions, alcohol liver disease and toxin-induced acute liver injury (Guy and Peters 2013) which could be due to different profiles of metabolizing enzymes expression such as cytochrome P540 (CYP) (Wolbold et al. 2003, Mennecozzi et al. 2015) and nuclear receptors expression (Rando and Wahli 2011) leading to varying sensitivity to drugs. Men, on the other hand, have a higher rate of primary liver malignancy. The ratio of hepatocellular carcinoma (HCC) between men and women is 3-4:1 (Naugler et al. 2007). Different hormonal signaling contributes to sexual dimorphism in liver malignancy. In female mice, estrogen prevents diethylnitrosamine (DEN)-induced HCC by inhibiting MyD88-mediated NF-κB transcriptional activity to reduce the level of tumor-promoting interleukin-6 (IL-6) (Naugler et al. 2007). In male mice, the activation of androgen receptor promotes hepatocarcinogenesis by promoting cell growth, increasing cellular oxidative stress to elevate cellular DNA damage and

inhibiting tumor suppressor P53 and its downstream targets (Ma et al. 2008). These sexual dimorphism effects are dependent on FOXA1/2 transcription factors that play differential roles in recruiting estrogen receptor α and androgen receptor to co-regulate the expression of their targets (Li et al. 2012).

The incidence of liver malignancy also differs among different races and ethnicities. In general, African Americans show high incidence and mortality rate for colorectal, cervical, prostate and liver tumors when compared to other races and are more frequently diagnosed with advanced cancer stage (Ward et al. 2004). When adjusted for census tract poverty level, African Americans and American Indians/Alaska Natives still show a lower 5-year survival rate when compared to non-Hispanic whites, indicating that socioeconomic status is not the only main factor for racial disparity in cancer incidence and mortality (Ward et al. 2004). American HCC patients are also more likely to present with regional and distant diseases at diagnosis (Sloane, Chen and Howell 2006). A recent study reported that a single nucleotide polymorphism on TP53 (P47S) gene, found to be specifically restricted in African-descent populations, could cause inefficient apoptosis induction when challenged with cisplatin and increased susceptibility to spontaneous tumorigenesis and metastasis (Jennis et al. 2016), thus cementing the contribution of genetic variations in promoting tumorigenesis and metastasis in these races/ethnicities.

1.5.3 Liver Metastases

Other than primary liver malignancy, liver is also one of the most common metastatic sites for breast, prostate, colon and skin cancers (Bubendorf et al. 2000, Minami and Kudo 2010, Tabariès and Siegel 2011).

There are several key factors that allow for the cancer cells to extravasate into the liver. First of all, the liver contains a complex network of blood circulation that delivers oxygenated blood via the hepatic artery and nutrient-enriched blood via the portal vein. The extensive networks of blood vessels, coupled with slow hepatic microcirculation in the sinusoidal capillaries potentiate cancer cell retention in the liver (Vidal-Vanaclocha 2011). Gut-derived venous blood also exposes the liver to various toxins. On average, the liver is exposed to 10-1000pg/mL of LPS, a major membrane component of gram negative bacteria (Lumsden, Henderson and Kutner 1988), that consequently put the liver under a basal level of inflammation (Parker and Picut 2005). The presence of external stimuli may induce chronic or acute inflammation that in turn activate liver resident cells (Liaskou, Wilson and Oo 2012) to cause various liver diseases such as hepatocellular carcinoma (Drucker et al. 2006), cirrhosis (Connolly et al. 2010) and alcoholic liver disease (Cohen and Nagy 2011). Additionally, the activation of the HSCs leads to excessive ECM secretion and cytokines/chemokines production to cause liver fibrosis. We posit that these events promote metastatic cancer cell growth in the liver (will be discussed in Chapter 3). Secondly, the liver endothelium lacks basement membrane, thus allowing tumor cells to access the extracellular matrix proteins in the space of Disse for motility and survival cues and facilitate efficient attachment to establish micrometastases (Elvevold, Smedsrod and Martinez 2008, Porquet and Huot 2011). Lastly, liver resident cells express high levels of surface molecules to enable them to uptake nutrients and clear gut-derived toxins and microbes. However, these surface molecules, such as cell adhesion molecules, endocytic receptors, toll-like receptors and oligosaccharides moieties (Vidal-Vanaclocha 2011), also play some roles in aiding cancer cell diapedesis from the lumen into the space of Disse (Porquet and Huot 2011).

The incidence of liver metastases is different among the primary tumor types. Most studies on liver metastases focus on colorectal, gastric and pancreatic metastases since the gastrointestinal (GI) tract and digestion accessory organs connect with liver through blood flow (Turdean et al. 2012). Accordingly, liver metastasis affects more than 50% of colorectal cancer patients during their lifespan (Misiakos, Karidis and Kouraklis 2011). However, it is important to note that liver metastases also occur at high frequencies in other solid tumors but are usually detected in the late stage of tumor progression. As discussed in previous sections, liver metastases also occur in high percentages in mainly CRPC patients and aggressive breast cancer patients.

In addition to different metastatic rates for different primary cancers, racial disparities in liver metastases have been reported. A Surveillance, Epidemiology and End Results (SEER)-based study on colorectal cancer reveals a higher percentage of metastases to the liver and lung in African American patients when compared to Caucasians, Asians and other ethnicities (Qiu et al. 2015). Interestingly, for gastric cancer patients, non-Hispanic whites showed higher incidence of liver metastases when compared to other ethnicities (Yao et al. 2005). Additionally, basal type or triple negative breast cancer, which is more prevalent in pre-menopausal African American women (Carey et al. 2006, Morris et al. 2007), shows greater propensity to metastasize to the liver and brain when compared to other breast cancer types (Yuan et al. 2014). In all, biological differences in these ethnic groups, together with environmental factors and socioeconomic status, may contribute to racial disparity of liver metastases.

Several lines of evidence insinuate sexual disparity in liver metastases where men are more prone to liver metastases (Yao et al. 2005, Manfredi et al. 2006, Hoyer et al. 2011) and, in some cases, show a poorer prognosis (Mazzaglia et al. 2007). Separate studies on two European

cohorts revealed a higher rate of liver metastases in men regardless of the origin of the primary tumor (Hoyer et al. 2011, Turdean et al. 2012). Furthermore, the frequency of both synchronous and metachronous colorectal cancer liver metastases are higher in men (Manfredi et al. 2006) suggesting a cancer-related predilection rather than metastases seeding into a diseased liver. In addition to colorectal carcinoma, a univariate analysis on patients with neuroendocrine liver metastases showed significant reduced survival in men when compared to women and multivariate analysis revealed that male sex was significantly associated with three times greater mortality risk (Mazzaglia et al. 2007).

In short, most solid primary malignancies progress to the liver at least in the later stages. Documented sexual and racial disparity in liver malignancy and metastases may pose critical implications on disease management and treatment approaches. The biological factors for the discrepancies in liver metastases between men and women and among different races/ethnicities are unclear but could be due to similar biological factors reported in primary liver cancer. Mouse models do not sufficiently replicate gender and ethnicity settings. Hence, reflective models are necessary to unravel the underlying causes and consequences of liver metastases.

1.5.4 Modeling the Liver for Cancer Study and Drug Discovery

All of the above indicate that liver metastasis is not simply ‘one disease’ and each specific setting should be separately addressed for better and efficacious targeted therapies. Further, the differences noted by sex and ethnicity/race of individuals argue for a diverse source of primary human cells to capture population heterogeneity *ex vivo*. Current studies on spontaneous liver metastases mainly utilize animal models that poorly replicate clinical conditions (Francia et al.

2011), while standard *in vitro* culture limits primary cell viability and functionality (Sosef et al. 2005).

Three-dimensional (3D) cell culture re-establishes the structural and signaling relationships of the tissues that are important for organ function. Several conditions in constructing the liver and modeling liver metastases ex-vivo need to be properly addressed in order to overcome the limitations associated with mouse models and standard 2D *in vitro* cultures. First, the liver itself must be reasonably recreated to allow for homeostatic functions such as drug metabolism. The *ex vivo* liver must also be responsive to external factors such as LPS that can promote metastasis and has been shown to be elevated in patients with liver metastases (Gul et al. 2012). Second, the tissue structure must be challenged with appropriate tumor cells that can recreate the metastatic phenotypes noted in persons, including both dormant and outgrowing nodules. This means a relatively comprehensive regeneration of the pathologically involved organ that will require reliable sources of human liver parenchymal and NPCs to recreate 3D *ex vivo* liver tissues on perfusable scaffolds that can maintain tissue integrity and function while minimizing the effects of the construct. Other considerations that should be included are common medium to accommodate heterogeneous cell types in the system and precise mechatronics to continuously supply nutrients at physiologic concentration and remove cellular wastes. Comprehensive requirements to ‘engineer’ the liver are extensively discussed elsewhere (Griffith, Wells and Stolz 2014). There are several platforms that have been constructed according to the aspects mentioned above such as LiverChip microphysiologic system (MPS), PEARL perfusion liver system and sequentially layered, self-assembly liver (SQL-SAL) platform. These platforms have been validated to improve and maintain liver

functions for extended period of times. The advantages and disadvantages of these platforms are reviewed extensively elsewhere by us and others (LeCluyse et al. 2012, Clark et al. 2016).

In addition to the platforms discussed previously, there are several liver platforms that have been recently developed including a novel microfluidic device that can simultaneously monitor oxygen consumption, glucose uptake and lactate production in real time (Bavli et al. 2016). The precise measurements of these parameters allow for accurate detection of early mitochondrial dysfunction that is critical in liver toxicity. This device, similar to other platforms, provides continuous perfusion and maintains hepatic functions for an extended period of time (Bavli et al. 2016). Another platform, metastasis-on-a-chip (MOC), connects gut and liver organoids through an elastic tube and allows for real-time monitoring of cancer migration from the gut to the liver (Skardal et al. 2016). The system also permits the investigators to manipulate the stiffness of the tumor foci separate from the stiffness of the tissue microenvironment (i.e. soft tumor foci and stiff gut/liver microenvironment and vice versa) which may be useful in studying the effects of microenvironment and ECM accumulation on tumor cell migration. Recently, bio-printing, a state-of-the-art technology to construct 3D structure using live cells into viable and functional tissues, has been utilized to construct 3D tissues including the liver for both static culture and perfused bioreactor (Robbins et al. 2013, Lee and Cho 2016, Ma et al. 2016, Nguyen et al. 2016). Table 1 summarizes some of the properties of these recent platforms.

These models however still lack several important parameters especially for reproductive organs malignancy and metastasis. The prime disadvantage of these models is the lack of multi-organ communication. Tumor metastasis occurs sequentially through hematogenous route to most secondary organs. Frequently, these cells colonized other organs such as bone and lung

before finally form liver metastases. Hence, to truly recapitulate liver metastases, multi-organ connection is paramount.

Additionally, most primary prostate and breast cancers are hormone-dependent. The hypothalamus-pituitary gland- gonadal gland (HPG) axis regulates the secretion of estrogen and testosterone hormones, the main drivers of breast and prostate cancer development. Moreover, pituitary gland also produces growth hormone and prolactin, both of which have been shown to implicate breast and prostate cancer tumorigenesis and metastasis. Furthermore, luteinizing, follicle-stimulating, growth and prolactin hormones play important roles in breast and prostate cancer progression and luteinizing hormone (LH) specifically is associated with metastasis and poor prognosis (Kluth et al. 2014, Subramani et al. 2017). It is important to note that although most of the prostate liver metastases are CRPCs, the cells still require androgen for growth which serves as the basis for the new generation of hormonal therapies to more efficiently inhibit androgen receptor activation in these CRPCs. This clearly shows that crosstalk between cells and organs is vital in cancer and metastasis development.

Lastly, inflammatory reactions are also key in tumor development and metastasis which is currently deficient in most human liver and other organ models. Inflammatory reactions from both resident and bone marrow-derived immune cells mediate both tumor suppression and tumor promotion (Brodt 2016), thus bolstering the requirement of inflammatory reactions in these model.

Current efforts aim at improving the systems by integrating several organoids or organ systems to more closely resemble human conditions. In addition, investigators are also utilizing different sources of hepatocytes including hepatocytes derived from induced-pluripotent stem

cells to model both genders and different ethnicities in an attempt to capture population diversity with the systems.

1.5.5 Conclusions

Metastasis is currently the main challenge in tumor management and therapy. Tumor cells, due to their inherent genetic instability and dysregulated cellular communications within tumor stroma, are able to metastasize and evade various treatment modalities. Tumor suppressor E-cadherin is crucial in blocking cell motility and invasion but may also play roles in promoting cancer cell seeding into secondary organs and protecting the cells against chemotherapeutic drugs. Liver metastases are common clinical observations and result in poor prognosis due to impaired liver functions. Current research methodologies for drug discovery and development are cost-inefficient, labor-intensive with deleterious toxicity. Proper human organ modeling might be the key to developing effective therapies against metastatic diseases.

Table 1: Recent 3D *Ex vivo* liver models

Platform	Cell types	Time	Features
LiverChip MPS	Fresh and cryopreserved primary human hepatocytes Fresh primary human NPCs	15-29 days	- Continuous and controlled perfusion - Spontaneously induces tumor dormancy (Wheeler et al. 2014) - Utilizes soft (PEG hydrogel) and stiff (polystyrene) scaffolds to more accurately model normal and diseased liver (Clark et al. 2017) - Establishes oxygen gradients that mimics liver physiology (Domansky et al. 2010) - Successfully predicts drug clearance and metabolism that correlates with clinical observations (Sarkar et al. 2015, Tsamandouras et al. 2017)
Bavli et al (Bavli et al. 2016)	HepG2/C3A derivative	28 days	- Measures oxygen consumption using tissue embedded two-frequency phase modulation phosphorescent microprobes - Simultaneously measures glucose uptake and lactate production using computer controlled microfluidic switchboard - Continuous/controlled perfusion

3D bio-printed liver	HepG2 and HUVEC Cryopreserved primary human hepatocytes iPS/ES cells	6-35 days	- Bio-mimetic perfusion (Lee and Cho 2016) - Allows for the fabrication of complex structure in only one-step 3D cell printing (Lee and Cho 2016) - Improves liver functions and iPS differentiation (Robbins et al. 2013, Ma et al. 2016)
MOC [Metastasis on a Chip] (Skardal et al. 2016)	HepG2 Human intestine epithelial cells (INT-407)	24 days	- Combines two organoids for early cancer metastasis study - Micro-peristaltic pump for controlled medium flow - Real-time microscopy to monitor cells migration - Tunable hydrogel system to modulate the stiffness of tumor foci and tumor microenvironment

2.0 THE ROLES OF CHEMOKINE RECEPTOR CXCR3 IN MESENCHYMAL-EPITHELIAL TRANSITION

This chapter contains materials from:

(Ma, Khazali and Wells 2015) "CXCR3 in carcinoma progression." Histol Histopathol 30(7): 781-792.

In the previous chapter, we have discussed the central roles of tumor suppressor E-cadherin in regulating tumor metastases. E-cadherin, ubiquitously expressed in almost all normal epithelial cells, is absent in high grade and metastatic solid tumors. Restoration of E-cadherin expression with drugs targeting epigenetic events is a promising metastasis prevention and therapeutic modality but has not shown significant progress especially for solid tumor treatment thus far. Moreover, the reversion of the cancer cell phenotype back to epithelial in the distant organs complicates the efforts of targeting metastatic disease and may require finely tuned treatment window and treatment modalities.

We sought to find alternative methods to regulate the expression of E-cadherin. Based on our previous studies on the roles of chemokine receptor in endothelial cells and other stromal cells, we found that the activation of CXCR3 by its ligands induces endothelial cell death and more importantly blocks cell migration. Thus, CXCR3 could be a potential protein of interest in regulating metastasis.

2.1 CHEMOKINE RECEPTORS AND LIGANDS

Chemokines, or chemotactic cytokines, are a superfamily of approximately 50 low molecular weight (8-15KDa) soluble cytokines that primarily act as chemoattractant to recruit leukocytes or other immune cells to inflammatory sites and to secondary lymphoid organs (Moser and Loetscher 2001). These chemokines bind to several seven-transmembrane G-protein coupled receptors and mediate various intracellular signaling. The chemokines are not simply immune regulators as they have been shown to play important roles in development, angiogenesis, hematopoiesis, atherosclerosis, inflammation, immune diseases and cancer progression (Luster 1998, Romagnani et al. 2004, Vandercappellen, Van Damme and Struyf 2008, Singh, Lillard and Singh 2011).

Chemokines are divided into 4 subgroups according to the number and position of conserved cysteines in the amino-terminal domains: C, CC, CXC and CX3C. The CXC chemokines are further divided into the presence or absence of glutamic acid-leucine-arginine sequence ("ELR" motif) which differently affect angiogenesis. Table 2 summarizes the receptors and their respective ligands. These ligand-receptor pairs usually involve in the development, trafficking and homing of leukocyte and hematopoietic stem cells. Excessive presence of leukocytes is one the main hallmarks of inflammation where the CC chemokines typically mediate chronic inflammation to recruit T-cells, monocytes/macrophages and eosinophils in autoimmune diseases while the CXC chemokines are mainly responsible for acute inflammation to recruit neutrophils in syndromes/diseases such as acute respiratory distress syndrome (Proudfoot 2002). An exception to this specialized chemokine-mediated inflammation is CXCR3 as this receptor is also found on T-cells during chronic inflammation (Proudfoot 2002). Chemokine receptors have also been implicated in other diseases such as cancer,

neurodegenerative diseases and vascular diseases. A particular emphasis on the roles of CXCR3 in solid cancers will be discussed in subsequent sections.

Table 2: Chemokine receptors and ligands

Receptors	Ligands
CXCR1	IL-8, GCP-2
CXCR2	GROαβγ, NAP2, ENA78, IL-8
CXCR3	PF4, MIG, IP10, I-TAC
CXCR4	SDF-1
CXCR5	BCA-1
CXCR6	CXCL-16
CCR1	RANTES, MIP-1α, MCP-3
CCR2	MCP-1, 2, 3, 4
CCR3	EOTAXIN, MCP-4, RANTES, MEC
CCR4	TARC, MDC
CCR5	MIP-1β
CCR6	MIP-3α
CCR7	ELC, SLC
CCR8	I-309
CCR9	unknown
CCR10	MEC, CTACK
CCR11	TECK
CX3CR1	FRACTALKINE
XCR1	LYMPHOTACTIN

2.2 CXCR3 RECEPTORS AND LIGANDS

2.2.1 CXCR3 Isoforms

CXCR3, like other CXC receptors, is a seven-transmembrane pass G protein-coupled receptor (GPCRs). CXCR3 was initially cloned and characterized as the selective receptor for CXCL9 and CXCL10 (Loetscher et al. 1996), and later mapped as a single-copy gene on chromosome Xq13 (Loetscher et al. 1998). It was renamed to CXCR3A after an alternative spliced isoform, CXCR3B, was discovered (Lasagni et al. 2003). CXCR3A mRNA encodes a protein of 368

amino acids with an unmodified molecular mass of 40,659 Dalton. CXCR3B has 415 residues which contains an extended extracellular domain at the N-terminus. Therefore, isolated detection of CXCR3A is difficult due to almost complete overlap with CXCR3B. Another spliced variant CXCR3Alt, a drastically altered C-terminal protein sequence compared to CXCR3A, has a predicted four- or five-transmembrane domain structure, differing from all known functional chemokine receptors. CXCR3Alt has 267 residues with a predicted size of 28,715 Dalton and displays a well-focusing band at ~33kDa on western blot analyses due to potential N-glycosylation on the extracellular region of the receptor. Despite severe structural changes, CXCR3Alt still localizes to the cell surface and mediates functional activity in the presence of CXCL11 (Ehlert et al. 2004). CXCR3A and CXCR3B mediate disparate signaling events to promote different cellular responses. Generally, CXCR3A appears to promote proliferation, cell survival, chemotaxis and invasion while CXCR3B appears to mediate growth suppression, apoptosis and angiostatic effects. Almost all human cells express both CXCR3A and CXCR3B, except for primary cultured human mesangial cells (HMC) that express only CXCR3A and human microvascular endothelial cell (HMvEC) that express only CXCR3B (Lasagni et al. 2003). Although most cells express both isoforms, the predominant isoform differs by cell types. In hematopoietic-derived cells, CXCR3A is the dominant isoform whereas CXCR3B predominates in differentiated epithelial cells and fibroblasts.

2.2.2 CXCR3 Ligands

CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CXCL4 (PF4) and its non-allelic variant CXCL4L1 are members of ELR-negative CXC chemokine subfamily. These chemokines bind to both CXCR3A and CXCR3B isoforms but at different affinities (Fulton 2009, Billottet,

Quemener and Bikfalvi 2013). CXCL9, CXCL10 and CXCL11 bind to both CXCR3A and CXCR3B, with all three chemokines show higher affinity for CXCR3A (Loetscher et al. 1996, Lasagni et al. 2003). Another ligand, CXCL4, only binds CXCR3B (Lasagni et al. 2003) but can also induce T-lymphocytes migration through CXCR3A-mediated signaling at higher (micromolar) concentration (Mueller et al. 2008). CXCL4L1, a variant of CXCL4 with only 3 amino acid residues substitution in the C-terminus, can bind to both isoforms and is more angiostatic than CXCL4 (Struyf et al. 2004). Human CCL21, in the absence of its primary receptor CCR7, has been reported to be a functional ligand for CXCR3 to induce chemotaxis of adult microglial cells where CXCR3 is predominantly expressed (Dijkstra et al. 2004). However, this observation has not been separately validated.

CXCL9, CXCL10 and CXCL11 are ubiquitously expressed. CXCL10 gene is highly expressed in the appendix and lymph nodes, moderately expressed in the colon, placenta, urinary bladder, liver, lung and thymus and lowly expressed in the prostate, kidney and brain (Gene [Internet] 2017). Both CXCL9 and CXCL11 gene expression show similar pattern but at significantly lower levels and CXCL9 is also highly in the lymph nodes (Gene [Internet] 2017, Gene [Internet] 2017). CXCL4 shows a distinct gene expression profile where it is highly expressed in the bone marrow, detectable in the spleen, placenta, lung and colon and negligible in other organs (Gene [Internet] 2017). CXCL4 gene is up-regulated in prostate cancers whereas CXCL10 and CXCL11 genes are down-regulated when compared to normal prostate cells. CXCL9 expression is negligible in all samples (Wu, Dhir and Wells 2012).

2.2.3 CXCR3 Signal Transductions

CXCR3A and CXCR3B mediate distinct signaling cascades depending on the cell types and the specific G protein coupled to the receptor (Lasagni et al. 2003, Kouroumalis et al. 2005). In general, CXCR3A signaling promotes cell proliferation and chemotaxis, whereas CXCR3B suppresses proliferation and migration and sensitizes cells to apoptosis. CXCR3A transfected-HMvEC exhibited a rapid, dose-dependent intracellular calcium flux in response to CXCL9-11 and CXCR3A-induced proliferation was pertussis toxic (PTX)-sensitive implying signaling via Gαi/o protein (Lasagni et al. 2003). CXCR3B transfectants showed much higher basal cAMP levels compared to mock transfectants which can be further increased upon CXCL9-11 and CXCL4 stimulation indicating receptor coupling to Gαs protein. A study using mice deficient in the Gαi2 and Gαi3 found that knocking out Gαi2 subunits abrogated CXCR3-induced lymphocytes chemotaxis, whereas knocking out Gαi3 increased lymphocytes migration and GTPγS binding (Thompson et al. 2007). These results suggest that Gαi2 subunits are required for CXCR3-mediated signaling and Gαi3 subunits inhibit CXCR3 signaling in mouse T-lymphocytes, though the translation to other cell types remain uncertain.

CXCR3B activation has been noted to exert angiostatic effects where CXCL10 binding results in PKA-dependent inhibition of m-calpain that prevents rear-end retraction for endothelial cells motility (Bodnar, Yates and Wells 2006). CXCR3B activation by CXCL4/PF4 also mediates angiostatic effect through P38/MAPK activation (Petrai et al. 2008). In keratinocyte, CXCL11 induces cell motility via signaling through PLC-β3, resulting in the activation of μ-calpain to allow for partial cell de-adhesion from the substratum (Satish et al. 2005). These findings were confirmed in prostate cancer cells where we found that CXCL4 and CXCL10 promoted cell motility and invasiveness of DU145 and PC3 cells through PLC-β3-mediated μ-

calpain activation but CXCR3B overexpression blocked m-calpain activation to suppress cell migration (Wu, Dhir and Wells 2012). Interestingly, although CXCR3A activity promotes endothelial cell motility for angiogenesis, the cleavage of the intracellular tail of the β3 integrin by μ-calpain following CXCR3A activation results in anoikis in endothelial cells (Bodnar et al. 2009). Of interest, pericytes produce CXCR3 ligands that then trigger anoikis in the immature vessels via the same μ-calpain pathway as noted above (Bodnar et al. 2013). Thus, CXCR3 signaling affects chemotaxis of normal cells in several different ways: 1) induce cell motility through CXCR3A-mediated μ-calpain activity but CXCR3A signaling may also induce anoikis due to integrin cleavage that blocks cell attachment that is crucial for cell survival 2) block the migration endothelial cell through CXCR3B-mediated m-calpain inhibition. CXCR3B activity appears to be the rate-limiting step for cell migration. The dominant isoform on a given cell will dictate the final outcome.

2.2.4 CXCR3 Roles in Cancers

CXCR3 has been examined in the context of cancer as increasing evidence shows that CXCR3 is expressed and functional in almost all cells, and is crucial in terminating migration during wound repair (Yates et al. 2008, Huen and Wells 2012) and angiogenesis (Bodnar, Yates and Wells 2006, Bodnar et al. 2013). CXCR3 was found upregulated in many primary and metastatic tumors such as breast, prostate, colon, colorectal, melanoma and ovarian cancer. Moreover, CXCR3 has been linked with poor prognosis in breast, melanoma and colon cancer patients.

2.2.4.1 CXCR3 in Prostate Cancer

The differential expression pattern of CXCR3 isoforms was found to correlate with the progression of prostate cancer. Previously, we published that CXCR3 expression was elevated in prostate cancer when compared to normal tissues (Wu, Dhir and Wells 2012). Specifically, CXCR3A mRNA was upregulated in prostate cancer specimens while CXCR3B mRNA was downregulated in these specimens and the ratio of CXCR3A/CXCR3B mRNA levels was increased in the invasive and metastatic DU145 and PC3 prostate cancer cells but not in the LNCaP cells compared to normal RWPE1 cells. High CXCR3A/3B ratio in the metastatic DU145 and PC3 cells resulted in CXCL10 and CXCL4-induced cell motility and invasion whereas cell motility was inhibited in RWPE-1 cells with low CXCR3A/3B ratio. We also found that ectopic expression of CXCR3B in DU-145 cells decreased cell motility and invasion. It was previously reported that overexpression of CXCL10 in LNCaP cells, where CXCR3B is the dominant isoform, inhibited cell proliferation and PSA production (Nagpal, Davis and Lin 2006). These two findings indicate that CXCR3B functions as anti-growth and anti-migratory isoform in prostate cancer. As mentioned before, CXCR3 is found primarily on the membrane of the normal cells but internalized in the cancer cells. It was postulated that intracellular expression might indicate progression into highly aggressive phenotype and induce intracellular tumorigenesis signaling (Engl et al. 2006). In all, these results suggest that a change from low to high ratio CXCR3A/CXCR3B promotes prostate cancer metastasis and stimulates cell migration and invasion (Wu, Dhir and Wells 2012).

2.2.4.2 CXCR3 in Breast Cancer

Breast cancer cells express both CXCR3 isoforms (Goldberg-Bittman et al. 2004, Datta et al. 2006, Li et al. 2011). Ma et al examined CXCR3 protein expression in a series of 75 stage I

or II breast tumors and detected CXCR3 in the cytoplasm and on the membrane of malignant cells from every patient whereas normal ducts were negative or weakly positive. They further reported a correlation between CXCR3 with the poor survival in breast cancer patients (Ma et al. 2009). Additionally, it was reported that the activated form of Ras, HA-Ras(12V) promoted CXCL10 transcription and downregulated the anti-growth isoform CXCR3B in human melanoma and breast cancer cell lines, MDA-MB-435 and MCF-7, with the combination of these two events resulted in enhanced breast cancer cell proliferation (Datta et al. 2006).

Several ways of preventing breast oncogenesis and metastasis through CXCR3 have been investigated. It was reported that prostaglandin E2 (PGE2) repressed CXCL9 and CXCL10 secretion in MCF-7 and MDA-MB231 cells and PGE2 repression could be inhibited by cyclooxygenase inhibitor to enhance intratumoral immune infiltration (Bronger et al. 2012). CXCR3 gene silencing and CXCR3 small molecule inhibitor, AMG487 were both effective in inhibiting lung metastases but did not affect the growth of local breast cancer in mouse model highlighting the role of CXCR3 in promoting breast cancer metastasis but not primary cancer incidence (Walser et al. 2006, Ma et al. 2009). To sum, signaling via CXCR3A promotes breast cancer proliferation and CXCR3B prevents cancer growth. Several therapeutic strategies have been explored including the use of COX2 inhibitor to promote immune cells tumor infiltration and the utility of CXCR3 gene silencing and small molecule inhibitor AMG487 to prevent metastasis.

2.2.4.3 CXCR3 in Colorectal Cancer

Clinical analyses of CXCR3 expression showed intense staining in 18-34% of colon cancer specimens with most of these CXCR3-positive patients were also diagnosed with lymph node metastases (Du et al. 2014). Kawada et al reported that, similar to breast cancer, patients

with CXCR3 expression presented with poorer prognosis compared to patients with negative CXCR3 expression or patients with CXCR4 or CCR7 expression. They also noted CXCR3 expression in some human colon cancer cell lines but documented similar metastasis rate for both CXCR3 expressing and non-expressing. Exogenously expressing CXCR3 in colon cancer cells resulted in greater tumor growth 4 weeks post rectal transplantation and increased metastatic tumor formation in the para-aortic lymph nodes at 6 weeks (59% vs 14%, $P<0.05$). Metastasis to the liver or lung, however, was rare and unaffected by CXCR3 expression (Kawada et al. 2007).

In clinical colorectal cancer (CRC) samples, CXCR3 expression level is significantly higher in metastatic foci within the lymph nodes (LNs) and liver compared to primary tumors. Some human CRC cell lines constitutively express all three known CXCR3 variants (Zipin-Roitman et al. , Rubie et al. 2008, Murakami et al. 2012). Similarly, CXCR3 activation *in vitro* and *in vivo* promoted cancer migration and growth and CXCR3 inhibition with AMG487 abrogated both responses. However, a separate study reported that CXCR3 antagonism only prevented lung metastases but not liver metastases as CXCR3 was only increased in lung nodules (Cambien et al. 2009). Knockdown of CXCR3 in SW620 cell line significantly reduced *in vivo* metastatic ability of the cells (Murakami et al. 2012). So, although the role of CXCR3 in colon cancer has been extensively studied, the specific roles of the isoforms are still not well characterized.

2.2.4.4 CXCR3 in Lung Cancer

In lung cancer, CXCR3 promotes cancer progression by modulating the expression of the receptor on immune cells or modulating the expression of CXCR3 ligands in the tumor cells. Unlike other cancers, CXCR3 is mainly negative in lung tumor cells but is highly expressed in

the infiltrating immune cells in non-small cell lung cancer clinical samples. Increased expression of CXCR3 in the tumor islets and stroma correlates with extended survival indicating immune cells recruitment for tumor killing (Ohri et al. 2010). Moreover, administration of IL-7 decreased tumor burden and was associated with increased CXCR3 expression on tumor associated T-cells, further corroborating the beneficial effects of CXCR3-mediated immune cells intratumoral recruitment (Andersson et al. 2009, Andersson et al. 2011). Restoration of CXCL10 secretion in human lung adenocarcinoma epithelial cell line A549 led to the inhibition of tumorigenesis independent of increased leukocyte infiltration (Arenberg et al. 1997). Thus, it is thought that in lung cancer CXCR3 and its ligands generally play roles in tumor killing via immune cells or inflammatory cells recruitment as opposed to direct effects on the tumor cells. The roles of CXCR3 isoforms are still unclear but the above evidence indicate signaling through CXCR3B in the tumor cells or activation of CXCR3A in the immune cells.

2.2.4.5 CXCR3 in Ovarian Cancer

Furuya et al reported differential expression of CXCR3 isoforms in endometriosis and ovarian cancers and CXCL4/CXCL4L1 expression in the tumor associated macrophages (TAMs). CXCR3A was found elevated in both ovarian cancer and endometriosis samples when compared to normal ovary while CXCR3Alt and CXCR3B were up-regulated and down-regulated in ovarian cancer, respectively when compared to endometriosis samples. CXCR3A was mainly expressed on the cancer cells and infiltrating lymphocytes whereas CXCR3B and CXCR3Alt were detected in the microvessels (Furuya et al. 2011). Angiostatic CXCL4 and CXCL4L1, on the other hand, were lower in the cancer lesions when compared to the corresponding endometriosis lesions found within the same cysts. Further analyses showed that CXCL4 was strongly expressed in CD68+ macrophages in the endometriosis samples but absent

in CD68+ macrophages within ovarian cancer lesions suggesting different functions of these cells (Furuya et al. 2007, Furuya et al. 2011, Furuya et al. 2012). These studies suggest that CXCR3A contributes to ovarian cancer tumorigenesis and lower expression of CXCR3B isoform and angiostatic CXCL4 in ovarian tumor may lead to impaired tumor angiogenesis.

2.2.4.6 CXCR3 in Renal Cancer

In renal cancer, CXCR3 is up-regulated and associated with poor prognosis (Johrer et al. 2005, Suyama et al. 2005, Klatte et al. 2008). Similar to our findings in prostate cancer, CXCR3A/CXCR3B ratio was found higher in renal cell carcinoma samples than in normal kidney samples, and total CXCR3 and CXCR3A expression was significantly higher in metastatic when compared to non-metastatic carcinoma samples (Utsumi et al. 2014). Calcineurin inhibitors (CNI), used to limit inflammation and allograft rejection, is known to promote the development and recurrence of several cancers. CNI mediated the progression of human renal cancer by downregulating CXCR3B and promoting proliferation through CXCR3A (Datta et al. 2008). A separate study found that CXCR3B overexpression significantly down-regulated the expression of anti-apoptotic heme oxygenase-1 (HO-1) in human renal cancer cells. Conversely, human renal cancer tissues expressing low amounts of CXCR3B significantly overexpressed HO-1 at both mRNA and protein level (Datta et al. 2010). In short, CXCR3B acts as anti-tumor isoform in renal cancer, similar to prostate and breast cancers.

2.2.4.7 CXCR3 in Melanoma

Normal melanocytes undergo a conversion from epithelial to mesenchymal phenotype during melanoma tumorigenesis. Evaluation of CXCR3 expression in patients with primary invasive cutaneous melanomas reveals significant association between CXCR3 positivity with

tumor thickness (>1mm) or invasiveness (Monteagudo et al. 2007). Thirteen primary melanoma-derived cell lines and five metastatic melanoma-derived cell lines expressed CXCR3 mainly in the cytoplasm but a small subpopulation (<2%) of the cells in six cell lines showed positive membrane staining. Additionally, most cell lines expressed high levels of CXCL9 and CXCL11 but not CXCL10. These results suggest that surface expression of CXCR3 is tightly regulated and intracellular receptor expression might be related to metastasis and poor prognosis (Pinto et al. 2014). Positive correlation between CXCR3 expression with melanoma invasion and metastasis has been documented and CXCR3 knockdown in mouse melanoma B16F10 cells markedly reduced metastatic frequency when compared to the parental cells (Kawada et al. 2004). In a highly invasive melanoma cell line BLM, CXCL9 triggered cell chemotaxis (Robledo et al. 2001). Lastly, it has been shown that tumor endothelial cells (ECs) secrete high levels of CXCL9 and CXCL10 in melanoma metastases to promote spontaneous migration of melanoma cells and disrupt endothelial barrier, resulting in an accelerated transendothelial migration (Amatschek et al. 2010). In summary, CXCR3 signaling conspires to promote melanoma invasion and dissemination of melanoma cells.

2.3 CXCR3B DRIVES E-CADHERIN RE-EXPRESSION TO FACILITATE PROSTATE CANCER LIVER METASTASIS

Tumor metastasis occurs in a series of sequential steps. The initial steps of metastasis require cancer cells to invade and migrate through the surrounding normal tissues and across blood barrier. These initial steps are potentiated by several mechanisms including the secretion of matrix degrading enzymes such as the MMPs and transition from epithelial to mesenchymal

phenotype (EMT). Cancer, once disseminated, is deadly and difficult to manage. Therefore, a potential alternative approach to treat metastatic disease is to prevent metastasis by restoring the physiological 'stop' signals that keep normal and dysplastic epithelial cells localized. E-cadherin is central in regulating cell migration and is reduced or absent in high grade primary tumors (Umbas et al. 1992, Oka et al. 1993, Wells, Yates and Shepard 2008). In prostate cancer cells, E-cadherin is downregulated due to reciprocal EGFR activity. E-cadherin is also crucial during MET to facilitate organ colonization during metastasis. Phenotypic reversion complicates the efforts of targeting E-cadherin in metastatic cells. Thus, further studies on E-cadherin regulation is required.

Cellular crosstalk, through various mediators, is important in driving metastasis (discussed in Chapter 1.0). Paracrine signals, as discussed in previous sections, have been found to also regulate cell motility migration (Wells et al. 2013). CXCR3 can both promote and block cell motility. Specifically, CXCR3B isoform has been reported to block endothelial cell migration. In metastatic prostate cancer, CXCR3A is the dominant isoform and can promote cell migration and invasion. Ectopic expression of CXCR3B can block ligand-induced cell migration and invasion.

Previous study indicates that CXCR3A is coupled to Gαi/o resulting in calcium flux to activate PLC and can be inhibited with pertussis toxin (Lasagni et al. 2003). Activation of CXCR3B, on the other hand, increases cAMP concentration to activate PKA (Lasagni et al. 2003). A recent publication gives an inkling on the connection between CXCR3 and E-cadherin expression where activation of PKA by forskolin or cholera toxin can increase homotypic E-cadherin interactions between cancer cells (Pattabiraman et al. 2016). Additionally, PKCδ, downstream of PLC-DAG and CXCR3A signaling cascades, reduces hemophilic E-cadherin

interactions to increase cell dissociation index (Chen and Chen 2009). Specifically, PKCδ phosphorylates threonine 790 (Thr790) on E-cadherin region I cytoplasmic domain which is important for β-catenin binding. The phosphorylation structurally and electrostatically hinders the binding of Asn430 of β-catenin, thus reducing the stability of the junctional complex, leading to E-cadherin internalization and degradation (Chen et al. 2016). Thus, these results imply that pro-migratory signaling cascade through CXCR3A may correlate with reduced E-cadherin whereas anti-migratory CXCR3B signaling may promote E-cadherin expression.

Direct link between CXCR3 and E-cadherin has not been reported and we hypothesized that CXCR3B mediates E-cadherin re-expression through PKA activation to facilitate metastatic cell seeding.

2.3.1 CXCR3B Increases E-cadherin Protein Level

In order to determine whether CXCR3 can directly affect E-cadherin expression, we firstly overexpressed CXCR3B in E-cadherin low DU145 prostate cancer cells. Overexpression of CXC3B was validated at both mRNA and protein level in which CXCR3B mRNA was up-regulated by 50-fold whereas CXCR3A remained unchanged (Figure 3a and b). Since there is no specific antibody for CXCR3A, only CXCR3B protein level was assessed in these CXCR3B-overexpressors. To validate this observation, we transfected DU145 cells with a different plasmid, pCMV-CXCR3B-flag tag and compared the expression of CXCR3A, CXCR3B and E-cadherin with lipofectamine-control transfectants. We verified that the overexpressors were positive for flag-tag (Figure 3e). Similar with previous results, plasmid transfection up-regulated CXCR3B at both mRNA and protein level but did not affect CXCR3A mRNA level (Figure 3c

and d). More importantly, E-cadherin was up-regulated at the protein level but not at mRNA in both of these transfectants.

2.3.2 CXCR3B is Elevated in DU145-Ecadherin^{high} Cells

DU145 cancer cells display heterogeneous E-cadherin expression. *In vitro* and *in vivo* characteristics of these cells are inherently different. The migration of DU145 E-cadherin^{high} (DU-high) cells was profoundly impeded (Putzke et al. 2011) and these cells were more resistant to chemotherapeutic drugs (Ma et al. 2016). To verify the correlation between CXCR3B and E-cadherin expression, we next compared the level of these proteins in DU145 E-cadherin^{low} (DU-low) and DU-high. No substantial differences were noted at mRNA level between DU-low and DU-high (Figure 4a). However, flow cytometry analysis on un-permeabilized cells showed high CXCR3B on the membrane for DU-high when compared to DU-low (Figure 4b). Additionally, cAMP level, downstream target of CXCR3B, was higher in DU-high than in DU-low even without 3-isobutyl-1-methylxanthine (IBMX) treatment which is a non-specific phosphodiesterase inhibitor that will raise intracellular cAMP level (Figure 4c).

We are currently validating our findings by knocking down CXCR3B in DU-high cells with CXCR3B shRNA. Initial finding suggested that knocking down CXCR3B resulted in reduced E-cadherin expression on DU-high cell membrane (Supplementary figure 1). However, further verification with technical replicates and proper control is still pending.

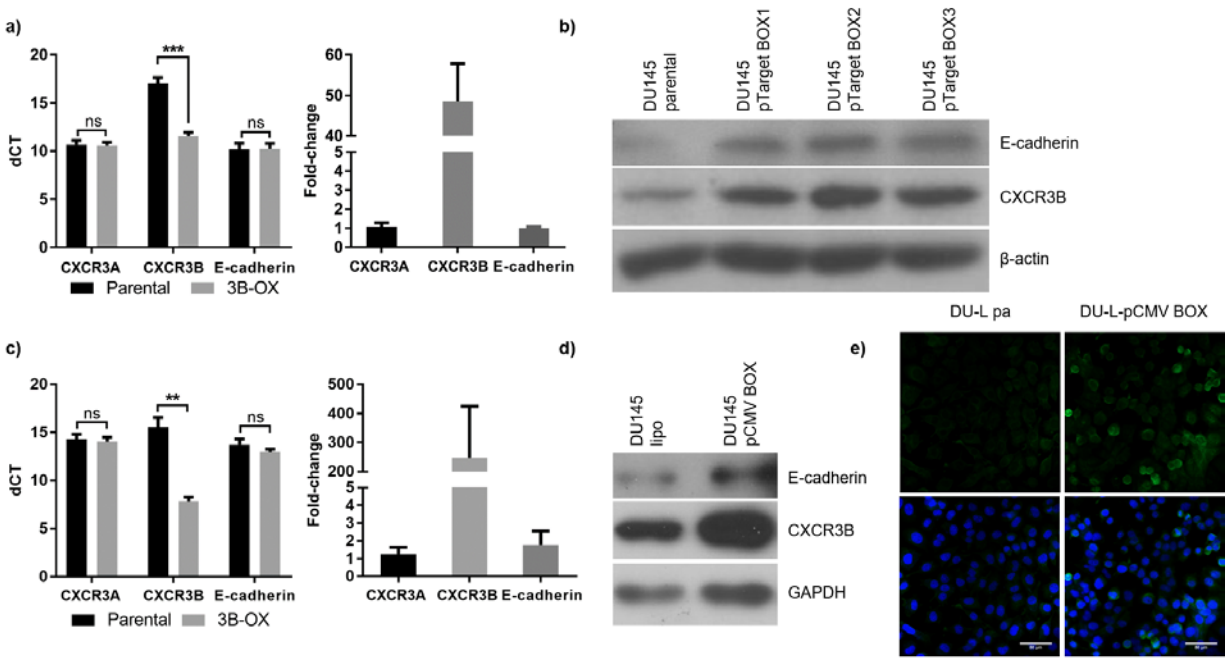


Figure 3: CXCR3B increases E-cadherin protein level.

Parental DU145 cells were transfected with plasmid (a,b) pTarget-CXCR3B and (c,d) pCMV-CXCR3B-flag. a,c) Average ΔCt and mRNA fold change of E-cadherin, CXCR3A and CXCR3B normalized to GAPDH with standard deviation, SD (n=4 for pTarget clone 2 in a and n=3 for pCMV in c). Student's t-test was used to test for statistical significance. P-value <0.01**, 0.001***. b,d) Total protein level of E-cadherin and CXCR3B. β-actin and GAPDH were used as loading control (n=2). e) Flag-tag immunofluorescence. Bar= 50µm.

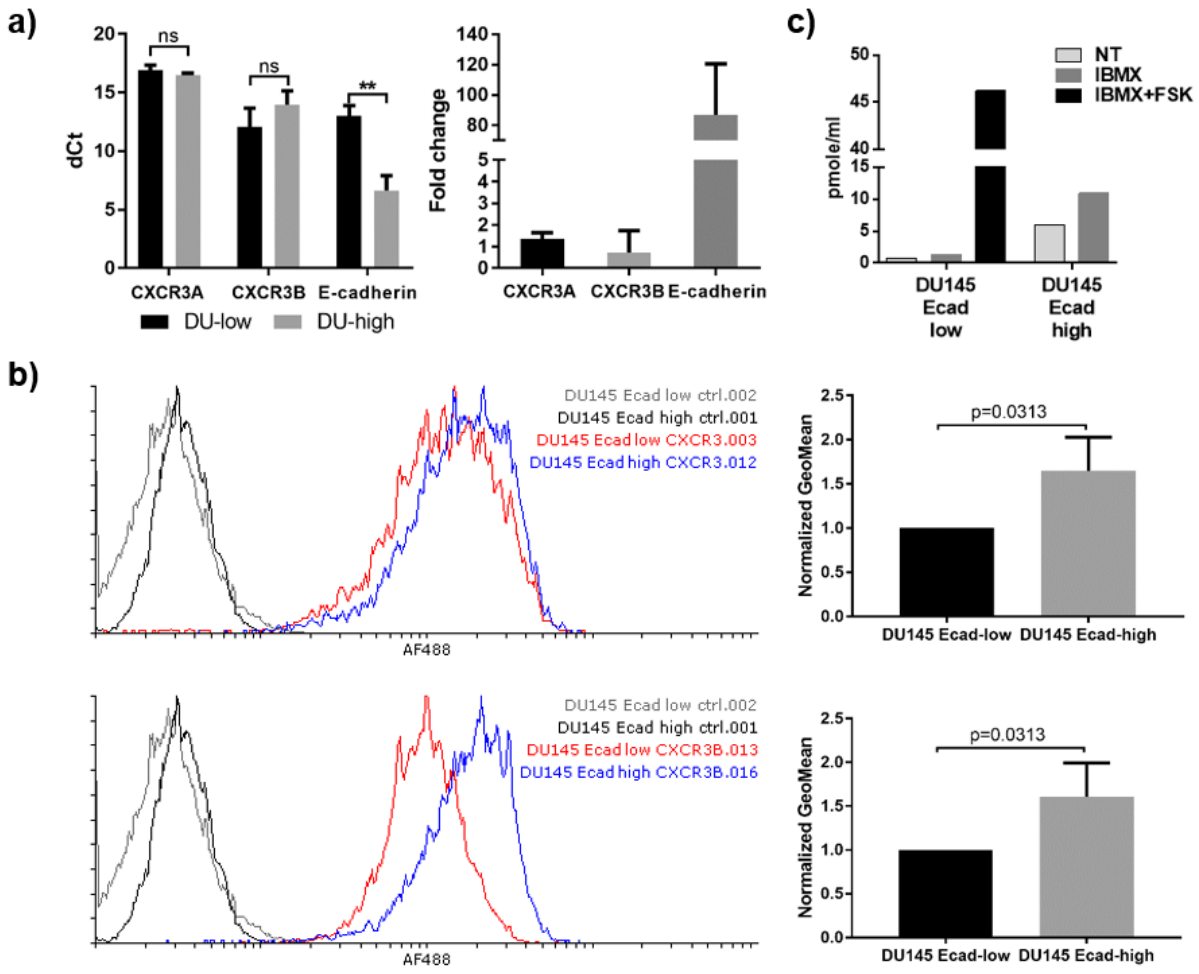


Figure 4: CXCR3B protein expression and activity are higher in DU-high cells.

a) Average ΔCt and mRNA fold change of CXCR3A, CXCR3B and E-cadherin normalized to GAPDH in parental DU-low and DU-high with SD (n=3). Student's t-test was used to test for statistical significance. P-value <0.01**. b) Average GeoMean of total CXCR3-AF488 and CXCR3B-AF488 expressed on unpermeabilized cells with SD (n=6). Wilcoxon matched-pairs signed rank test was used to test for statistical significance. c) Average cAMP level determined using ELISA. Forskolin (Fsk) treatment served as the positive controls (n=1).

2.3.3 DU145 Cells Increase CXCR3B Expression in Liver Metastases

Metastatic prostate cells and tissues have been shown to express low level of CXCR3B relative to CXCR3A (Wu, Dhir and Wells 2012) (Figure 3 and c). We previously reported that hepatocytes can induce E-cadherin expression in DU145 prostate cancer cells (Yates et al. 2007) and previous *in vitro* results suggest that CXCR3B correlates with E-cadherin expression. Thus, based on these observations, we hypothesized that the cancer cells would up-regulate CXCR3B expression in metastatic liver nodules. To test this hypothesis, we performed intrasplenic injection of DU-low cells and compared the level of CXCR3B protein expression in DU-low cells in the spleen and in the metastatic cells in the liver. As previously mentioned, almost all human cells including the hepatocytes and spleen cells express both isoforms of CXCR3. DU-low cells occupying most of the spleen were mostly negative for CXCR3B except for one mouse where DU-low cells express low-intermediate level of CXCR3B in the spleen. In the liver, metastatic DU-low formed multiple liver nodules with heterogenous CXCR3B levels but most of the nodules (77%) were positive for CXCR3B (Figure 5).

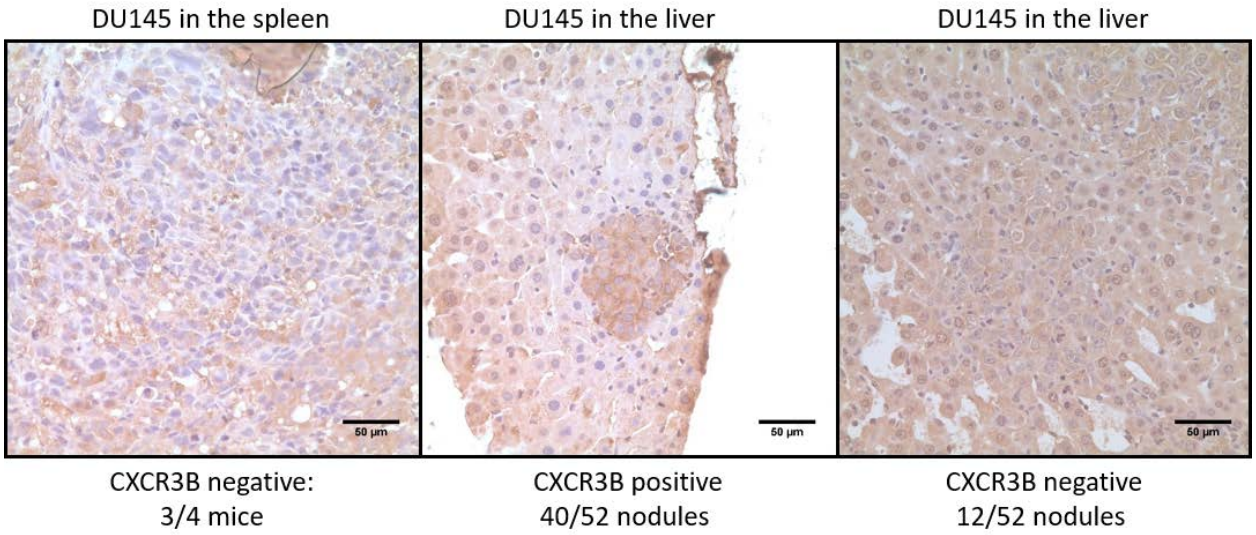


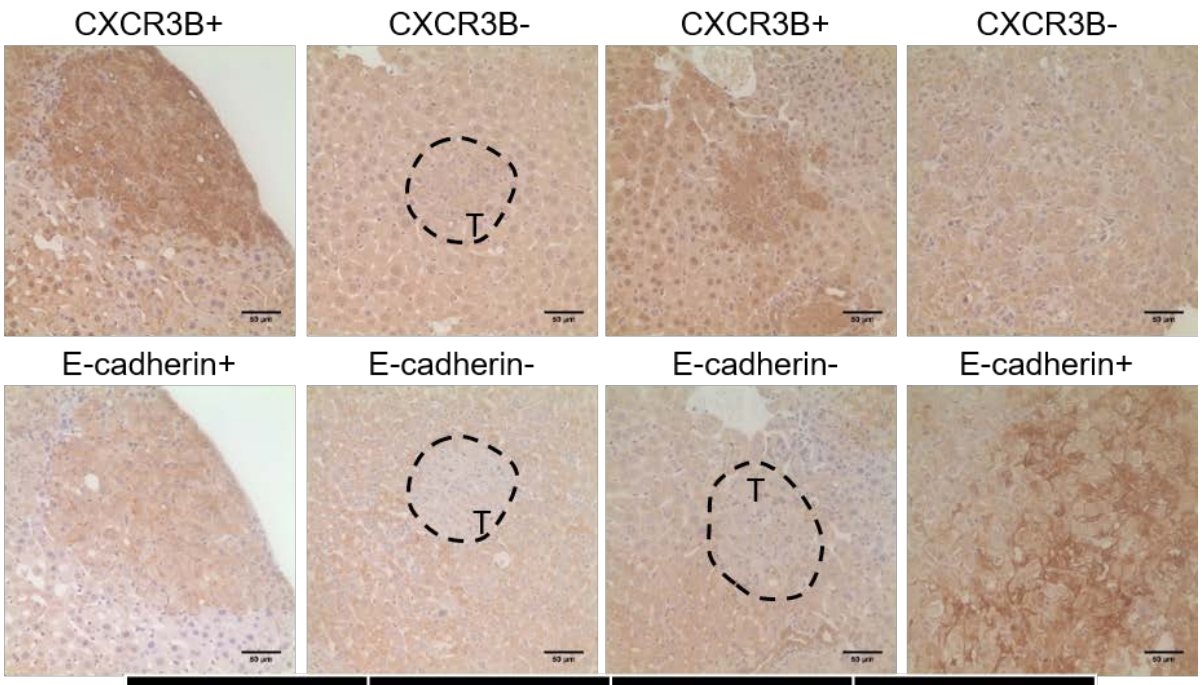
Figure 5: DU145 cells elevate CXCR3B expression in liver nodules.

Representative images of CXCR3B immunohistochemistry (IHC) on DU145 spleen and liver sections. IHC for CXCR3B was performed on the spleen and the derived liver metastases (n=4 mice). A representative image was acquired for the spleen sections whereas at least ten separate nodules were imaged for each liver section. The numbers of spleen and liver nodules that are positive or negative for CXCR3B were counted and documented. Bar= 50µm.

2.3.4 CXCR3B Correlates with E-cadherin in DU145 and PC3 Cells *In vivo*

Next, we performed immunohistochemistry on liver metastases of DU-low and PC3 cells to verify the correlation between CXCR3B and E-cadherin *in vivo*. Liver nodules of metastatic prostate cancer cells displayed heterogeneous expression of both CXCR3B and E-cadherin (Figure 6 and 7). The expression of CXCR3B and E-cadherin were grouped into positive or negative with most of the nodules were either CXCR3B-/E-cadherin- or CXCR3B+/E-cadherin+. Contingency table analysis revealed significant correlation between CXCR3B and E-cadherin in DU-low cells *in vivo* (Figure 6). Similar trend was also observed for PC3 liver metastases but borderline significant (Figure 7).

a)



b)

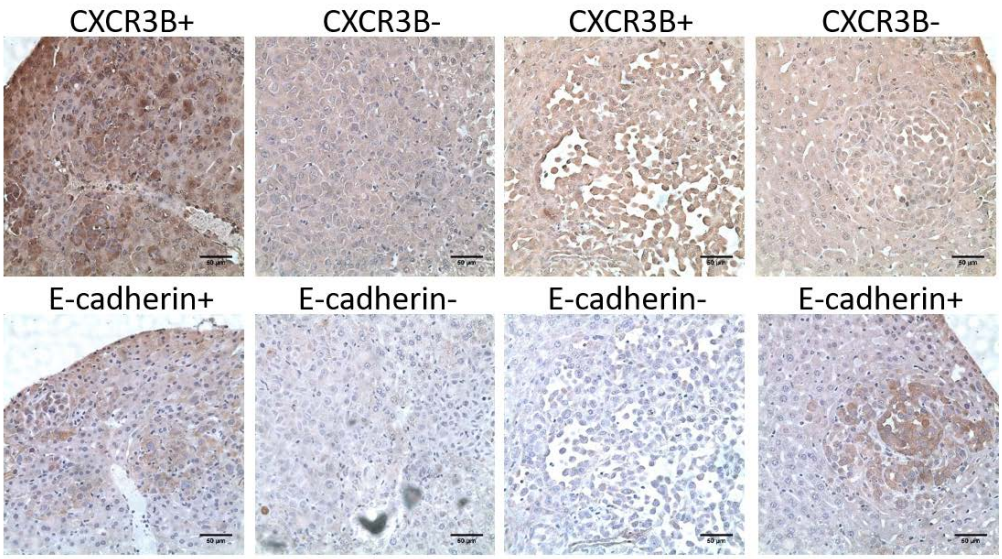
DU145-low	CXCR3B+	CXCR3B-	Total
E-cadherin+	29	3	32
E-cadherin-	11	9	20
Total	40	12	52

Fisher's exact test p-value: 0.0056

Figure 6: CXCR3B significantly correlates with E-cadherin in DU145 cells *in vivo*.

a) Representative images of CXCR3B and E-cadherin immunohistochemistry on DU-low liver sections. At least 10 liver nodules were imaged for each liver section (n=3 mice). Bar= 50µm. b) 2x2 contingency data analysis using Fisher's exact test.

a)



b)

PC3	CXCR3B+	CXCR3B-	Total
E-cadherin+	14	5	19
E-cadherin-	3	7	10
Total	17	12	29

Fisher's exact test p-value = 0.046

Figure 7: CXCR3B significantly correlates with E-cadherin in PC3 cells *in vivo*.

a) Representative images of CXCR3B and E-cadherin immunohistochemistry on DU-low liver metastases. At least 10 liver nodules were imaged for each liver section (n=2 mice). Bar= 50µm. b) 2x2 contingency data analysis using Fisher's exact test.

2.3.5 CXCR3B Expression in Metastatic Liver Nodules Increases Chemoresistance.

Next, we sought to determine the functional aspect of CXCR3B expression in DU145 liver nodules. We previously published that increased expression of E-cadherin in prostate and breast cancer cell lines increased cell resistance against chemotherapeutic drugs *in vitro* and *in vivo* (Chao et al. 2012, Ma et al. 2016). Thus, we hypothesized that the correlation between CXCR3B and E-cadherin in these metastatic liver nodules also promoted chemoresistance. The cancer cells were challenged with paclitaxel two weeks after inoculation. The nodules were stained for

CXCR3B, E-cadherin and cleaved caspase-3, a marker of apoptosis. Interestingly, we found that most of these CXCR3B/E-cadherin double positive nodules were also cleaved caspase-3 negative indicating elevated resistance against the chemo drug (Figure 8).

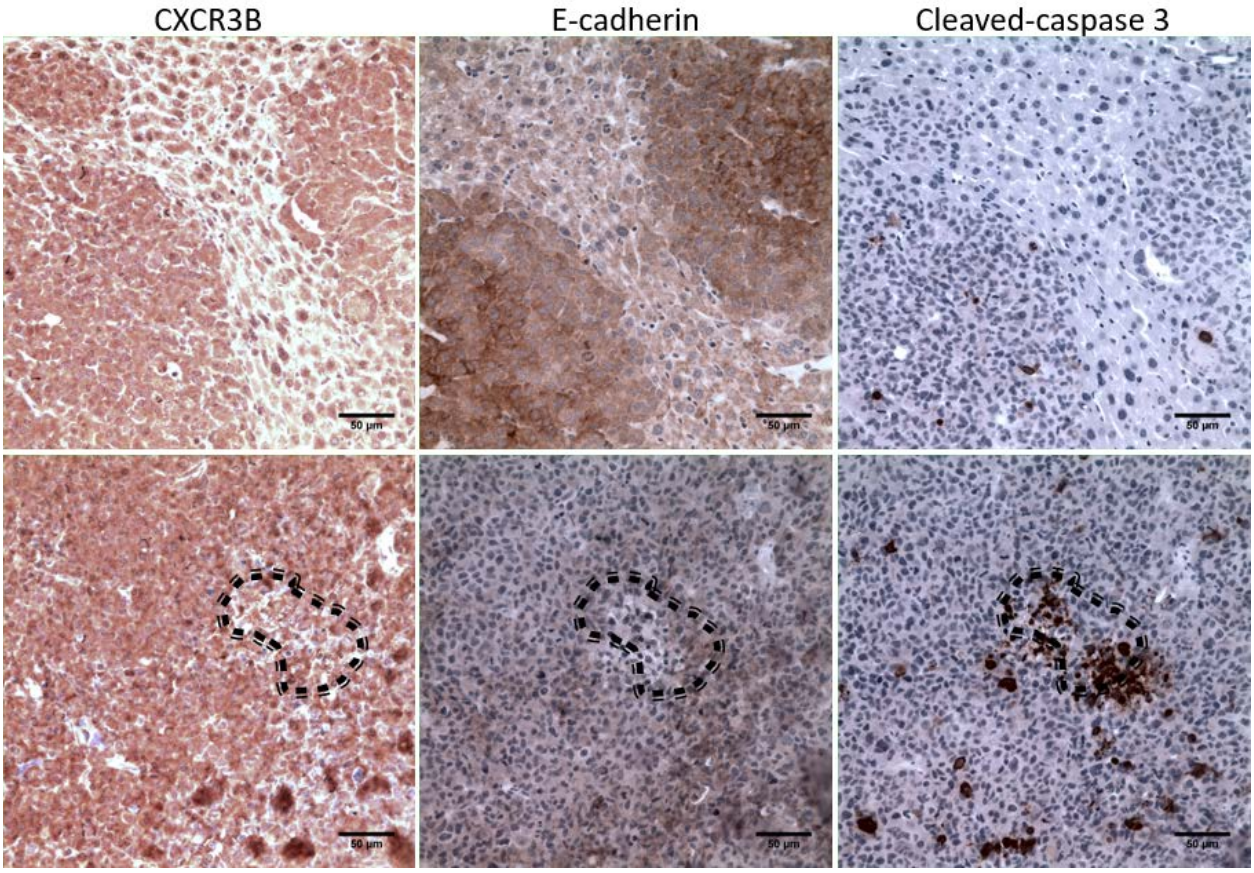
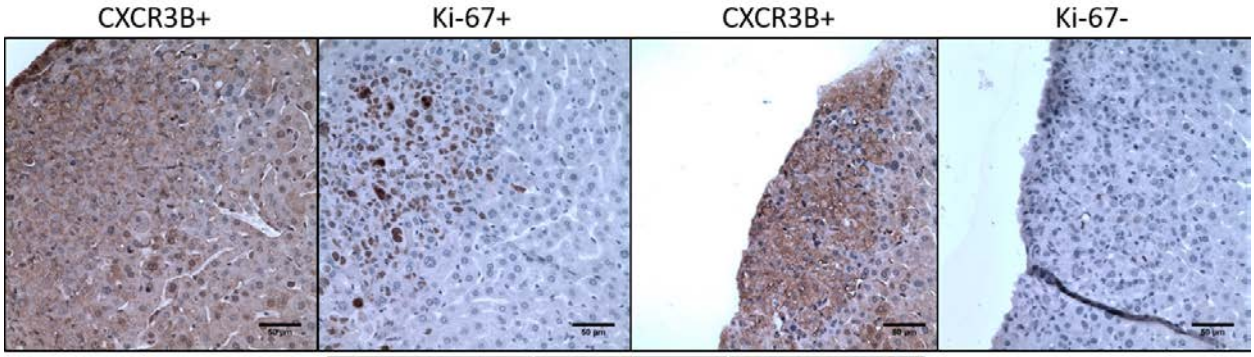


Figure 8: CXCR3B expression promotes chemoresistance in DU-low liver nodules.

Representative images of CXCR3B, E-cadherin and cleaved caspase 3 immunohistochemistry on DU-low liver nodules. Top panels show positive CXCR3B and E-cadherin and negative cleaved caspase-3 expression in DU-low liver nodules. Bottom panels show negative CXCR3B and E-cadherin expression and positive cleaved caspase-3 expression in the dotted circles. Bar= 50µm.

2.3.6 Elevated CXCR3B does not Promote Cancer Dormancy

CXCR3B overexpression in breast cancer MCF7 cells was found to increase P38 phosphorylation and reduce ERK phosphorylation (Balan and Pal 2014), a signature of dormant tumor cells (Aguirre-Ghiso et al. 2003). CXCR3B has also been noted to limit cancer growth. We theorized that CXCR3B expression induced cancer cells to undergo dormancy, rendering the cells insensitive to chemotherapeutic drugs. To test the hypothesis, Ki-67 protein level was examined in DU-low liver nodules. Ki-67 is a protein that is present in the nucleus of actively dividing cells (G1, S, G2 and M phase) but not in quiescent cells (G0 phase). Ki-67 staining revealed that both CXCR3B negative and positive tumor nodules were mostly positive for Ki-67, indicating that these nodules were not dormant.



DU145-L	CXCR3B pos	CXCR3B neg
Ki67 pos	26	6
Ki67 neg	6	0

Fisher's exact test= 0.5623

Figure 9: CXCR3B expression does not promote tumor dormancy in the liver.

Representative images of Ki-67 and CXCR3B positive and negative staining on DU-low liver nodules. at least ten liver nodules were imaged for each section (n=4 mice). 2x2 contingency analysis with Fisher's exact test was performed for statistical significance. Bar= 50µm.

2.3.7 Discussions and Conclusions

CXCR3 signaling mediates disparate effects on cellular motility and migration through CXCR3A and CXCR3B isoforms. CXCR3A is the predominant isoform in immune cells and is crucial for recruitment whereas CXCR3B is the dominant isoform in epithelial cells that regulates cell motility. CXCR3B is down-regulated in metastatic prostate cancer cells and overexpression of this isoform blocked ligand-induced cell migration (Wu, Dhir and Wells 2012). Similarly, E-cadherin is down-regulated in high grade tumors and metastatic cancer cells (Umbas et al. 1992) and re-expression of E-cadherin also reduced or blocked cancer migration (Chen et al. 1997).

Herein, we, for the first time, demonstrated that CXCR3B expression correlated with E-cadherin expression where overexpression of CXCR3B led to E-cadherin up-regulation in DU-low cells and CXCR3B knocked-down diminished membranous E-cadherin in DU-high. We then validated our observation *in vivo* where CXCR3B was up-regulated and significantly correlated with E-cadherin expression in DU-low and PC3 liver metastases. Functionally, CXCR3B expression appeared to elevate chemo-insensitivity in DU-low liver nodules.

Previous works on CXCR3B isoform mainly focused on its roles in limiting cancer growth and proliferation or in inducing cancer cell death. This is the first study to establish the correlation between CXCR3B and E-cadherin in tumor metastasis setting. However, there are several findings that require further discussion and validations. First of all, we did not observe elevated E-cadherin gene expression following CXCR3B overexpression. E-cadherin expression in cancer cells is regulated via two known mechanisms thus far. In prostate cancer cells, E-cadherin is post-transcriptionally regulated following EGFR activation that leads to catenin phosphorylation and junctional complexes instability. We are currently investigating the

mechanism for CXCR3B-mediated E-cadherin expression and we postulate CXCR3B inhibit catenin phosphorylation to maintain the stability of junctional complexes. Secondly, the translation of these findings to other cancer types may be limited as E-cadherin promoter hypermethylation could be the main mechanism for E-cadherin downregulation in other metastatic cancer cells. Our initial finding showed that CXCR3B overexpression seemed to not affect E-cadherin level in MDA-MB231 cancer cells at both mRNA and protein (Supplementary figure 2). Current data, however, are inconclusive as direct measurement of promoter methylation in the CXCR3B overexpressing cells is still pending.

We also reported here that CXCR3B expression resulted in increased E-cadherin expression and elevated chemo-resistance *in vivo*. However, increased chemo-resistance might be solely due to hepatocyte-mediated E-cadherin re-expression as previously published (Chao et al. 2012, Ma et al. 2016). Therefore, it is plausible that CXCR3B effects could be secondary to liver protection.

We also found that increased CXCR3B did not induce the cancer cells to undergo dormancy even though CXCR3B overexpression in a different cell line resulted in low pERK/pP38 dormancy signature. A plausible explanation for this observation is that the *in vivo* model used in this study was not an appropriate dormancy model. Previous *in vivo* dormancy models utilized cancer clones with limited proliferative capacity or poorly metastatic cancer cells to induce latent cancer cells *in vivo* or manipulated experimental methodology by injecting low number of cancer cells to promote dormancy (Naumov et al. 2002, Panigrahy et al. 2012). The presence of dormant cells in our *in vivo* model may be masked by the overwhelming numbers of metastatic cells. Moreover, the ratio of phosphor-ERK and phosphor-P38 has not been validated

in CXCR3B-overexpressing DU-low cells. Further analysis with appropriate model may be required to truly establish the connection between CXCR3B and tumor dormancy.

2.3.8 Future works

The overarching hypothesis for this study is that CXCR3B mediates E-cadherin re-expression through PKA activation to facilitate metastasis cell seeding.

Thus, to completely test this hypothesis, we will need to validate whether the activation of CXCR3B with IP-10/CXCL10 and PF4/CXCL4 increases PKA level and importantly promotes E-cadherin expression in DU-low and PC3 cells. IP-10 and PF4 are chosen because IP-10 is moderately expressed in the liver whereas MIG and I-TAC are lowly expressed in the liver as mentioned in preceding sections. We are also interested in PF4 as it specifically activates CXCR3B only at low concentration. CXCR3B activation will be validated via cAMP ELISA and we will measure membranous E-cadherin re-expression via immunofluorescence. cAMP inhibitor H89 will serve as the negative control.

Secondly, we would like to verify our findings with the effects of CXCR3A activation in DU145-high cells. We postulate that this will decrease E-cadherin protein level and this finding will substantially bolster our current hypothesis.

Thirdly, to test the final part of our hypothesis, we will determine cancer seeding efficacy between DU-high and DU-high shCXCR3B cells onto hepatocyte monolayer *in vitro* as previously established (Chao et al. 2012). Briefly, hepatocyte monolayer will be seeded 24 hours prior to RFP-labeled cancer cell seeding and RFP signal will be measured four hours post seeding and heterotypic E-cadherin interaction will be assessed via immunofluorescence.

Lastly, although we initially examined this observation in the context of prostate cancer liver metastases, we were not able to obtain human specimens of paired primary-liver metastases for prostate cancer patients. As previously mentioned in Chapter 1, prostate cancer liver metastases are rarely diagnosed but affect up to 48% of the patients at autopsy. Thus, prostate cancer liver metastases specimens are scarce. We, fortunately, were able to obtain some primary-liver metastases pairs from colorectal cancer patients and we will use these samples to validate *in vitro* and *in vivo* our observations with these clinical specimens.

2.3.9 Conclusions

Here, we report a novel association between CXCR3B and E-cadherin expression. During liver colonization, both CXCR3B and E-cadherin are up-regulated. This correlation may improve chemoresistance and is postulated to also enhance cancer seeding. Further works are required to truly establish the mechanism(s) and the functional effects of CXCR3B overexpression in cancer cells. Inhibition of CXCR3B may be useful to block liver or distant organ colonization.

Supplementary information, materials and methods for this chapter are available in Appendix B.

3.0 CANCER CELL SURVIVAL IN LIVER MICROENVIRONMENT

Metastasis, although deadly, is a very inept process. As discussed in the previous chapters, metastasis requires a myriad of processes and changes on the cancer cells. In addition, complex interactions network within tumor microenvironment also influences the formation of metastatic nodules. In general, there are three main outcomes for the cancer cells following extravasation from the blood vessels. First of all, some of the invading cancer cells will undergo apoptosis due to lack of tumor angiogenesis to supply nutrients (Naumov, Akslen and Folkman 2006) or elimination by immune cells in the distant organs (Teng et al. 2008). Secondly, some of the highly aggressive cancer cells can rapidly proliferate even in the face of all these obstacles. Lastly, a subpopulation of the cancer cells may undergo growth arrest. Experimental observations point to the survival of cancer cells in the new microenvironments as the rate-limiting step for metastasis (Valastyan and Weinberg 2011). Quiescent cancer cells are of interest as they can evade the rate-limiting step of metastasis only to come back years later. This chapter will focus on tumor dormancy and the factors that trigger emergence.

3.1 CANCER CELL DEATH AND METASTATIC PROLIFERATION

The first outcome following extravasation is cell death. Naumov et al demonstrated that although most of the injected cancer cells could survive in the circulation and extravasate into

mouse liver, up to 1% of poorly metastatic cancer cells and up to 4% of highly metastatic cancer cells underwent apoptosis (Naumov et al. 2002). Using a real-time imaging method to detect brain metastases, Kienast et al reported that 5-35% of the extravasated cancer cells underwent apoptosis (Kienast et al. 2010). In addition, apoptosis continued even in the newly form micrometastases (Luzzi et al. 1998). Cell death induction is due to the activity of the organs' resident cells or the infiltrating immune cells. For example, upon cancer cell invasion, reactive astrocytes express high level of Fas ligand (FasL) and convert plasminogen into plasmin. The plasmin then cleaves membrane-bound FasL into soluble FasL that induces apoptosis of the metastatic cells (Valiente et al. 2014). Similarly, in the liver, Kupffer cells limit cancer growth by phagocytosing the extravasated cancer cells, secreting TNFα and mobilizing other immune cells to target the cancer cells (Brodt 2016).

The second outcome following cancer cells extravasation is aggressive proliferation to form micrometastases and macrometastases. However, this is actually a rare event where experimental evidence showed that less than 7% of extravasated melanoma and lung carcinoma form macrometastases in the distant sites (Luzzi et al. 1998, Kienast et al. 2010). These rapidly proliferating macrometastases, as indicated by high percentage of Ki67 positive cells within the tumor nodules (Luzzi et al. 1998, Naumov et al. 2002) are hypothetically sensitive to currently chemotherapy drugs which serves as the basis for chemotherapy in metastatic patients but can develop resistance within a short period of times.

3.2 CANCER DORMANCY

The third outcome for the extravasated tumor cells is tumor dormancy which will be the main focus of this chapter. Dormancy is the cause of delayed recurrence after patients are seemingly "cured" from primary tumors. Research into tumor dormancy is gaining interest since the tumors are particularly difficult to detect and target.

3.2.1 Dormancy: Definition

Currently, there is no consensus definition for tumor dormancy. In clinical context, tumor dormancy refers to asymptomatic and undetectable microscopic tumor cells or nodules that emerge locally or in distant organs following a long latency period after primary treatments (Hayat 2013). The latency period for dormant tumor is typically beyond 5 years and could be up to 20 years (Aguirre-Ghiso 2007). In experimental setting, dormant tumor may refer to 1) the growth tumor mass that are kept in check by balanced proliferation and cell death or 2) the quiescent tumor cells that enter the G0 of cell cycling stage

3.2.2 Dormancy: Clinical and Experimental Evidence

Clinical evidence for tumor dormancy has long been documented. Demicheli et al reported a bimodal recurrence pattern in patients treated with mastectomy only. The bimodal pattern was more pronounced in patients with distant metastases when compared to local-regional metastases. The first peak of recurrence was at around 18 months after the surgery. This could be due to highly metastatic and aggressive tumor cells or failure in completely resecting the tumor

cells. The second peak occurred at around 60 months (~5 years) after the surgery and was postulated to be due to tumor dormancy. This bimodal pattern was verified with a second set of patients consisting of 2233 patients treated with lumpectomy (Demicheli et al. 1996) and in a Norwegian cohort (Dillekås et al. 2014). Karrison et al reported that although they did not observe a similar bimodal pattern, they found a significant excess mortality rate up to 20 years post primary treatment and argued for breast cancer curability to be beyond 20-25 years (Karrison, Ferguson and Meier 1999). Another study reported that about 5% of patients treated with mastectomy ± radiation therapy suffered recurrence, mostly at distant sites, more than 10 years post-surgery (Lee 1985). Lastly, Fisher et al reported that the recurrence percentage of patients treated with mastectomy alone, lumpectomy alone or lumpectomy plus irradiation after five years and beyond was about 10%, 13% and 13%, respectively (Fisher et al. 2002).

Experimentally, using live-cell imaging to track tumor cells, Kienast et al reported that for lung carcinoma and melanoma brain metastases, 3-4% of the cells underwent long term dormancy (Kienast et al. 2010). Naumov et al observed that 80% of poorly metastatic murine mammary cancer cells remained undivided whereas 30% of highly metastatic murine mammary cancer cells were detected as undivided, solitary cells in the liver after 25 days (Naumov et al. 2002). Similarly, 40% of melanoma cells presented as solitary cells two weeks following extravasation into the liver (Luzzi et al. 1998).

3.2.3 Dormancy: Mechanisms

Several mechanisms have been proposed to contribute to tumor dormancy. The first mechanism is regulated tumor growth via immunosuppression. Lymphocyte infiltration into tumor nodules to limit tumor growth has been well documented (Teng et al. 2008). The role of

immunosuppression in tumor dormancy was first described using B-cell lymphoma (BCL_1)-derived immunoglobulin immunization which protected mice from splenomegaly for beyond 60 days and slowly relapsed over a period of 610 days (Vitetta et al. 1997). Importantly, emergence from tumor dormancy in this BCL_1 model was achieved with T-lymphocytes depletion or IFNγ neutralization (Farrar et al. 1999).

The second mechanism for tumor dormancy is through the depletion of nutrient and pro-tumor factors due to lack of tumor angiogenesis. Initial study revealed minimal intratumoral CD31 expression in microscopic tumors (1-2mm) while macrometastases showed well-organized vessel, indicating the role of angiogenesis in driving tumor escape from dormancy (Naumov, Akslen and Folkman 2006). Consistently, decreased expression of thrombospondin-1, an angiogenesis inhibitor, resulted in sustained dormancy of breast cancer in the lung (Ghajar et al. 2013). Spontaneous angiogenic switch had been documented to trigger tumor growth following prolong periods of dormancy (months) in several mouse models (Naumov, Akslen and Folkman 2006).

Lastly, tumor dormancy can also be achieved at cellular level in which the growth of individual cancer cells is arrested at G0/G1 phase. Several reports suggest that quiescent cells are the main contributor to tumor dormancy. Injection of both poorly (D2.0R) and highly metastatic (D2A1) cell lines resulted in a large number of solitary, dormant and Ki67 negative cells in the liver ten days after cell inoculation (Naumov et al. 2002). Similarly, less than 3% of solitary melanoma cells found in the liver two weeks after injection were positive for either Ki67 or TUNEL, indicating that balanced cell death and proliferation was not the main contributor to tumor dormancy (Luzzi et al. 1998). Modeling cancer growth with *in silico* Markov chain Monte Carlo mathematical model, aiming to assess the contribution of balanced proliferation in clinical

tumor dormancy, revealed a narrow survival probability window (49.7-50.8%) for the cancer cells to remain dormant after 1218 cell divisions. Based on this model, majority of the initial 1000 metastatic cells were predicted to die out or grow out if tumor cells constantly undergoing balanced cell death and proliferation for a period of 5-10 years (Taylor et al. 2013).

Dormant tumor nodules in breast cell lines and patient samples have been typed for transcriptomes and the following molecules are upregulated with dormancy: STAT3, SREBF1, IGFBP5, BHLHE41, NR2F1 and SOX9, whereas the following are downregulated: IL8, NT5E, PLAT, FOSL1 and ODC1. Specifically, BHLHE41 and NR2F1 were verified to be crucial for tumor dormancy (Kim et al. 2012). NR2F1, an orphan nuclear receptor, is downregulated in human cancers, correlates with prolonged disease-free survival and is part of retinoic acid receptor (RAR) pathway. Further analyses revealed that NR2F1 was highly expressed in dormant D-Hep3 cells but hypermethylated in rapidly-proliferating T-Hep3 and FaDu cells. NR2F1-induced dormancy was dependent on SOX9, a downstream target of NR2F1 and is known to be involved in regulating cell growth and differentiation (Sosa et al. 2015).

3.2.4 Dormancy: Modeling Human Tumor Dormancy *Ex vivo*

One of the main obstacles in studying tumor dormancy is lack of reliable human models. Mouse models are a great tool to study tumor dormancy. Spontaneous tumor dormancy models in mice have been reported (Luzzi et al. 1998, Kienast et al. 2010, Ghajar et al. 2013, Magnus et al. 2014). However, animal models poorly recapitulate human pathology (Francia et al. 2011). In addition, in some cases, there are some species-specific differences such as the expression of cytochrome p450 and nuclear factors that are important in assessing drug metabolism and toxicity and may affect cancer growth in the liver (Androutsopoulos, Tsatsakis and Spandidos

2009, Uno et al. 2009). Moreover, most mouse models used in cancer research are immunocompromised thus eliminating one of the important factors in tumor growth induction and regulation.

Modeling metastases with 3D organotypic *ex vivo* culture systems might address some of the shortcomings of animal models. Thus far, there are several systems available to model metastases to the liver, bone, female reproductive tract, lung, and brain (Bersini et al. 2014, Clark et al. 2017, Xiao et al. 2017). These models are equipped with various mechanical and electronic components and employ organ-specific human cells to accurately mimic human physiology such as menstrual cycle and drug metabolism, thus engendering more valuable information than animal models (Tsamandouras et al. 2017, Xiao et al. 2017). Moreover, these models allow for effortless real-time monitoring and early time point analysis to detect singular cancer cells. Importantly, some of these models are tunable for multiple organs construction (Skardal et al. 2016, Xiao et al. 2017). The 3D *ex vivo* liver microphysiologic system LiverChip^{TM}, has been shown to promote spontaneous tumor dormancy in the liver which can then be activated to grow out with physiologic levels of growth factors (Wheeler et al. 2014, Clark et al. 2017). We employed this model in our subsequent study.

3.2.5 Dormancy: Emergence

Several factors and mechanisms driving tumor escape from dormancy have been described. Initiation of angiogenesis, mediated by tissue factor and epoxyeicosatrienoic acids among other factors, drives tumor cells emergence from dormancy (Panigrahy et al. 2012, Magnus et al. 2014). Tumor angiogenesis also provides dormant cancer cells with periostin and transforming growth factor-beta (TGFβ) to grow (Ghajar et al. 2013). In addition to angiogenesis-related

signals, activation of ERK, downstream of urokinase plasminogen activator receptor (uPAR) activity leads to breast, prostate and skin cancer cells outgrowth from dormancy on chick chorioallantoic membrane (Aguirre-Ghiso et al. 2003). Escape from dormancy was also documented in cancer cells cultured on collagen-I enriched surfaces to mimic fibrotic environment. The outgrowth was potentiated through SRC-ERK-induced proliferation (Barkan et al. 2010). However, these factors have not been validated with human samples.

Inflammation has long been postulated to be associated with tumorigenesis (Coussens and Werb 2002). Elevated concentrations of C-reactive protein and serum amyloid-A were found to be associated with reduced overall and disease-free survival in breast cancer patients, independent of race and tumor stage at diagnosis (Pierce et al. 2009). Cancer patients with a history of rheumatoid arthritis (RA) show worse prognosis and overall survival than those without RA history, especially for patients with breast cancer, small intestinal cancer and squamous cell carcinoma (Ji et al. 2011). To elucidate the mechanism of inflammation-induced metastasis, spontaneous autoimmune arthritic mice were inoculated with 4T1 murine breast cancer cells and showed higher bone and lung metastases than in control mice. This effect was potentiated by mast cells through c-kit-SCF interaction that increased cancer migration and homed the migrating cancer cells to bone and lung (Das Roy et al. 2013). Increased pro-inflammatory cytokines such as TNF, IL-6 and IL-17 were also reported in this model resulting in three-fold increase of lung metastasis (Das Roy et al. 2009).

This chapter will focus on two pro-inflammatory chemokines: CXCL8/interleukin-8 (IL-8) and CCL2/monocyte chemo-attractant protein 1 (MCP-1). IL-8 is one of the pro-inflammatory cytokines secreted by various cells primarily to recruit immune cells to an infection or injury site. IL-8 binds to CXCR1 and CXCR2 and the IL-8-receptor axis has been found to be implicated in

a variety of tumor types (Freund et al. 2003). MCP-1, as the name suggests, primarily serves as chemoattractant for immune cells and other cells expressing CCR2 receptor. Similar to IL-8, increased MCP-1/CCR2 expression has also been associated in several cancers including breast cancers (Fang et al. 2012).

3.3 STELLATE CELL-DERIVED IL8 PROMOTES CANCER ESCAPE FROM DORMANCY IN THE LIVER

Liver is one of the main metastatic sites for a number of cancer cells including the breast (Tabariès and Siegel 2011). However, research on breast cancer dormancy and metastasis in the liver is relatively scant when compared to bone or lung metastasis. We have previously reported that normal liver cells can impose a phenotypic reversion of the cancer-related epithelial-mesenchymal transition (EMT) back to epithelial phenotype during mesenchymal-epithelial transition (MET) on breast cancer cells (Chao, Shepard and Wells 2010). E-cadherin re-expression results in smaller tumor nodules and resistance against chemotherapy-induced apoptosis both *ex vivo* (Chao et al. 2012) and *in vivo* (Ma et al. 2016). These results suggest that cancer cells can undergo dormancy in the liver.

The liver consists of hepatocytes and non-parenchymal cells (NPC) of several different cell types including liver endothelial cells, Kupffer cells and hepatic stellate cells (HSCs). We previously published that co-culture with human primary NPC augmented breast cancer cells growth whereas co-culture with primary human hepatocytes only restricted cancer growth (Taylor et al. 2014). We further showed that stressed endothelial cells promoted tumor growth through epidermal growth factor receptor (EGFR) activation (Taylor et al. 2014), consistent with

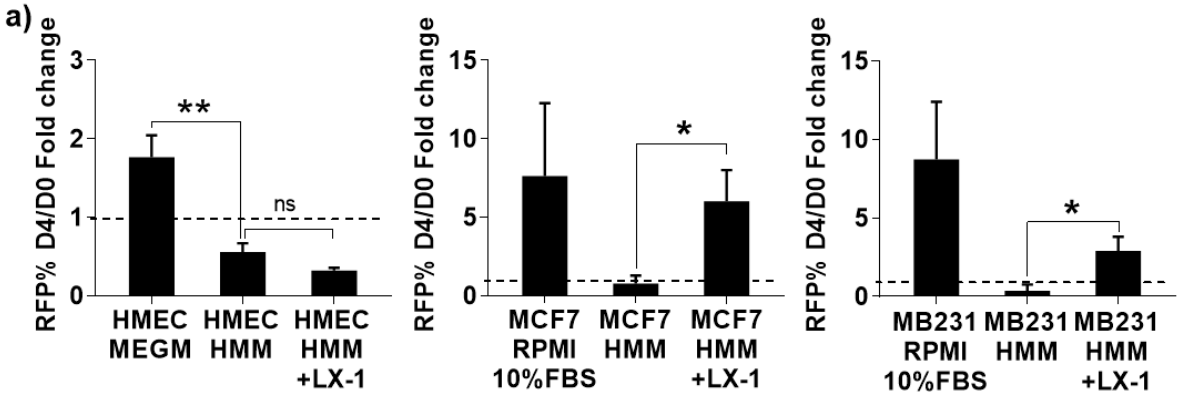
the finding of micrometastases outgrowth at the tips of angiogenic sprouts (Ghajar et al. 2013). Kupffer cells, the liver resident macrophages, display phenotype plasticity, with the alternative M2 macrophages, but not the M1 or M0, significantly promoted tumor growth (Yang et al. 2016). In this chapter, we queried the role of human HSC in tumor growth and tumor escape from dormancy in human liver microphysiologic setting.

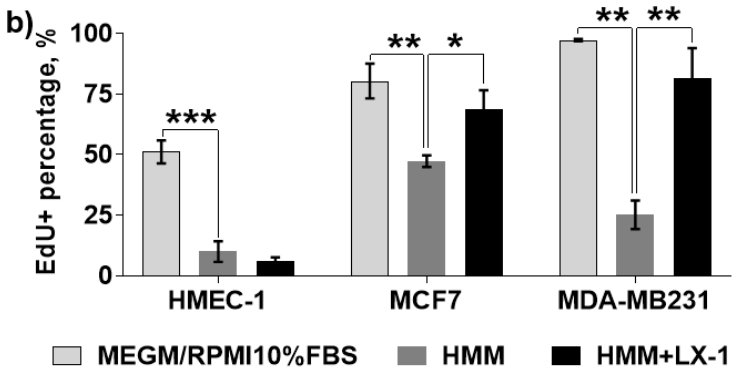
HSCs are stromal cells that store and process vitamin A in their non-activated state in normal livers (Friedman 2008). Upon activation following liver injury or infection, the cells transdifferentiate into myofibroblast and actively secrete collagen matrix, thus linking injury to chronic inflammation and fibrosis, a suitable setting for cancer outgrowth. Recent findings suggested that external cues firstly triggered Kupffer cell-mediated inflammatory responses that in turn activated the stellate cells to relay and exacerbate the inflammatory reactions to the hepatocytes (Fujita et al. 2016). We posited that these HSC-mediated inflammatory reactions can lead to tumor escape from dormancy in the liver.

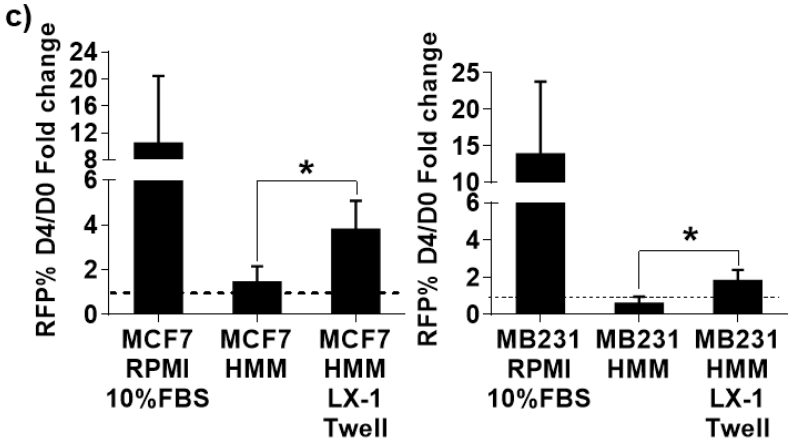
3.3.1 Hepatic Stellate Cells Drive Cancer Cell Growth and Proliferation

To determine whether hepatic stellate could indeed induce tumor growth and proliferation, RFP-labeled MCF7 and MDA-MB231 cancer cells were co-cultured with human hepatic stellate cells, LX1, in serum-free hepatocyte maintenance medium (HMM) *in vitro*. Cancer growth was measured by increased RFP signal (fold-change > 1) and proliferation was measured with 5-ethynyl-2'-deoxyuridine (EdU) assay which is a thymidine analog that is incorporated into DNA during S-phase synthesis. We found that MCF7 and MDA-MB231 growth were impeded in the minimal HMM media used for liver cultures, but the breast cancer cell numbers increased by 3 to 6-fold when co-cultured with LX1 cells (Figure 10a and b). The co-cultured samples also

showed 1.5 to 3-fold higher percentages of EdU positive cells when compared to HMM negative control (Figure 10c). This growth induction, however, was not observed in normal HMEC-1 breast cells, as the cells did not grow or proliferate in LX1 co-culture but elicited significant proliferation in the MEGM growth media, demonstrating cell viability (Figure 10). The stellate cells were not profoundly affected in the serum-free culture condition as some of the cells were still undergoing cell cycling as indicated by non-RFP EdU positive staining in these fibroblast-like cells after 4 days of culture (Figure 11a). We validated stellate cell-induced MCF7 growth with other stellate cell lines namely HSC-T6 and TWNT-1 cells. These two cell lines imparted significant growth advantage on MCF7 under restrictive serum-free HMM medium similar to endothelial cells-induced outgrowth (TMNK-1 cells) that we published previously (Taylor et al. 2014).


a)
RFP% D4/D0 Fold change
3
2
1
0
**
ns
HMEC MEGM
HMEC HMM
HMEC HMM +LX-1
15
10
5
0
*
MCF7 RPMI 10%FBS
MCF7 HMM
MCF7 HMM +LX-1
MB231 RPMI 10%FBS
MB231 HMM
MB231 HMM +LX-1


b)
EdU+ percentage, %
100
75
50
25
0
***
**
*
**
**
HMEC-1
MCF7
MDA-MB231
MEGM/RPMI10%FBS
HMM
HMM+LX-1


c)
RFP% D4/D0 Fold change
24
20
16
12
8
6
4
2
0
*
MCF7 RPMI 10%FBS
MCF7 HMM
MCF7 HMM LX-1 TwoII
25
20
15
10
6
4
2
0
*
MB231 RPMI 10%FBS
MB231 HMM
MB231 HMM LX-1 TwoII

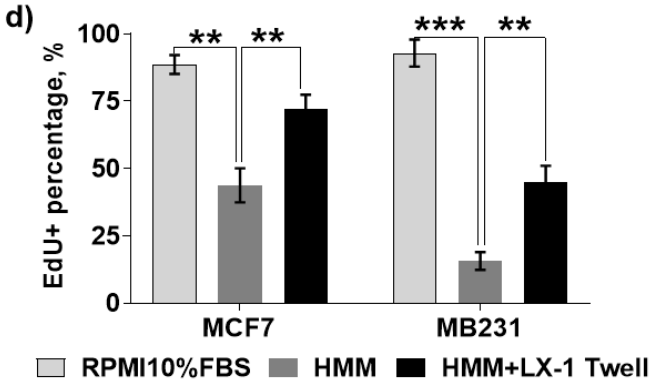

d)
EdU+ percentage, %
100
75
50
25
0
**
**
***
**
MCF7
MB231
RPMI10%FBS
HMM
HMM+LX-1 TwoII

Figure 10: LX1 promotes breast cancer cell growth and proliferation in vitro.

Serum-free HMM and complete media (RPMI 10%FBS or MEGM) served as negative and positive controls, respectively. Average RFP% area fold-change (10a) and average EdU incorporation percentage (10b) with standard deviation (SD) of normal breast HMEC-1, MCF7 and MDA-MB231 cells in LX-1 co-culture (n=3). Total number of cells counted/condition in (10b) is as follows: HMEC-1 MEGM= 262 cells, HMEC-1 HMM= 140 cells, HMEC-1 LX1= 175 cells, MCF7 RPMI 383 cells, MCF7 HMM= 92 cells, MCF7 LX1= 376 cells, MB231 RPMI= 749 cells, MB231 HMM= 86 cells and MB231 LX1= 533 cells. Average RFP % area fold change (10c) and average EdU incorporation percentage (10d) with SD of MCF7 and MDA-MB231 in LX-1 transwell separate culture. n=4 for MCF7 and n=3 for MDA-MB231. Total number of cells counted/condition is as follows: MCF7 RPMI= 1031 cells, MCF7 HMM 212 cells, MCF7 LX1 Twell= 463 cells, MB231 RPMI= 979 cells, MB231 HMM= 138 cells and MB231 LX1 Twell= 183 cells.

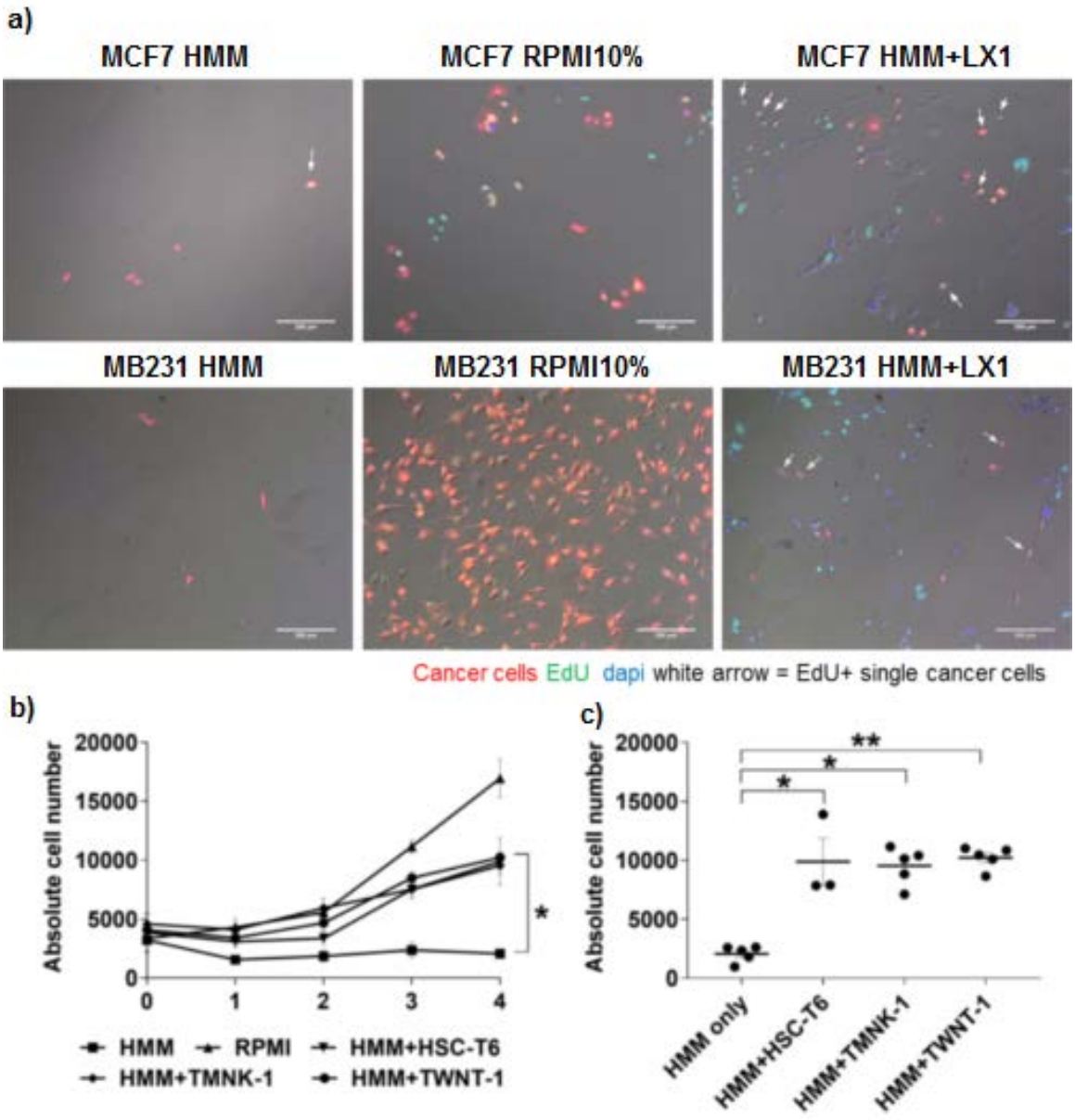


Figure 11: Individual cancer cells survive and proliferate in stromal cells co-culture after 96 hours.

a) Day 4 representative images of MCF7 (top row) and MDA-MB231 (bottom row) co-cultured with LX1 cells. Proliferating individual cancer cells were marked with white arrows. Scale bar= 200µm. b, c) Flow cytometry analysis of MCF7 growth in co-culture conditions with complete RPMI and serum-free HMM as positive and negative controls, respectively. b) Average absolute MCF-7 count in different co-culture conditions with standard error of the mean (SEM) for n=4. b) Kruskal-Wallis one-way ANOVA and c) multiple comparison t-tests were used to compute the statistical significance. P-values <0.05* and <0.01**.

3.3.2 Activated Stellate Cells Stellate Cell Impart Higher Proliferative Induction than Quiescent Stellate Cells

In normal livers, hepatic stellate cells are in a quiescent/non-activated state. Activation induces the stellate cells to undergo several major changes including increased proliferation and contractility, fibrogenesis and loss of retinoid. All hepatic stellate cell lines under normal culture on plastic surfaces, including LX1 and LX2 cells, are in the activated state, marked by the presence of αSMA fibers and pro-collagen 1 and HSP47 gene expression (Figure 12 and 13). In order to determine whether the activation state of the hepatic stellate cells affects tumor induction, the stellate cells were cultured on laminin-coated soft (0.3kPa) and stiff (40kPa) polyacrylamide (PA) gels and the conditioned-media from these two conditions were used to culture MCF7 and MDA-MB231 cells. LX-1 cells could not properly revert and survive on the soft gels (unpublished observation). So, we opted to use LX2 cells which were derived from LX1 clones that are more stable and could grow in low serum conditions (Xu et al. 2005). We firstly verified that LX2 cells also exerted similar growth and proliferative induction on the cancer cells but not on the normal HMEC-1 cells (Figure 14a and b). The LX2 cells reverted to non-activated state when culture on laminin-coated 0.3kPa PA gel as marked by reduced expression of lamin A/C, less cell spreading and lacked of and disorganized α-SMA fibers when compared to the cells cultured on 40kPa gels (Figure 14c and d). Interestingly, cancer cells cultured with conditioned-media from soft 0.3kPa gel culture failed to significantly increase the percentage of EdU incorporation in MCF7 and MDA-MB231, unlike those cultured in the conditioned media from the stiff 40kPa gels (Figure 14e). Thus, our results indicated that the HSC-mediated growth induction was due to soluble factors secreted by the activated stellate cells.

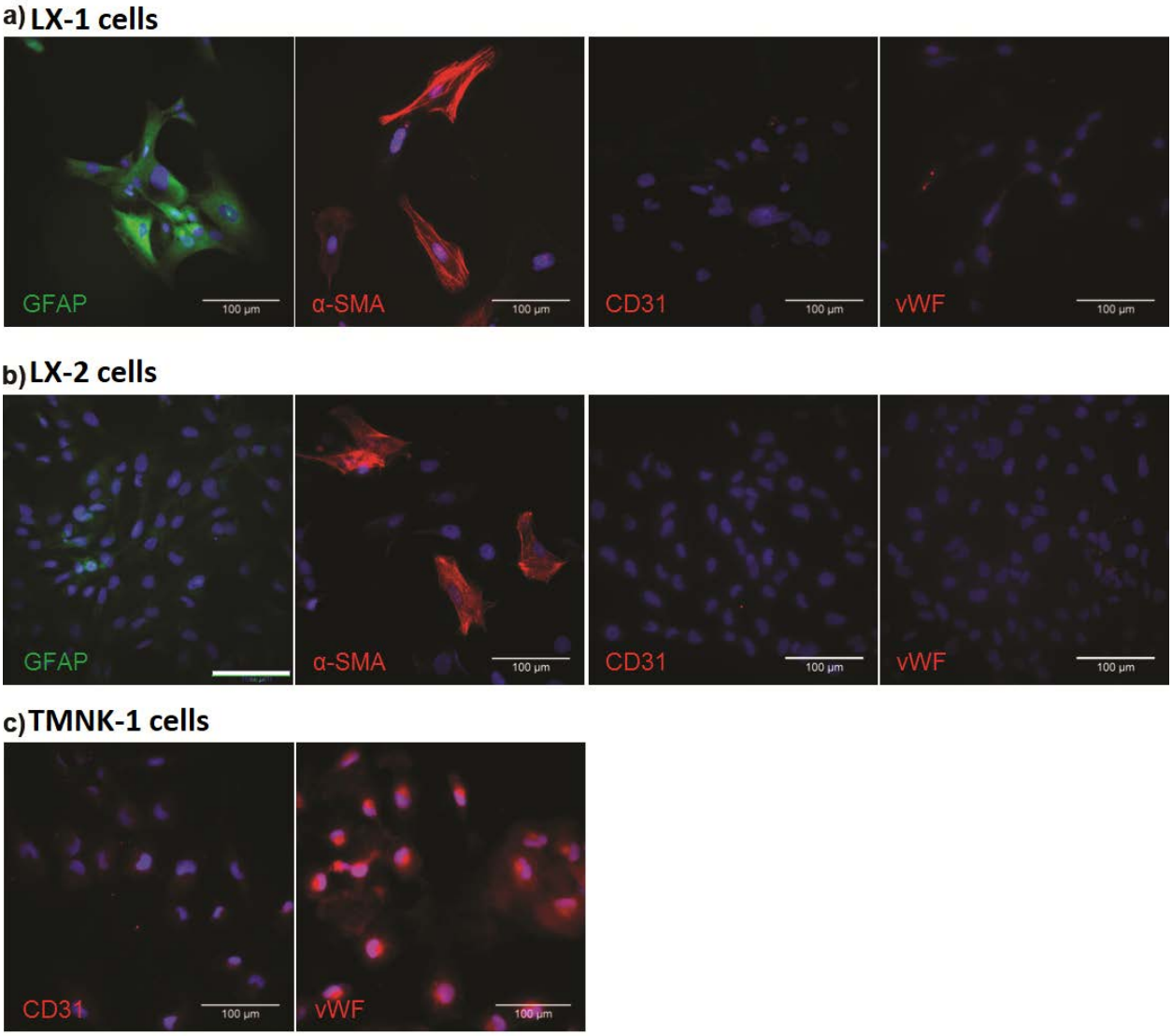


Figure 12: LX1 and LX2 cells express stellate cell markers.

LX1 (a) and LX2 (b) cells were cultured and stained for stellate cell and endothelial cell markers. DAPI was used as a counterstain. c) TMNK-1 cells, human liver endothelial cells, were used as positive controls for the endothelial cell markers, CD31 (Santa Cruz Biotechnology) and vWF (Santa Cruz Biotechnology). Scale bar= 100µm.

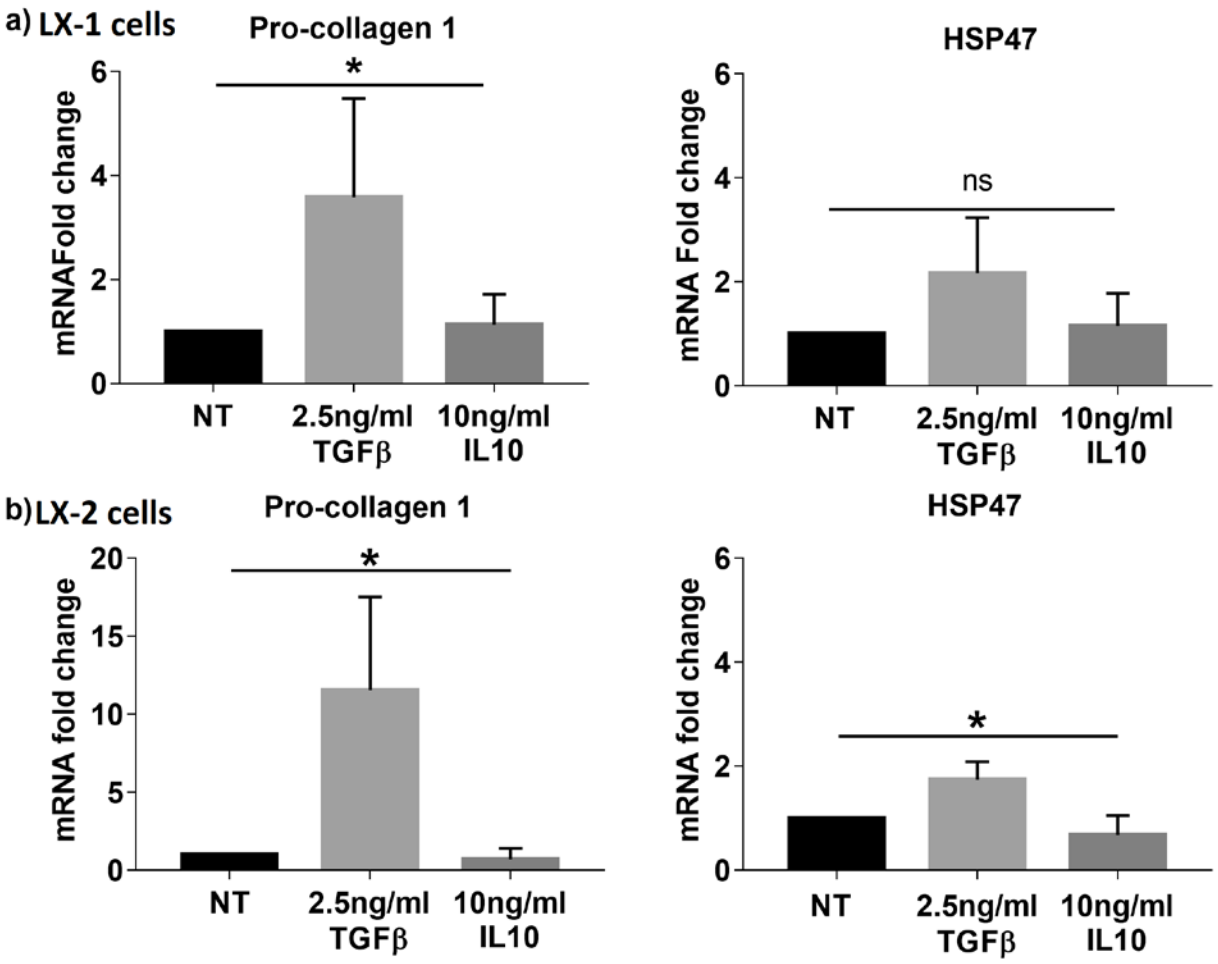


Figure 13: LX1 and LX2 cells show typical stellate cell response to TGFβ treatment by increasing pro-collagen and HSP47 gene expression.

LX1 (a) and LX2 (b) cells were cultured in full medium overnight followed by serum-starvation for 6 hours. Cells were then treated with 2.5ng/ml TGFβ (R&D) or 10ng/ml IL-10 (R&D) for 24 hours in serum-free DMEM medium (Corning). Each bar shows the average fold change of pro-collagen 1 and HSP47 gene expression with SD from n=4. One-way ANOVA was used to determine the statistical significance with p-value <0.05*.

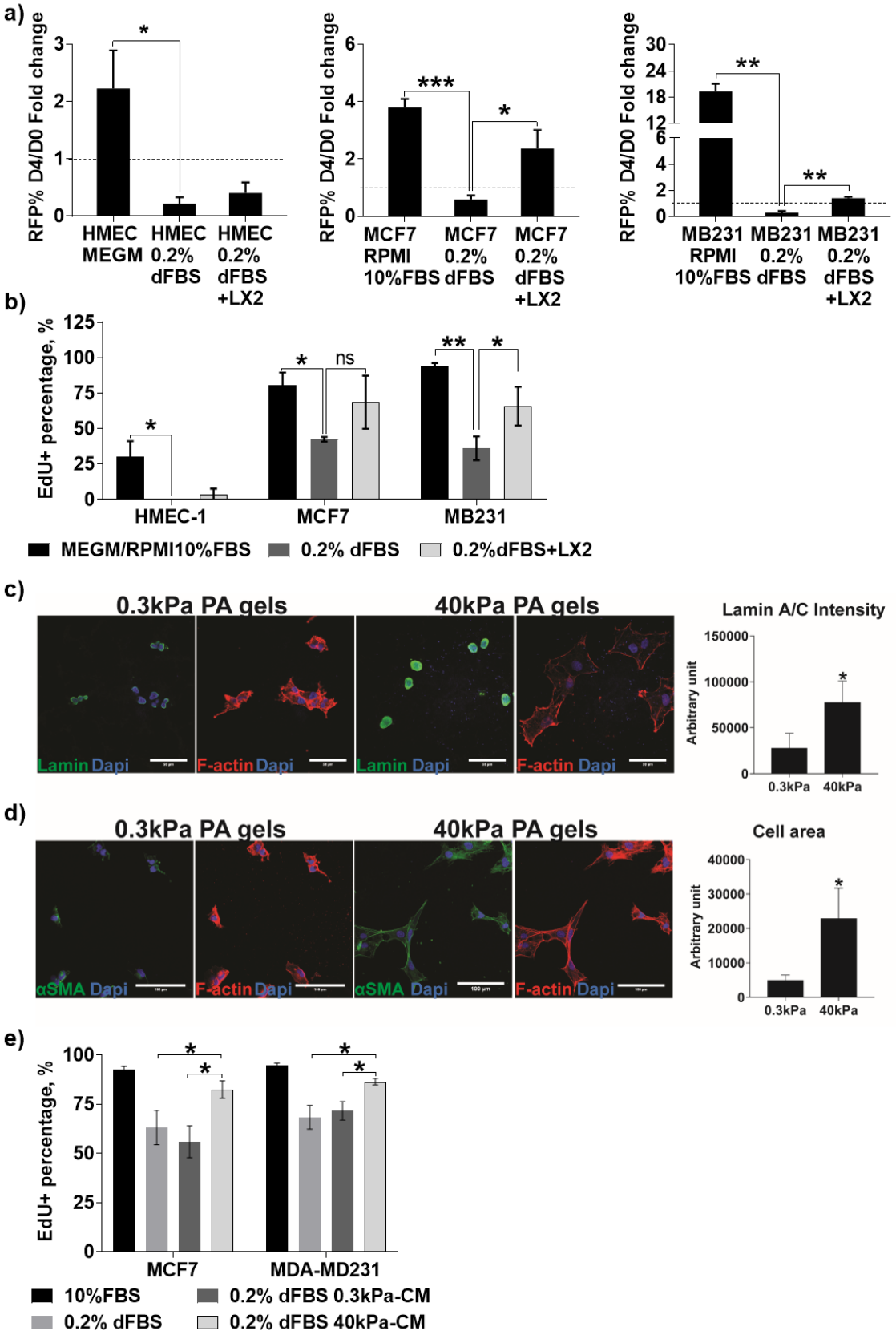

a)
RFP% D4/D0 Fold change
HMEC MEGM
HMEC 0.2% dFBS
HMEC 0.2% dFBS +LX2
MCF7 RPMI 10%FBS
MCF7 0.2% dFBS
MCF7 0.2% dFBS +LX2
MB231 RPMI 10%FBS
MB231 0.2% dFBS
MB231 0.2% dFBS +LX2
b)
EdU+ percentage, %
HMEC-1
MCF7
MB231
ns
MEGM/RPMI10%FBS
0.2% dFBS
0.2%dFBS+LX2
c)
0.3kPa PA gels
40kPa PA gels
Lamin Dapi
F-actin Dapi
Lamin A/C Intensity
Arbitrary unit
0.3kPa
40kPa
d)
αSMA Dapi
Cell area
e)
MCF7
MDA-MD231
10%FBS
0.2% dFBS
0.2% dFBS 0.3kPa-CM
0.2% dFBS 40kPa-CM

Figure 14: Activated stellate cells trigger higher proliferative induction than less-activated stellate cells.

DMEM 0.2% dialyzed FBS and RPMI 10% FBS serve as negative and positive controls, respectively. Average RFP % area fold change (14a) and average EdU incorporation percentage (14b) with standard deviation (SD) of HMEC-1, MCF7 and MDA-MB231 cells in LX-2 co-culture (n=3). Total number of cell counted/condition in 14b: HMEC-1 MEGM= 463 cells, HMEC-1 0.2%dFBS= 162 cells, HMEC-1 LX2= 181 cells, MCF7 10%FBS= 520 cells, MCF7 0.2%dFBS= 157 cells, MCF7 LX2= 454 cells, MB231 10%FBS= 812 cells, MB231 0.2%dFBS= 226 cells and MB231 LX2= 540 cells. 14c) Representative images of LX-2 lamin A/C immunofluorescence and average lamin A/C intensity with SD (n=4). Scale bar= 50um. 14d) Representative images of LX-2 αSMA immunofluorescence and average cell area with SD (n=3). Scale bar=100um. 14e) Average EdU incorporation percentage in MCF7 and MDA-MB231 cultured with 100% conditioned-media (CM) from 0.3kPa and 40kPa LX-2 cultures for 48 hours (n=3). Total number of cells counted/condition is as follows: MCF7 10% FBS= 341 cells, MCF7 0.2% dFBS= 243 cells, MCF7 0.3kPa-CM= 316 cells, MCF7 40kPa-CM= 366 cells, MB231 10% FBS= 366 cells, MB231 0.2% dFBS= 208 cells, MB231 0.3kPa-CM= 255 cells, MB231 40kPa-CM= 234 cells.

3.3.3 Activated Stellate Cells Secrete Soluble Inflammatory Factors (IL-8, MCP-1, IL-6, and GRO)

Our previous transwell and conditioned-medium experiments suggested that the activated stellate cells induced tumor growth by secreting soluble factors. In addition, some of the proliferating cancer cells in the LX1 co-culture samples appeared to be contact-independent (white arrows in Figure 11).

Therefore, we next utilized protein array and Luminex assays to determine the cytokines and growth factors secreted by the activated stellate cells and by primary human NPC. Protein array analyses revealed high level of IL-8, IL-6, GRO-βγ and MCP-1 secretion by the LX1 cells but not by the cancer cells *in vitro* (Figure 15a and b). The complete map for the protein array is available as supplementary figure 4 in Appendix C. Less activated LX-2 cells, cultured on

0.3kPa PA gels, secreted lower levels of these pro-inflammatory cytokines especially IL8 (Figure 15c). Interestingly, primary human NPC, cultured on stiff scaffolds in 3D liver MPS, secreted higher level of IL-8, MCP-1, IL-6 and IP-10 when compared to primary hepatocytes alone (Figure 15d).

Based on these results, we postulated that inflammatory chemokines, namely IL-8 and MCP-1, could be responsible for hepatic stellate cell-induced cancer growth.

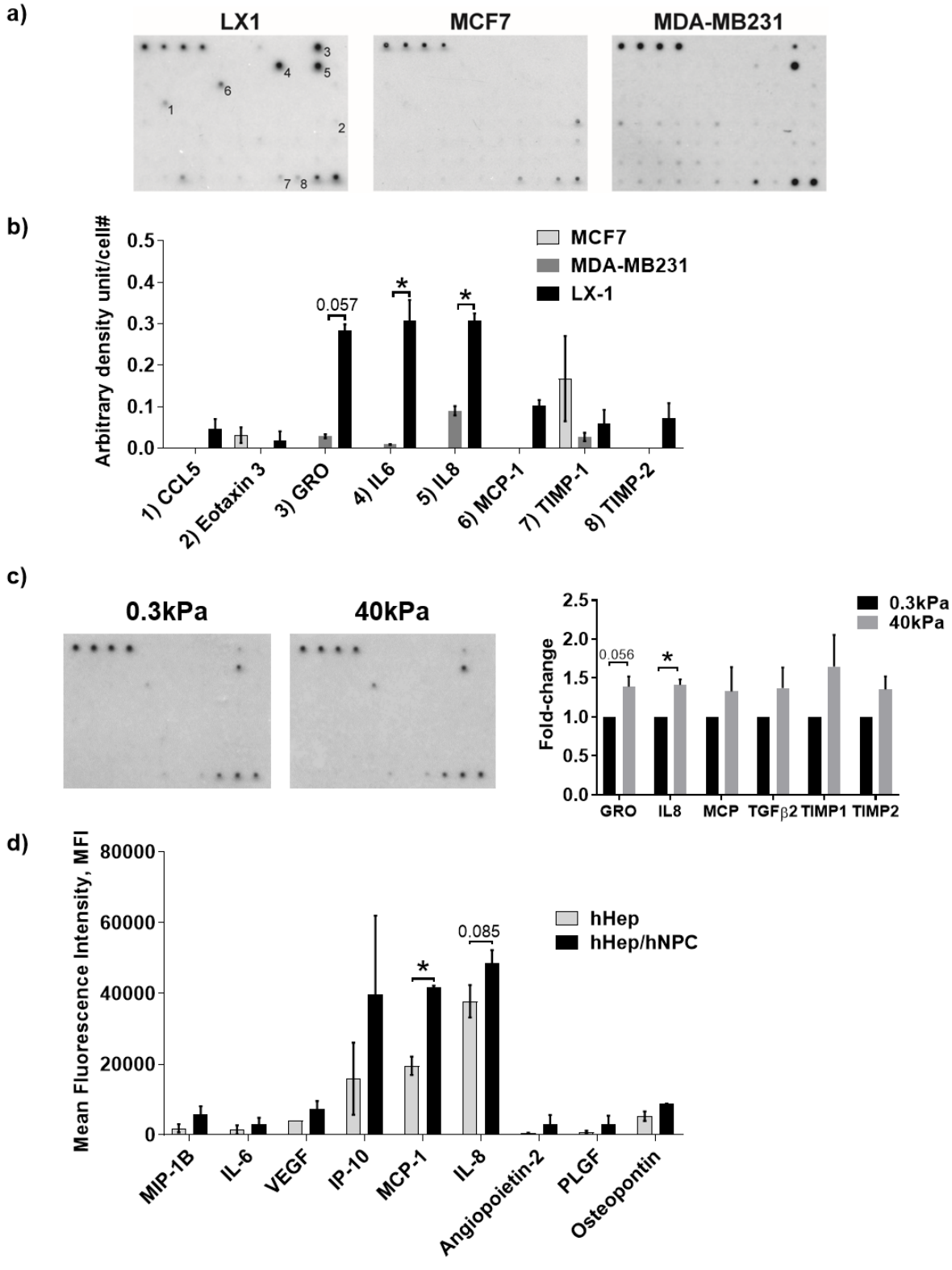


Figure 15: Activated HSCs and primary liver NPCs secrete high level of IL-8 and MCP-1.

a) Representative images of protein array analysis on cells cultured in HMM and b) average density of selected cytokines normalized to cell number with SD (n=2). c) Representative images of secretome analysis of LX-2 cells cultured on 0.3kPa and 40kPa PA gels in DMEM+0.2% dialyzed FBS and average normalized spot density fold-change (40kPa/0.3kPa) of selected cytokines with SD (n=4). d) Average mean fluorescence intensity (MFI) of chemokines/cytokines with standard error of the mean measured using Luminex assay on supernatants from primary human hepatocytes ± NPC cultured in 3D Liver MPS in serum-free William's E medium (n=2 donors).

3.3.4 IL-8 Promotes Cancer Growth *in vitro* and Tumor Escape from Dormancy in the *ex vivo* Liver MPS potentially via ERK activation

Next, we tested whether these chemokines could directly affect cancer cell growth in vitro. In serum-free medium (SFM), MCF7 cells did not respond to IL-8 although the epidermal growth factor (EGF) positive control showed significant growth induction (Figure 16a).

In MDA-MB231 cells, EGF treatment did not promote cell growth but significantly alleviated serum-deprived growth arrest (Figure 16b). Likewise, the addition of IL-8 in SFM significantly sustained MDA-MB231 cell when compared to the negative control (Figure 16b). MCP-1 did not affect the growth of MCF7 and MDA-MB231 (Figure 16a, b), suggesting that its effects, if any, would be indirect for the cancer cells.

To verify the effects of IL-8 in promoting cancer cell growth and survival in vitro, cancer cells cultured with LX-1-transwell insert were treated with CXCR2 neutralizing antibody. Similar with our previous results, LX-1 transwell insert augmented cancer growth by 2-3 folds (Figure 16c, d). Blocking CXCR2 did not affect MCF7 growth and proliferation (Figure 16c) but significantly decreased LX-1-induced MDA-MB231 cancer growth and proliferation (Figure 16d).

We verified the effects of IL-8 on dormant tumor cells in the 3D *ex vivo* liver MPS, a near physiological situation that can determine effects of isolated stimuli (Wheeler et al. 2014). MDA-MB231 cells were treated with doxorubicin to eliminate proliferating cells as established (Wheeler et al. 2014). LPS+EGF treatment, serving as positive controls, induced the dormant cells to significantly grow and proliferate (Figure 17b and c). Addition of 0.25µg/ml of IL-8 into the physiologic medium also promoted tumor cell escape from dormancy as indicated by two-fold increase in RFP% area and increased EdU incorporation in about 50% of the cells, similar to the level seen in the positive controls (Figure 17b and c). There were also some EdU positive cells in the doxorubicin-only negative controls but most of these cells were on the stiff polystyrene bridges between the pores of the scaffold, an area that does not allow for dormancy as previously observed (Clark et al. 2017). IL-8 induced cancer cell proliferation even within the soft liver tissue (green arrow heads in Figure 17a right panel). Prolonged cell culture and treatments with cytokine, growth factors, chemotherapy drugs or LPS did not adversely affect the health and functions of the hepatic tissues as the injury markers, AST and ALT, remained at low levels throughout the whole experiment and the cells maintained blood urea nitrogen production and CYP enzymes metabolic activities (Figure 18).

Previously, it was reported that a high ERK/P38 ratio indicated tumor outgrowth whereas low ERK/P38 ratio was associated with growth arrest in several tumor cell lines (Aguirre-Ghiso et al. 2003). We verified that, in MDA-MB231 cells, IL-8 treatment increased the level of phosphorylated ERK and the addition of SCH772984, an ERK-specific inhibitor, blocked IL-8 induced ERK phosphorylation (Figure 19b middle and right panel). Augmented ERK phosphorylation correlated with a significant increase in cancer cell survival when IL-8 was added into SFM which was reversed with SCH772984 treatment (Figure 19b left panel). The

effects on MCF7, however, were less minimal (Figure 197a). This suggests that inherent E-cadherin signaling in the MCF7 confers much of the ERK signaling (Ma et al. 2016).

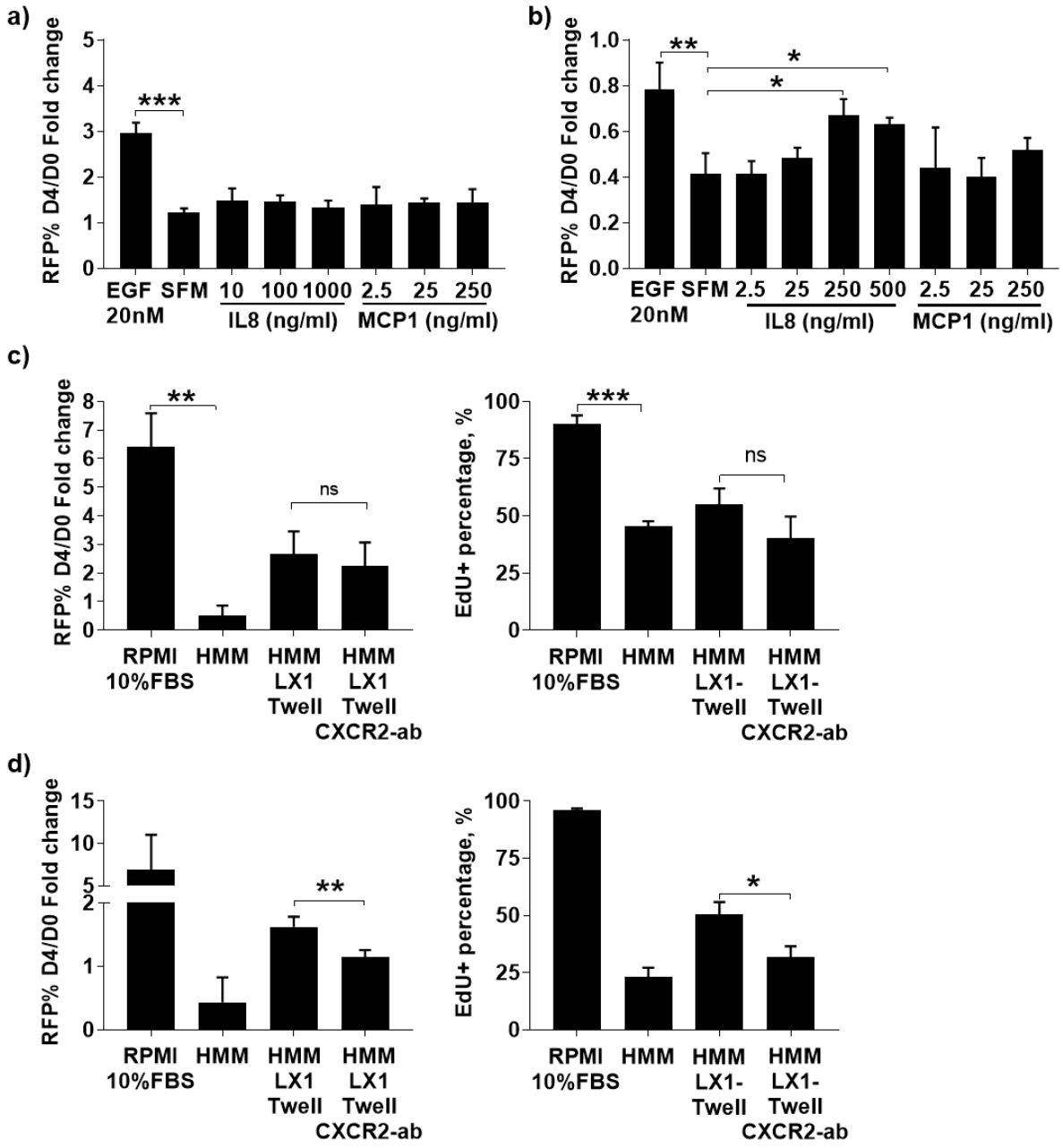


Figure 16: IL-8, but not MCP-1, alleviated serum starvation-induced growth arrest in MDA-MB231 cells in vitro.

Serum-free medium (SFM) and SFM+20nM EGF served as the negative and positive controls, respectively. Average RFP% fold-change with SD of MCF7 (16a) and MDA-MB231 (16b) cells with chemokine addition into SFM at the indicated concentration (n=4). Average RFP% fold-change and average EdU incorporation percentage with SD of MCF7 cells (16c) and MDA-MB231 (16d) cultured with LX-1-transwell insert ± 1µg/ml CXCR2 antibody (n=3). Total number of cells counted/condition in (16c and 16d) is as follows: MCF7 10%FBS= 1031 cells, MCF7 HMM= 242 cells, MCF7 LX1 Twell= 761 cells, MCF7 LX1 Twell+ CXCR2mAb = 815 cells, MB231 10%FBS= 1125 cells, MB231 HMM= 234 cells, MB231 LX1 Twell= 352 cells and MB231 LX1 Twell+ CXCR2mAb= 338 cells.

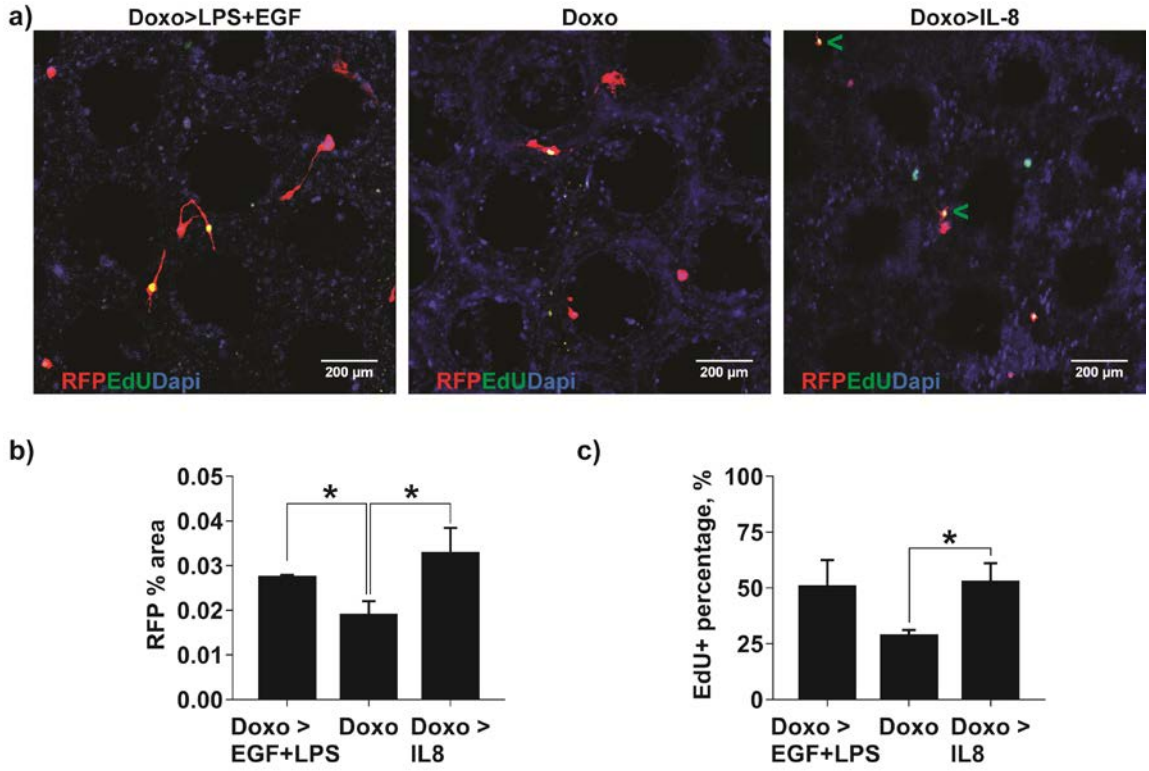


Figure 17: IL-8 drives cancer cell emergence from dormancy in the ex vivo MPS.

a) Representative images of scaffolds with doxorubicin followed by LPS+EGF treatments (positive controls), doxorubicin (negative controls) and doxorubicin followed by 0.25µg/ml IL8 treatment. Green arrow heads point to proliferating cancer cells in the soft liver tissues. Scale bar=200µm. Average RFP % area (b) and average EdU positive percentage (c) with SD of duplicate samples from three donors in three separate experiments (n=3). Total number of cells counted/condition for 17c: Doxo>LPS+EGF= 160 cells, Doxo =164 cells and Doxo>IL8=137 cells.

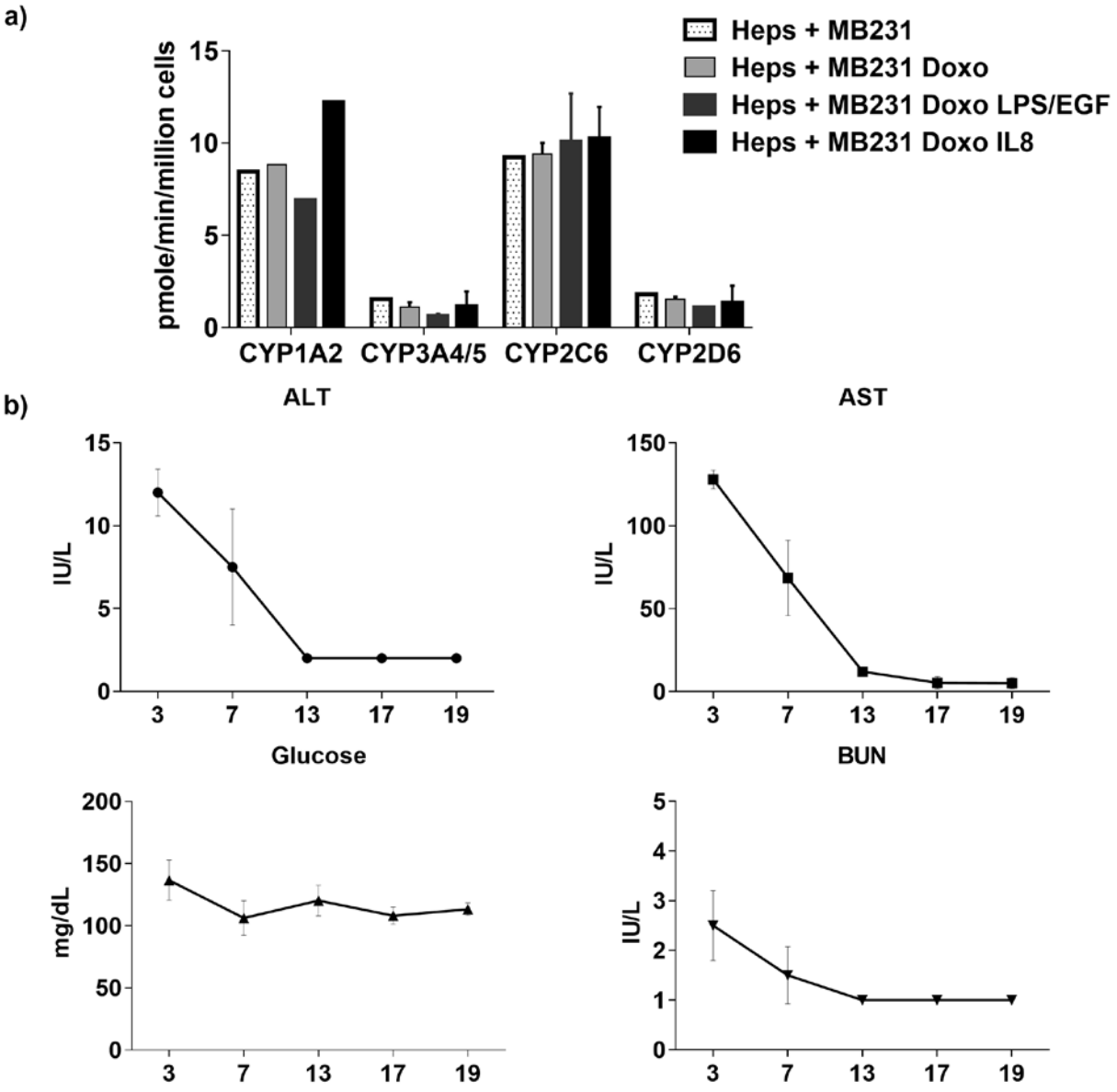


Figure 18: Hepatocytes were functional and healthy in the MPS until day 19.

a) CYP enzymes activity measured using LC-MS/MS. N=2. b) Clinical chemistry analyses measuring the production of liver injury markers (AST and ALT), nitrogen and glucose consumption by primary hepatocytes seeded with MDA-MB231 cells on day 3, 7, 13, 17 and 19. Each point shows the average values of duplicate samples from two donors.

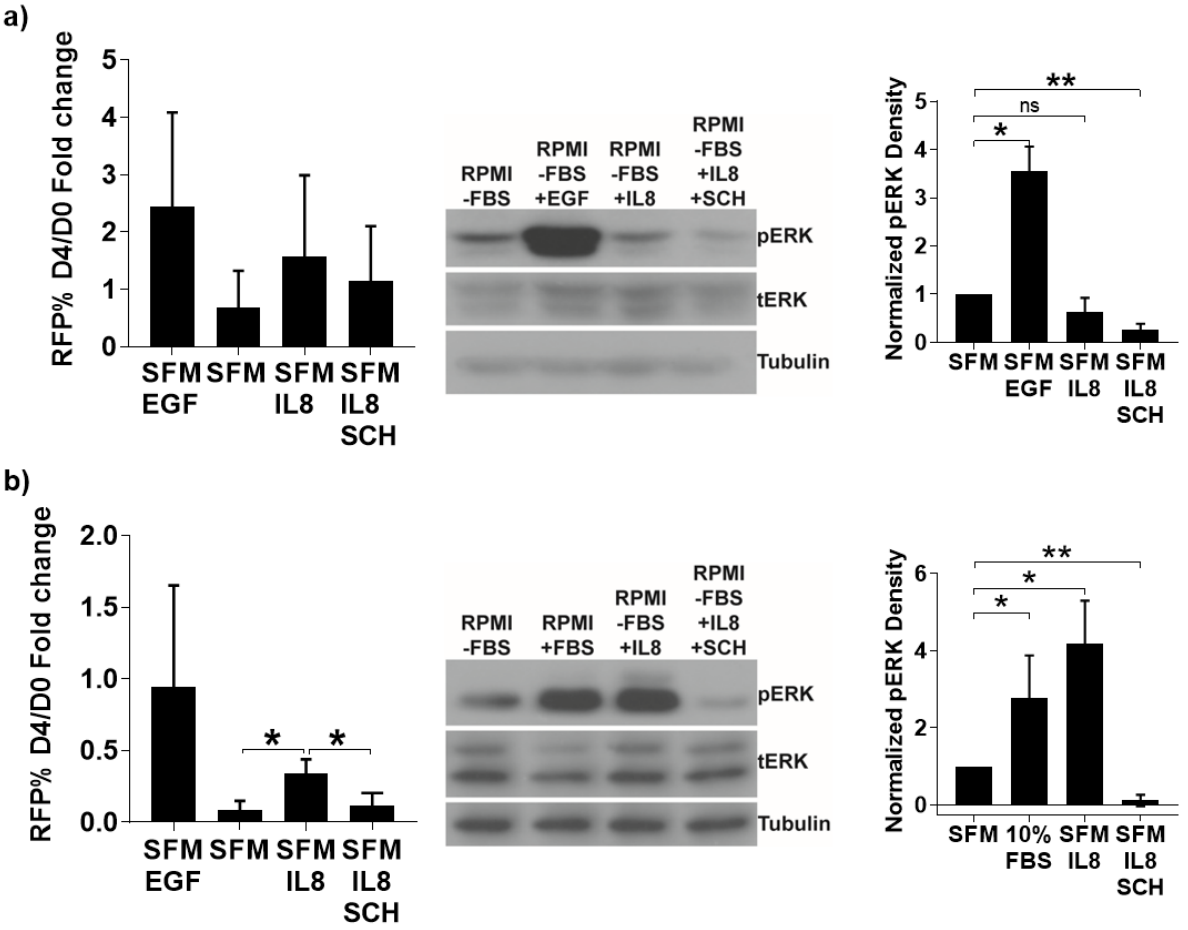


Figure 19: IL-8 induces ERK phosphorylation.

Serum-free RPMI medium (SFM) and SFM+EGF/RPMI+10% FBS serve as negative and positive controls, respectively. Average RFP% growth fold-change (left panel), immunoblot for phosphor-ERK (pERK), total ERK (tERK) and tubulin (middle panel) and pERK density quantification (right panel) of MCF7 (6a) and MDA-MB231 (6b) cells following 4 days of IL-8 treatments with SD (n=3, n=4 for MCF7 immunoblot). Treatments: 1µg/ml IL-8 for MCF7, 0.25µg/ml IL-8 for MDA-MB231, 20nM EGF and 0.5µM SCH772984.

3.3.5 Discussions

The factors that drive cancer to re-emerge after long latency periods are still unclear. We hypothesized that inflammation can trigger metastatic cancer emergence in the liver. Liver

inflammation can activate the stromal cells, including the HSC, to produce growth factors or inflammatory mediators that in turn cause dormant cancer cells to grow. Here, we found that activated human stellate cells could indeed drive breast cancer cell growth and proliferation by secreting tumor-promoting soluble factors. We also found that activated stellate cells and primary human NPC secreted inflammatory cytokines, primarily IL-8/CXCL8, to directly promote tumor growth and emergence from dormancy.

IL-8 has been reported to increase cancer cell motility, invasion and metastasis by inducing cancer cells to undergo epithelial-mesenchymal transition (Fernando et al. 2011). However, the role of IL-8 in cancer cell proliferation remains controversial. Several studies reported that IL-8 could not induce cell proliferation *in vitro* (Freund et al. 2003, Yao et al. 2007) and repressed *in vivo* cancer growth via neutrophil recruitment (Hirose et al. 1995, Lee et al. 2000). However, it was also reported that IL-8 increased cyclin D1 expression as well as PI3K/Akt/mTOR activation in DU145 and PC3 prostate cancer cells, and blocking IL-8 expression abrogated prostate cancer cell proliferation (MacManus et al. 2007). Moreover, IL-8 significantly promoted the proliferation of NSCLC (Luppi et al. 2007) and colon cancer cell (Lee et al. 2012) *in vitro* and *in vivo*. As we found that IL-8 is significantly increased by stressed stellate cells and stimulated liver MPS, we queried whether IL-8 could sufficiently induce cell proliferation to facilitate tumor escape from dormancy. Dormant cancer cells usually exist as single cells or small clusters. Therefore, to mimic this observation, we sparsely seeded cancer cells in serum-free medium for *in vitro* cultures and subjected these cells to IL-8 treatment to determine the induction of cancer cell growth and proliferation. However, in line with previously published results, we found that IL-8 treatment did not confer significant growth advantage to the cancer cells *in vitro* and could merely maintain MDA-MB231 cell survival in serum-free

conditions when compared to the negative controls. However, we found that IL-8 imparted a significant increment in cancer cell growth and proliferation in the 3D *ex vivo* liver MPS, in a microenvironment that is richer in signals than the 2D co-culture. Blocking CXCR2, a receptor that can be activated by the highly secreted cytokines IL-8 and GRO, substantially reduced cytokine-induced proliferation. Thus, IL-8/CXCR2 may be crucial in driving tumor outgrowth from dormancy.

It is important to note that other chemokines and cytokines might also contribute either directly or indirectly to cancer cell escape from dormancy since neutralization of CXCR2 did not fully abrogate cancer growth in the transwell assays (Figure 17c and d). These factors could compensate CXCR2 blocking and create feed-back loops to increase the secretion of other inflammatory cytokines and growth factors by other cells in the liver. For example, unlike IL-8, we found that MCP-1/CCL2 was highly and exclusively secreted by the liver NPC but did not induce significant cancer growth. It was previously reported that breast cancer cells and tissues expressed high levels of both MCP-1 and its receptor CCR2 which conferred survival, but not proliferative, advantage to the breast cancer cells (Fang et al. 2012). MCP-1 can indirectly affect tumor outgrowth by recruiting inflammatory monocytes and facilitating efficient tumor cell extravasation for metastatic seeding (Qian et al. 2011) or inducing monocyte polarization into the alternative, pro-tumor M2 phenotype (Roca et al. 2009, Sierra-Filardi et al. 2014). Linking this with our finding of M2-like macrophages driving a mesenchymal reversion (Yang et al. 2016) would provide an indirect mechanism for promoting emergence. IL-6 is another pro-inflammatory cytokine secreted by both primary human NPC and LX1 stellate cells and has been shown to induce MCF7 growth and spheroid formation (Sansone et al. 2007). Another candidate could be TGFβ, a potent inducer of HSCs activation and IL-8 secretion. Interestingly we

observed a slight induction of TGFβ2 secretion by the LX-2 cells cultured on the stiff 40kPa gels when compared to soft 0.3kPa gels (Figure 15c).

In this study, we strove to closely recapitulate human physiology. However, the isolation of stellate cells from primary human NPC fraction proved to be quite challenging due to limited availability of the cells and lability during isolation. We therefore opted to use human NPC hepatic cell lines. The human LX1 and LX2 stellate cell lines were established in the laboratory of Dr. Scott Friedman in Mount Sinai School of Medicine, NY using a viral vector for immortalization (Xu et al. 2005). We validated that both cell lines expressed hepatic stellate cell markers such as αSMA and GFAP but not endothelial cell markers CD31 or vWF (Figure 12). The cells also secreted TIMP-1 and TIMP-2 as shown in the protein array (Figure 15). Finally, the cells showed typical stellate cell response to TGF-β1 by increasing pro-collagen 1 and HSP47 gene expression (Figure 13). The key effect of hepatic stellate cells on cancer cell growth was validated with TWNT-1 and HSC-T6 cell lines where cancer cell growth induction was found to be significantly augmented and was comparable to TMNK-1 endothelial cell-induced outgrowth (Figure 11b and c) as published previously (Taylor et al. 2014).

In addition to the stromal cell line, we also used tumor cell lines, MCF7 and MDA-MB231 cells. MDA-MB231 was established from triple negative breast cancer (TNBC) cells whereas the MCF7 cells were established from pleural effusion of a luminal-type breast cancer (Holliday and Speirs 2011). There are several results and experimental settings that need to be addressed regarding these two cell lines. First of all, although MDA-MB231 cells are the more aggressive cancer cells than the epithelial-like MCF7 cells, our co-culture and transwell assays using HMM showed higher fold change in MCF7 growth when compared to MDA-MB231. We observed that the MDA-MB231 cells are highly dependent on the serum as evident by the

dramatic increased of raw RFP% area in the RPMI+10% FBS positive controls (4-7%) when compared to MCF7 positive controls (less than 2%). The negative effect of serum free media could be rescued by co-culturing the cancer cells with stellate cells or by treating the cancer cells with IL-8 *in vitro* and *ex vivo*. Second, we observed weaker growth in the transwell assays when compared to co-culture experiments. This is likely due to dilutional effects where higher medium volume is required for the transwell assay and cell growth area in the transwell insert (0.33cm2) is five-times less than 24-well plate (1.9cm2). Third, less prominent effects were observed in IL-8 treatment on MCF7. This is due to lower level of CXCR2 expression in MCF7 cells when compared to MDA-MB231 cells. CXCR1 is not expressed by both cell types (our unpublished data) (Freund et al. 2003). Thus, the effects of IL-8 and CXCR2 blocking in MCF7 cells may not be biologically relevant. Fourth, the cytokine array indicated that MDA-MD231 cells also secreted IL-8. However, the secretion was lower than LX1 when normalized to their respective cell numbers. It must be noted that these studies were performed under conditions wherein the single cancer cells were initially <1% of the total cells in the 3D liver MPS. In the setting of tumor dormancy, the cancer cells are usually scant in number. Therefore, we presumed that the surrounding stromal cells were the major contributor of IL-8 or any other factors. Lastly, only MDA-MB231 cells were used in the 3D *ex vivo* liver MPS. TNBC shows a higher propensity to metastasize to the liver (Yuan et al. 2014). Unlike the MCF7 cells, MDA-MB231 cells could spontaneously metastasize to various organs *in vivo* (Holliday and Speirs 2011). Thus, we opted to use the MDA-MB231 cells in the 3D *ex vivo* liver MPS to match *in vivo* and clinical observations. The cells were treated with doxorubicin to eliminate proliferating cells so that the effects of IL8 treatment observed will be specific to the remaining dormant cells.

Hepatic stellate cells are one of the main components of the liver NPC. The cells remain quiescence under physiologic condition. A recently published paper showed that activated stellate cells could revert to dormant phenotype when cultured on soft matrix *in vitro* (Caliari et al. 2016). In addition, several reports suggested that laminin could prevent the activation of primary hepatic stellate and also reversed the activation status of some stellate cell lines (Friedman 2008, Stone et al. 2015). The roles and mechanisms for HSC-induced tumor growth in mouse models have been reported (Zhao et al. 2011, Coulouarn et al. 2012). However, since there are various processes and complex interactions between stellate cells and their microenvironment, it may be difficult to pinpoint the specific effects of stellate cell activation in driving tumor growth. Thus, in order to directly compare tumor growth induction by quiescent and activated stellate cells, we cultured LX2 cells on 0.3kPa PA gel to model physiologic stiffness of the liver and 40kPa PA gel to model liver tissue with fibrosis/cancer. Here, the LX2 cells reverted to a quiescence state *in vitro* as marked by the reduced expression of nuclear lamin A/C and lack of α-SMA fibers on soft PA gels, similar with published observation (Swift et al. 2013, Caliari et al. 2016) and resulted in reduced cancer proliferation induction. The percentages of EdU incorporation for these experiments were higher than other results due to shorter experimental period but significant differences in proliferation induction were still observed. The fold-change of cytokine secretion, though marginal, is replicable and statistically significant. Similar results were reported where pro-inflammatory cytokines were slightly upregulated in activated primary rat HSCs when compared to quiescent HSCs (Jiang, Parsons and Stefanovic 2006).

In summary, we showed here that human stellate cells and their derived IL-8 could induce breast cancer growth and proliferation. As this factor is expressed upon stressors

activating the stellate cells, this could link systemic inflammation to breast cancer emergence and outgrowth. Furthermore, we found that IL-8 prolonged *in vitro* cell survival and potentiated cancer growth and proliferation in the 3D *ex vivo* liver MPS tumor dormancy model through ERK activation. This aspect of survival could also explain the generalized chemoresistance of metastases. This study suggests that preventing liver inflammation or specifically inhibiting key inflammation inducers might be beneficial to cancer patients to prevent delayed tumor recurrence or to re-establish chemo-responsiveness.

3.3.6 Future Works

Firstly, we speculate that MCP-1 indirectly cause tumor escape from dormancy. Thus, one of our future directions is to investigate whether MCP-1 can activate liver NPC to secrete more pro-inflammatory and growth factors in the 3D *ex vivo* liver MPS and whether the activation is sufficient to promote tumor outgrowth. Although we mentioned that MCP-1 knock-down could tip macrophage polarization towards M2 phenotype, several conflicting data showed otherwise. Thus, we will verify the effects of MCP-1 in macrophage polarization to hopefully accede with our previous findings where we found significant tumor induction in M2 macrophage co-culture.

Secondly, our results imply that inflammation may drive tumor outgrowth. Therefore, it is interesting to see whether inflammation prevention or resolution can lead to favorable outcomes. Most of the clinical studies focus on the effects of anti-inflammatory drugs in primary cancers prevention. A recent study on more than 120,000 participants revealed that frequent long-term aspirin intake (even at low doses) can reduce the risk of gastrointestinal cancers especially colorectal cancers but the effects on other primary solid cancers and importantly

metastatic cancers are still unclear (Cao et al. 2016). The Liverchip will easily allow us to compare tumor emergence from dormancy with or without inflammatory insults and whether inflammation resolution can impede metastatic outgrowth.

3.3.7 Conclusions

Activated hepatic stellate cells induce tumor growth and escape from dormancy by secreting inflammatory cytokines. IL-6, IL-8, GRO and MCP-1 are highly secreted by activated HSC and activated primary human NPCs. IL-8 directly promoted cancer growth and escape from dormancy potentially through ERK activation. MCP-1, on the other hand, does not directly affect cancer growth. Limiting liver inflammation could be beneficial in preventing delayed liver metastases or emergence from dormancy. These results, taken together with our previously published papers, indicate important roles of human NPCs in driving tumor escape from dormancy via several different mechanisms. Synergistic effects of these mechanisms are still unknown and could provide vital information on the predominant factor(s) for tumor escape from dormancy in the liver.

Supplementary information, materials and methods for this chapter are available in Appendix C

4.0 SUMMARY OF FINDINGS

Tumor suppressor E-cadherin is critical in tumor metastasis. Forced expression of this junctional complex protein in primary tumor cells may prevent tumor dissemination. However, this option may not engender favorable outcome since cancer cell shedding occurs early during tumorigenesis. Hence, metastatic cascades may have already been initiated in up to 70% of the patients at diagnosis (Hunter, Crawford and Alsarraj 2008). Instead of preventing metastasis, tumor suppressor E-cadherin ironically may promote cancer metastasis by establishing heterotypic interaction between cancer and normal cell during colonization. Most of these disseminated cancer cells, however, are rapidly cleared by the body through various mechanisms leaving only a small percentage of these metastatic cells to readily form micro- and macro-metastases in secondary organs upon extravasation. Currently available chemotherapeutic drugs can sufficiently target these proliferating cells. However, a significant percentage of cancer patients harbor viable but indolent metastatic cells. These cells can be stimulated to recur decades after primary treatment in tumor-permissive microenvironment.

We report here for the first time that chemokine receptor CXCR3, specifically through CXCR3B isoform, can positively regulate the expression of E-cadherin in prostate cancer cells *in vitro* and *in vivo*. Knocking-down CXCR3B resulted in diminished membranous E-cadherin expression. Similar to our previous study where E-cadherin re-expression was induced with EGFR inhibition or with hepatocyte co-culture, CXCR3B-mediated E-cadherin re-expression

elevated resistance of prostate tumor nodules against chemotherapeutic drugs. The induction of E-cadherin expression may be dependent on cAMP-mediated signaling through CXCR3B. Of interest, tumor cells expressing both E-cadherin and CXCR3B do not appear to be dormant thus, nullifying our hypothesis that CXCR3B mediates chemo-insensitivity by inducing tumor dormancy.

In our effort to understand tumor dormancy and escape from dormancy, we utilized a novel 3D *ex vivo* liver model which is currently being commercialized by CN Bio Innovation Ltd. Our initial investigations revealed that liver non-parenchymal cells promoted tumor outgrowth whereas liver parenchymal cells regulated tumor growth. Specifically, liver endothelial cells induce tumor growth via EGFR signaling whereas anti-inflammatory M2 macrophages promote tumor growth via EMT induction. Herein, we found that activated stellate cells secrete inflammatory cytokines mainly IL-8 to drive tumor growth and proliferation *in vitro*. IL-8 also mediates tumor escape from dormancy in the 3D *ex vivo* liver MPS potentially via ERK activation.

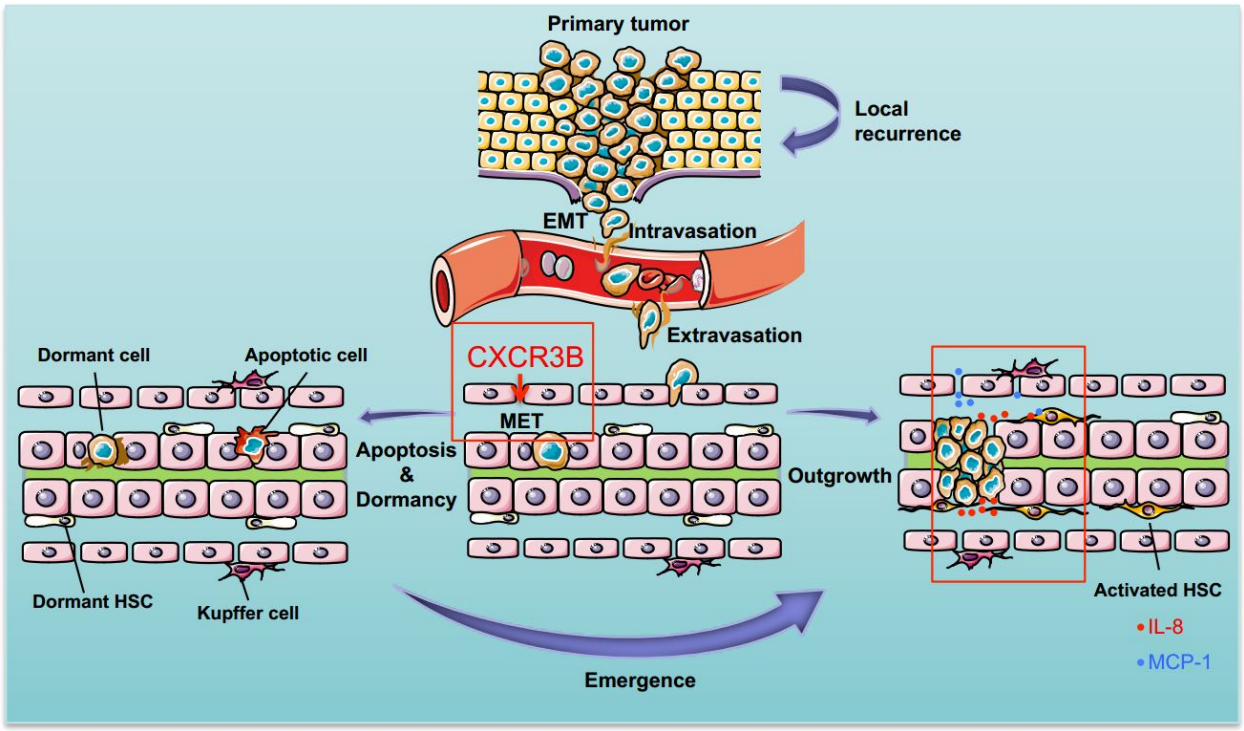


Figure 20: Summary of findings.

Main findings from this study are boxed (red) in the schematic above. The first part of this study establishes the novel role of CXCR3B in driving E-cadherin re-expression that may facilitate tumor cells seeding into liver parenchyma. The reversion may also protect the cancer cells from the cytotoxic effects of chemo drugs. As previously mentioned, disseminated metastatic cancer cells may grow out, die or remain dormant during distant organ colonization. The second part of this study identifies IL-8, primarily secreted by the activated hepatic stellate cells, to be part of the mechanisms promoting tumor escape from dormancy in inflamed liver. MCP-1 does not directly affect cancer cell growth and proliferation. We postulate that MCP-1 indirectly induces tumor growth by recruiting and stimulating immune cells to secrete more pro-inflammatory factors. MCP-1 may also mediate M2 macrophage polarization that has been shown to increase tumor cell proliferation (Yang et al. 2016).

4.1 SIGNIFICANCE

4.1.1 Significance in Clinical Setting

In the current paradigm, E-cadherin is a tumor suppressor protein that theoretically is crucial in inhibiting cancer cells from escaping primary sites. Current efforts utilize drugs targeting epigenetic regulators to induce E-cadherin expression that may prevent primary cancer from escaping the primary sites. However, these efforts thus far engender limited success. Our results offer an alternative way, via CXCR3B, to induce E-cadherin expression.

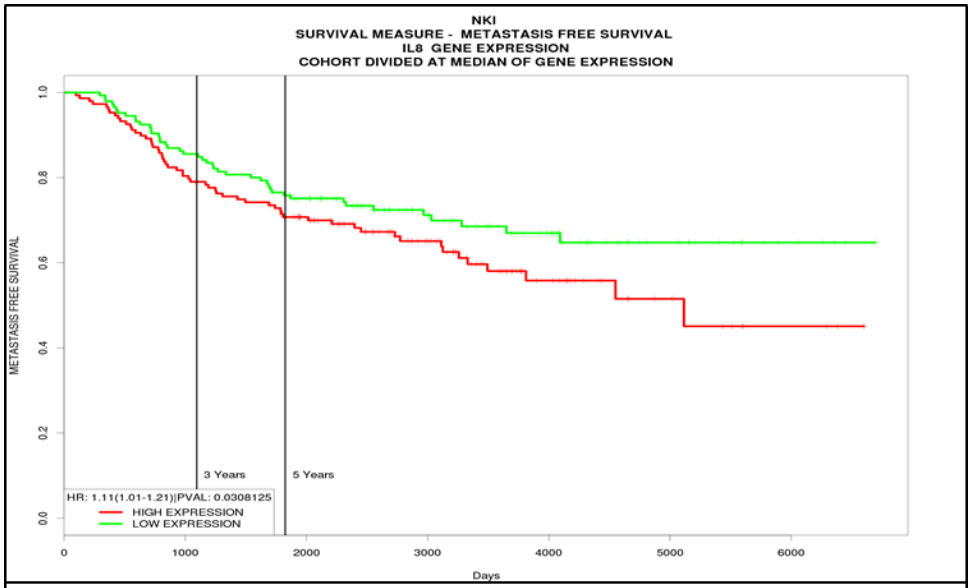
However, in a complex tumor metastasis process, we and others found that E-cadherin, during MET, is essential for metastatic colonization by protecting cancer cells against chemotherapeutic drugs. Thus, re-instating E-cadherin expression could be counterproductive. Nonetheless, our findings here shed light on an alternative way to regulate E-cadherin expression and inhibiting CXCR3B could be useful in preventing cancer cell seeding into secondary organs.

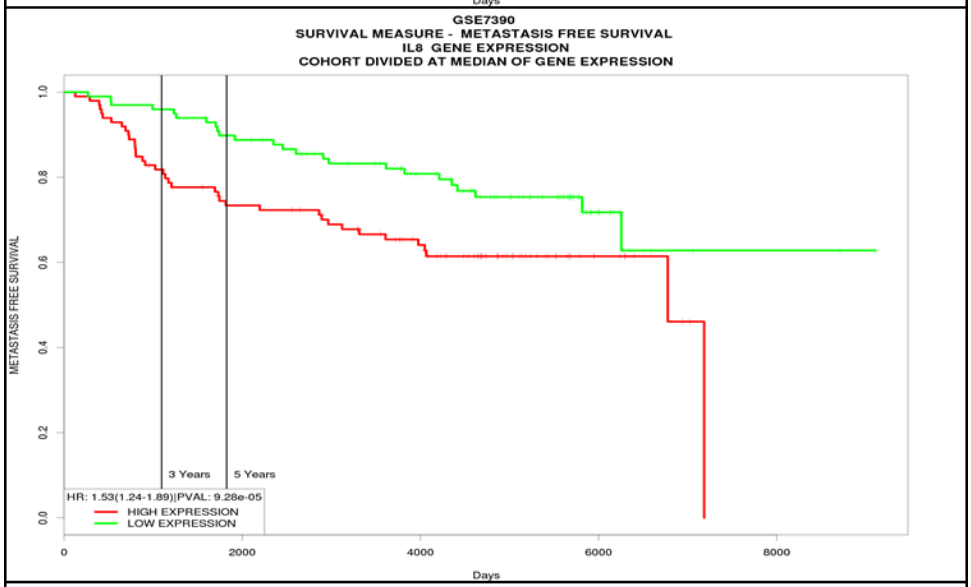
The concept of tumor dormancy is well-accepted based on clinical evidence from various studies. Tumor dormancy provides an alternative therapeutic strategy for metastatic diseases that is to keep the tumor cells in a dormant state which will prevent metastatic tumor burden and organ failure. This approach will shift this deadly disease to a chronic one. Dormancy also offers a long therapeutic window before the tumor manifests. Our study highlights pro-inflammatory signaling through IL-8/CXCR2 that can drive tumor escape from dormancy. Prognosis analyses show, in general, shorter metastasis free survival in breast cancer patients with high IL-8 expression when compared to patients with low IL-8 gene expression. Nine datasets are available for analysis and were plotted separately. Two out of the nine datasets, NKI and GSE7390, show statistically significant results in which higher IL-8 expression leads to shorter metastasis-free

survival (Figure 21). Five other datasets show similar trend but are not statistically significant and the rest (two out of nine datasets) show no difference in metastasis-free survival with regard to IL-8 expression. Hence, it is clinically relevant to target IL-8 for metastasis prevention.

4.1.2 Significance in Research Setting

The second part of this study utilizes a novel all human 3D liver MPS to closely mimic physiologic conditions and recapitulate human disease. Existing models require extensive manipulations such as treatment with high dose of drugs to establish dormant cancer cells (Li et al. 2014) and modified immunocompromised mouse models (Naumov et al. 2002, Panigrahy et al. 2012) which are not appropriate for research in inflammation-related study. Animal models, while proven to be very essential in understanding the mechanisms of most human physiology and pathology, are limited to end point analyses thus missing the early cues of pathogenesis (Wheeler et al. 2013) and detection of early metastasis and microscopic dormant tumor in vivo is technically challenging (Brackstone, Townson and Chambers 2007). Moreover, for this study specifically, animal model is not suitable because the rodent liver anatomy and functions such as the cytochrome p450 profile are different from human liver (Androutsopoulos, Tsatsakis and Spandidos 2009). Thus, this study helps to validate and establish a useful and relevant human tumor dormancy model.


NKI
SURVIVAL MEASURE - METASTASIS FREE SURVIVAL
IL8 GENE EXPRESSION
COHORT DIVIDED AT MEDIAN OF GENE EXPRESSION
METASTASIS FREE SURVIVAL
0.0
0.2
0.4
0.6
0.8
1.0
3 Years
5 Years
HR: 1.11(1.01-1.21)|PVAL: 0.0308125
HIGH EXPRESSION
LOW EXPRESSION
0
1000
2000
3000
4000
5000
6000
Days


GSE7390
SURVIVAL MEASURE - METASTASIS FREE SURVIVAL
IL8 GENE EXPRESSION
COHORT DIVIDED AT MEDIAN OF GENE EXPRESSION
METASTASIS FREE SURVIVAL
0.0
0.2
0.4
0.6
0.8
1.0
3 Years
5 Years
HR: 1.53(1.24-1.89)|PVAL: 9.28e-05
HIGH EXPRESSION
LOW EXPRESSION
0
2000
4000
6000
8000
Days

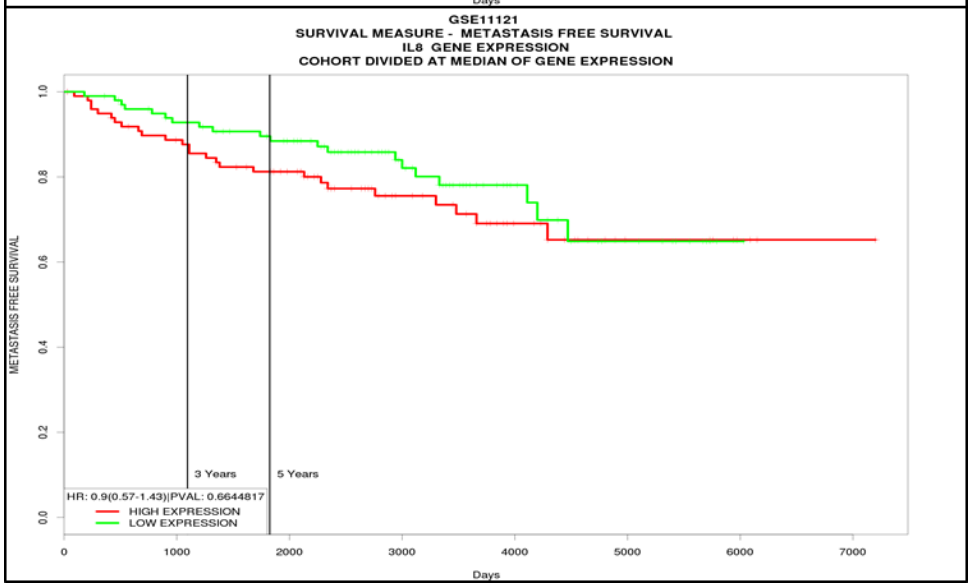

GSE11121
SURVIVAL MEASURE - METASTASIS FREE SURVIVAL
IL8 GENE EXPRESSION
COHORT DIVIDED AT MEDIAN OF GENE EXPRESSION
METASTASIS FREE SURVIVAL
0.0
0.2
0.4
0.6
0.8
1.0
3 Years
5 Years
HR: 0.9(0.57-1.43)|PVAL: 0.6644817
HIGH EXPRESSION
LOW EXPRESSION
0
1000
2000
3000
4000
5000
6000
7000
Days

Figure 21: High IL-8 gene expression correlates with reduced metastasis free survival in breast cancer patients.

Metastasis-free Kaplan-Meier survival plots were generated based on median IL8 gene expression using publicly available software and data online (Goswami and Nakshatri 2013). Nine datasets are available for analysis but only datasets with sample size (n) of about 100 per group are shown here. Metastasis-free survival between patients with high (red) and low (green) IL-8 expression in NKI cohort (top), GSE7390 cohort (middle) and GSE11121 cohort (bottom) were analyzed and plotted. The NKI contains 295 samples and the study was performed to uncover a gene-expression signature as a predictor of survival in breast cancer. GSE7390 contains 198 samples. In this study, IL8 is one of the 76-gene prognostic signature. GSE11121 contains 200 samples and was initially performed to study prognostic impact of humoral immune system in node-negative breast cancer patients.

4.2 IMPLICATIONS

Re-expression of E-cadherin following CXCR3B overexpression paves an alternative way to regulate tumor metastasis. Primary tumors express higher level of CXCR3B in comparison to metastatic cells. Thus, the effects of CXCR3B on primary tumors may be greater. Hence, CXCR3B-mediated E-cadherin re-expression may serve as a potential method to re-instate the "stop" signal in tumor metastasis. Further studies are required to determine whether CXCR3B can be effectively and selectively activated which may have beneficial implication in preventing primary tumor dissemination.

In a complex tumor metastasis setting, the utility of CXCR3B activation to suppress primary tumor migration may be limited as the prospective treatment window is narrow since metastasis tends to occur early on and most of the patients typically harbor indolent micrometastases at diagnosis. In addition to the correlation between CXCR3B and E-cadherin, our results also show that CXCR3B is up-regulated in liver metastases. Thus, inhibiting

CXCR3B or its downstream signaling components might prevent metastatic cancer phenotypic reversion that might be able to reduce cancer cell seeding into liver parenchyma.

In our subsequent study, we report that IL-8 may play significant role in promoting tumor escape from dormancy. This finding has a huge implication as currently, several monoclonal antibodies targeting IL-8 or its receptors have been developed and evaluated in cancer patients. Reparixin, a non-competitive inhibitor of CXCR1/2, has been shown in preclinical studies to deplete tumor-initiating cells, reduce *in vivo* tumor growth and augment cytotoxic effects of paclitaxel in triple negative breast cancer cell lines and xenografts (Brandolini et al. 2015). ABX-IL8 and HuMax-IL8 are two monoclonal antibodies that directly bind and neutralize IL-8. Preclinical studies show immense benefits of these two antibodies in reducing invasion, limiting tumor angiogenesis, inhibiting MMP-2 and increasing cancer cell apoptosis (David et al. 2016). These promising effects however hinge on clinical validations where Phase I clinical trial of HuMax-IL8 on patients with advanced malignant solid tumors had just recently been completed and another clinical trial evaluating progression free survival in triple negative breast cancer patients treated with paclitaxel alone or paclitaxel with Reparixin is currently underway (ClinicalTrials.gov [Internet] 2015, ClinicalTrials.gov [Internet] 2015). Therefore, it is interesting to see whether these monoclonal antibodies can produce positive effects in preventing tumor escape from dormancy or improving metastasis-free survival rate in patients.

4.3 INNOVATIONS

4.3.1 Novel Role of CXCR3B

This is the first study to demonstrate the correlation between CXCR3B and E-cadherin expression both *in vitro* and *in vivo*. Previous studies on CXCR3 isoforms focused on their direct effects on cancer cell growth, proliferation and invasion as well as the indirect effects on tumor cells through immune cell recruitment (summarized in Chapter 2.2.4). The mechanism(s) and functional aspects of this correlation are still under investigation but is postulated to increase cancer cell seeding and increase chemoresistance.

4.3.2 Novel Cell Culture Technology

One of main innovations in this study is the utilization of 3D *ex vivo* Liver MPS that more closely mimics human liver microenvironment than standard 2D cultures. We are able to successfully recapitulate metastatic pathogenesis, specifically in modelling tumor dormancy in the liver, without extensive manipulation on the cancer cells or on the liver resident cells while maintaining normal liver functions for an extended period of times.

4.3.3 Novel mechanisms for tumor emergence from dormancy

Several mechanisms for tumor escape from dormancy have been proposed including tumor angiogenesis, suppressed immune regulation and activation of integrin signaling cascades. The role of IL-8 in driving cancer proliferation is still debatable. Nonetheless, we demonstrate here

that IL-8 could promote cancer cell growth and proliferation. Importantly, we show that IL-8 also mediates tumor cell escape from dormancy.

Appendix A

PUBLICATIONS AND PRESENTATIONS

A.1.1 Publication(s)

- Ma, B., Khazali, A., & Wells, A. (2015). CXCR3 in carcinoma progression. Review. *Histol Histopathol, 30*(7), 781-792. doi:10.14670/HH-11-594.
- Khazali AS, Clark AM, Wells A. (2017). A Pathway to Personalizing Therapy for Metastases Using Liver-on-a-Chip Platforms. Stem Cell Rev. 2017 Jun;13(3):364-380.
- Khazali AS, Clark AM, Wells A. (2017). Inflammatory cytokine IL-8/CXCL8 promotes tumor escape from dormancy. British Journal of Cancer (in revision).
- Khazali AS, Ma B, Wells A. (2017). Divergent roles of chemokine receptor CXCR3 isoforms in regulating E-cadherin expression. (in preparation)

A.1.2 Poster abstract and presentation

- Khazali AS, Clark AM, Wheeler SE, Wells A. Breast Cancer Emergence from Dormancy can be Activated by Hepatic Stellate Cells. FASEB J April 2016 30:698.8

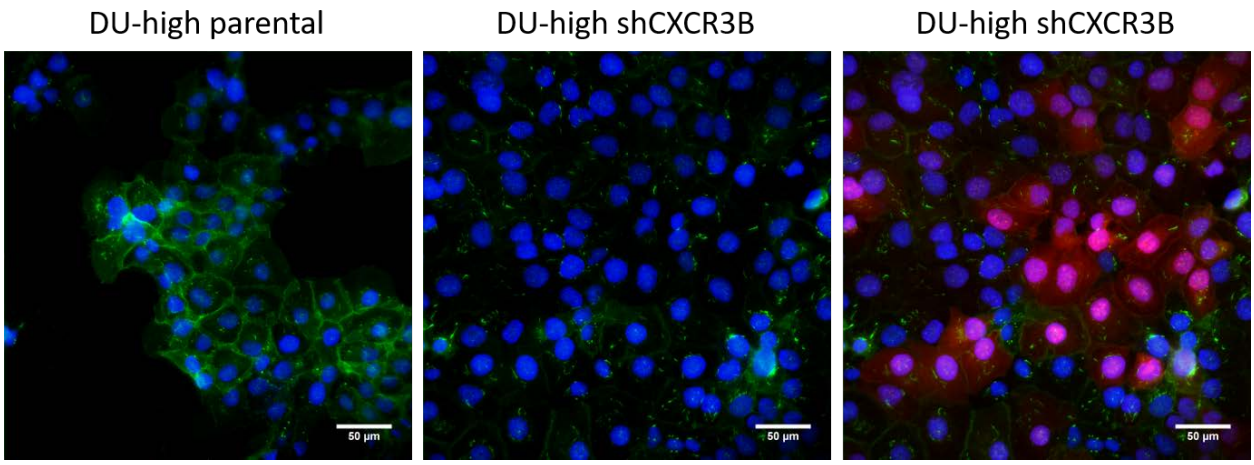
Appendix B

SUPPLEMENTARY INFORMATION: CXCR3B DRIVES E-CADHERIN RE-EXPRESSION TO FACILITATE PROSTATE CANCER LIVER METASTASIS

B.1 SUPPLEMENTARY FIGURES

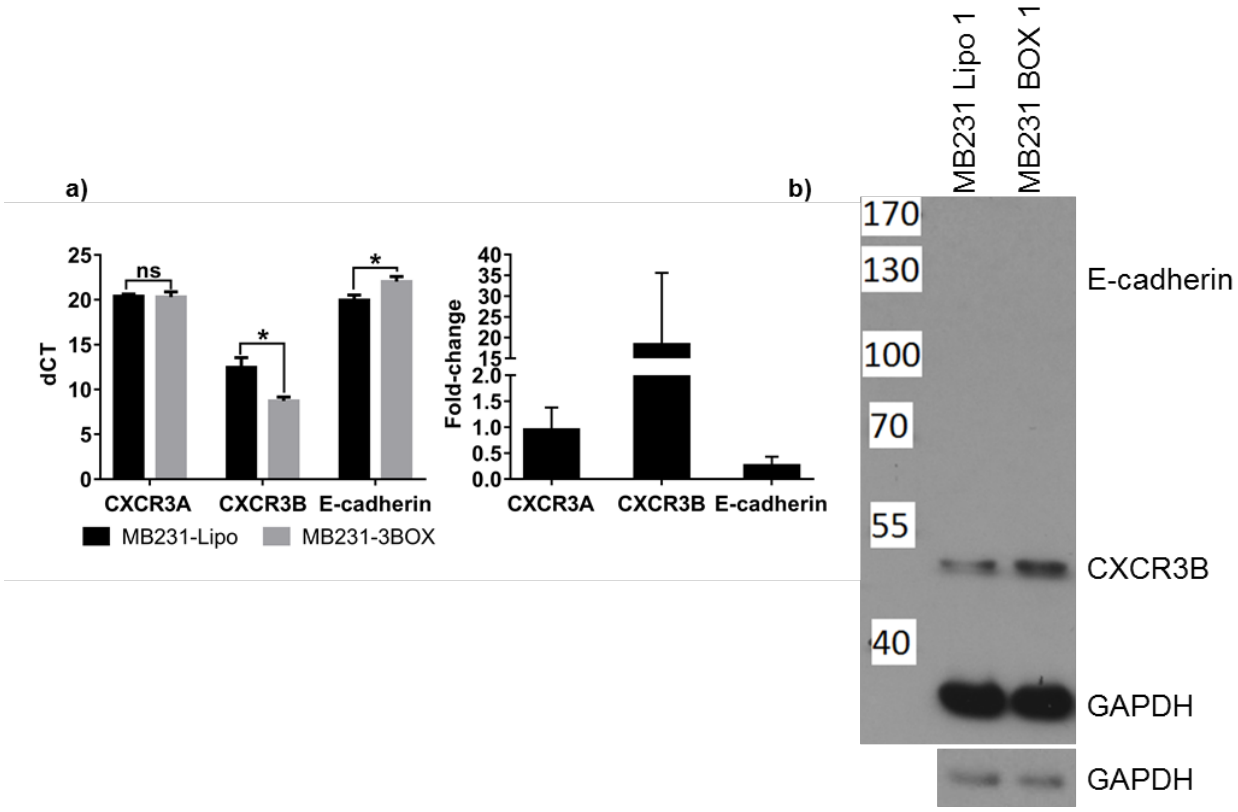
Table 3: Cell lines used in CXCR3 study

Cell line	Tissue	CXCR3 isoform	E-cadherin
DU145 E-cadherin^{low} (DU-low)	Prostate carcinoma Metastatic site: brain	High CXCR3A Low CXCR3B	Low
DU145 E-cadherin^{high} (DU-high)	Prostate carcinoma Metastatic site: brain	High CXCR3A Low CXCR3B	High
PC3	Prostate adenocarcinoma Metastatic site: bone	High CXCR3A Low CXCR3B	Low
LNCaP	Prostate adenocarcinoma Metastatic site: lymph node	Low CXCR3A High CXCR3B	High



Supplementary figure 1: CXCR3B knock down decreased E-cadherin on cell membrane.

Immunofluorescence of E-cadherin (green) on parental DU-high (left panel) and DU-high transfected with p-RFP-CS-CXCR3B-shRNA (middle panel: DAPI and E-cadherin-AF488 merge; left panel: DU-RFP+DAPI+E-cadherin-AF488 merge. Bar=50µm.



Supplementary figure 2: CXCR3B overexpression does not affect cells with hypermethylated E-cadherin.

a) Average ΔCt values with SD (top) and average mRNA fold change (bottom) of CXCR3A, CXCR3B and E-cadherin normalized to GAPDH in MDA-MB231 cells transfected with pTarget-CXCR3B. N=3. Student's t-test was performed to determine statistical significance with p-value<0.05*. b) Immunoblot of CXCR3B and E-cadherin and GAPDH as a loading control. N=4.

B.2 MATERIALS AND METHODS

B.2.1 Cell culture

Parental DU145 E-cadherin^{low} and E-cadherin^{high} cells were cultured in DMEM with 4.5g/L glucose, L-glutamine and sodium pyruvate (Corning) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco). PC3 cells were cultured in F12K medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. MDA-MB231 cells were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin.

B.2.2 Transfection

pTarget-CXCR3B was a gift from Dr. P. Romagnani from Italy. pCMV-ddk-CXCR3B and CXCR3B-specific shRNA plasmids were purchased from Origene. Cell were seeded and cultured for an overnight. Transfection was performed using Lipofectamine-2000 (Invitrogen) according to manufacturer's protocol for 4-6 hours and allowed to recover in complete medium for 72 hours. CXCR3B overexpressing cells were subsequently cultured in complete DMEM medium with 1mg/ml G418. CXCR3B knocked-down cells were sorted for RFP expression and cultured in complete DMEM medium with 5μg/ml puromycin.

B.2.3 Immunoblot

Cancer cells were seeded and cultured until the cells reached 90% confluency. Ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors was used to harvest cell lysates. BCA assay (Thermo Fisher) was performed to determine protein concentration. Proteins were separated using SDS-PAGE and transferred to PVDF membrane (Millipore). Primary antibody incubation was performed at 4^{o}C for an overnight and secondary antibody was performed at room temperature for an hour. Protein bands were detected using chemiluminescent substrates (Thermo Fisher) on autoradiography films with SRX-101A developer (Konica Minolta).

B.2.4 Quantitative reverse-transcription PCR (qRT-PCR)

Cells were seeded and cultured until 90% confluency. RNA was harvested using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Reverse transcription was performed using Quantitect Reverse Transcription kit (Qiagen) and qRT-PCR was performed using Fast SyBr Green master mix (Invitrogen) on MX3000P Stratagene machine (Agilent Genomics). The primers used were as follows: 5'-GAGTCAACGGATTTGGTCGT-3' (GAPDH forward), 5'-TTGATTTTGGAGGGATCTCG-3' (GAPDH reverse), 5'- AGCCCAGCCATGGTCCTTGA-3' (CXCR3A forward), 5'- CTGTAGAGGGCTGGCAGGAA-3' (CXCR3A reverse), 5'-TGCCAGGCCTTTACACAGC-3' (CXCR3B forward), 5'- TCGGCGTCATTTAGCACTTG-3' (CXCR3B reverse), 5'- CTGTGCCCAGCCTCCATGTTTT-3' (E-cadherin forward) and 5'-CTGGATAGCTGCCCATTGCAAGTTA-3' (E-cadherin reverse). Gene expression fold-change was normalized against GAPDH and calculated using ΔΔCt method.

B.2.5 Flow cytometry

Cells were cultured for an overnight in complete DMEM medium. The following day, cells were trypsinized, washed and fixed with 4% paraformaldehyde (Electron Microscopy Sciences). Cells were then incubated with primary antibody for 30 minutes at room temperature, followed by secondary antibody incubation for 30 minutes at room temperature with PBS washing in between the incubation periods. Sample incubated in secondary antibody alone was used a negative control. Samples were run on FACS Calibur (BD Biosciences).

B.2.6 Immunofluorescence

Cells were cultured on heat-sterilized coverslips. The cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences), permeabilized with 0.5% Triton-X 100 (Sigma) and blocked with 5% bovine serum albumin (Sigma) for one hour at room temperature. Cells were then incubated with primary antibodies for an overnight at 4^{o}C. Then, the cells were rinsed with PBS and incubated with secondary antibodies for an hour at room temperature. Cells were imaged using Olympus BX40 microscope or Olympus Fluoview 1000 microscope.

B.2.7 cAMP ELISA

Cells were cultured and serum starved for 24 hours. Next, the cells were treated with 5uM forskolin for positive control. No treatment samples were used as the negative controls. 100µM 3-isobutyl-1-methylxanthine, a non-selective phosphodiesterases inhibitor was added to all culture conditions. Samples were treated with the chemokine for 2 hours in the incubator.

Samples were then washed with PBS and extracted with 0.1M HCl at room temperature for 10 minutes. ELISA was performed and analyzed according to manufacturer's protocol.

B.2.8 Intrasplenic inoculation and chemotherapeutic drug treatment

The animal studies and procedures were approved by the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Institutional Animal Care and Use Committees of Veteran's Administration Pittsburgh Health System. Seven-week-old male NOD/SCID gamma mice (00557, Jackson Laboratory) were anesthetized with ketamine/xylazine. Long-acting buprenorphine was used as pain reliever. Sterile surgery was performed to expose the spleen. Half a million of cancer cells were injected into the spleen using 27-gauge needle. Running stitch with absorbable suture was performed to close the omentum and metal wound clips were used to close the skin wound. Paclitaxel (Fresenius Kabi) was administered at 10mg/kg body weight via i.p every 2 days for a total of 5 rounds starting from 2.5 weeks post-injection. Mice were sacrificed after 5 weeks using a carbon dioxide chamber according to AVMA Guidelines on Euthanasia.

B.2.9 Colorectal cancer human specimens

Paired colon cancer and liver metastases tissues were obtained from the University of Pittsburgh, Tissue and Research Pathology Services/ Health Sciences Tissue Bank which receives funding from P30CA047904. The use of these tissues and slides was approved by the University of Pittsburgh institutional review board.

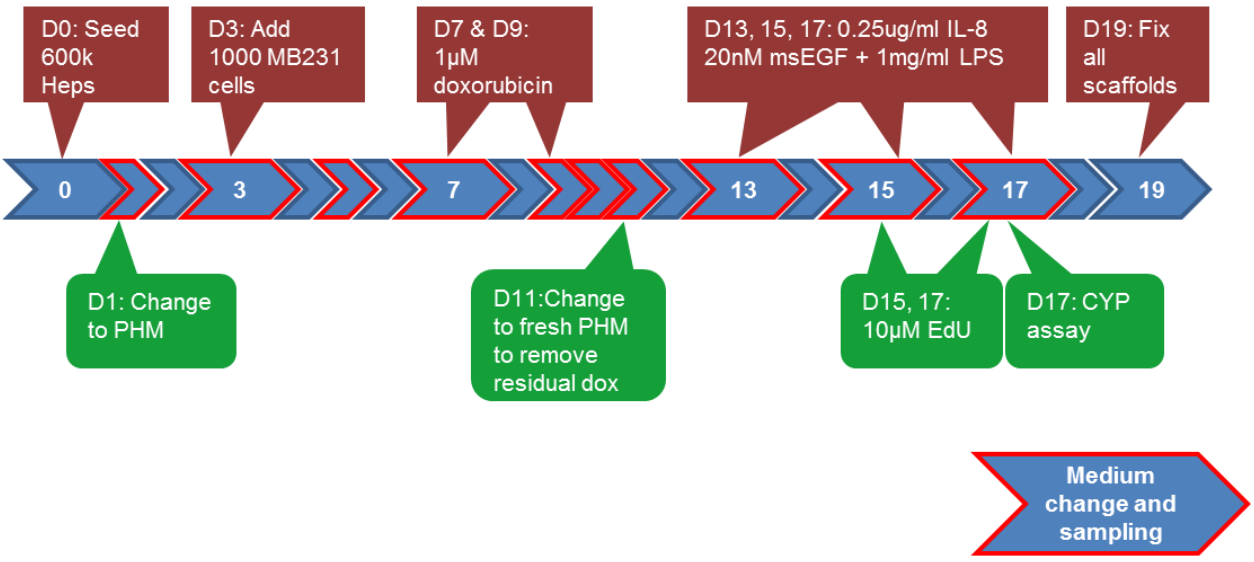
B.2.10 Reagents

The following antibodies and reagents were used throughout the study: mouse anti-human CXCR3B specific antibody (Proteintech), rabbit anti-human GAPDH (Cell Signaling), rabbit anti-human B-actin (Sigma), mouse-anti ddk tag (Origene), mouse anti-human E-cadherin (Invitrogen)-for immunofluorescence and rabbit anti-human E-cadherin (Cell Signaling)-for immunoblotting.

Appendix C

SUPPLEMENTARY INFORMATION: STELLATE CELL-DERIVED IL-8 PROMOTES TUMOR ESCAPE FROM DORMANCY IN THE LIVER

C.1 SUPPLEMENTARY FIGURES



Supplementary figure 3: 3D *ex vivo* MPS experimental flow.

An overview of *ex vivo* MPS experimental workflow. Primary hepatocytes were seeded in the scaffolds on day 0 in William's E medium supplemented with the Hepatocytes Thawing and Plating Supplement Pack (Life Technologies). The medium was change to physiologic medium on day 1 and every other day. Cancer cell were

seeded on day 3. Doxorubicin treatment was performed on day 7 for 72 hours. LPS+EGF or IL8 treatments were performed starting on day 13 until day 19. 10μM EdU was added on day 15 for 96 hours. CYP assays were performed on day 17. Supernatants were collected during medium change. CYP analyses were performed by our collaborator, Dr. Venkataramanan and clinical assays were performed by the University of Pittsburgh Medical Center certified clinical laboratories.

	A	B	C	D	E	F	G	H	I	J	K
1	POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GRO a/b/g	GRO alpha (CXCL1)
2	I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
3	IL-12 p40/p70	IL-13	IL-15	IFN-gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
4	MIP-1 delta	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
5	OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta 8-1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
6	FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha	NAP-2 (CXCL7)	NT-3
8	NT-4	OPN (SPP1)	OPG (TNFRSF11	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

Supplementary figure 4: Ray Biotech array map.

Ray Biotech ACH-CYT-5 Protein array map. Main chemokines or cytokines secreted by the LX1 cells are boxed in red.

Table 4: Cell lines used in HSCs/IL8 study

Cell line	Tissue/origin	Note
LX1	Human hepatic stellate cells (immortalized, nontransformed)	(Xu et al. 2005)
LX2	Human hepatic stellate cells (immortalized, nontransformed)	(Xu et al. 2005)

TWNT-1	Human hepatic stellate cells (immortalized, nontransformed)	(Watanabe et al. 2003)
TMNK-1	Human liver endothelial cells (immortalized, nontransformed)	(Matsumura et al. 2004)
HSC-T6	Rat hepatic stellate cells (immortalized, nontransformed)	(Vogel et al. 2000)
HMEC-1	Human mammary epithelial cells (normal, nontransformed, non-immortalized)	Lonza
MCF-7	Human breast adenocarcinoma Luminal A type Metastatic site: pleural effusion	E-cadherin positive ER+/PR+/HER2-; EGFR+ CXCR1 negative; CXCR2 low (IL8) CCR2 positive (MCP-1) TLR4 positive (LPS)
MDA-MB231	Human breast adenocarcinoma Basal/triple negative Metastatic site: pleural effusion	E-cadherin negative ER-/PR-/HER2-; EGFR+ CXCR1 negative; CXCR2 positive (IL8) CCR2 positive (MCP-1) TLR4 positive (LPS)

C.2 MATERIALS AND METHODS

C.2.1 Cell culture

RFP-labeled MDA-MB231 and MCF7 cells were maintained in RPMI-1640 Glutamax (Gibco) supplemented with 10% FBS, 1X penicillin/streptomycin (Gibco) and puromycin (5μg/ml for MDA-MB231 and 1μg/ml for MCF7). Mammary epithelial growth medium (MEGM) was prepared according to manufacturer's protocol (Lonza) and used to maintain human mammary epithelial cells (HMEC-1) (Lonza). Human hepatic stellate cells (LX1 and LX2) and rat stellate cells (HSC-T6), gifts from Dr. Scott Friedman (Mount Sinai School of Medicine, NY), were cultured in 4.5 g/l of glucose DMEM without sodium pyruvate (Lonza) supplemented with 10% FBS (2% FBS for LX2) and 1X penicillin/streptomycin. TWNT-1 (human stellate cell line) and TMNK-1 (human endothelial cell line) cells, gifts from Dr. Alex Soto-Gutierrez (University of Pittsburgh, PA), were cultured in 4.5 g/l of glucose DMEM with L-glutamine and sodium pyruvate (Corning) supplemented with 10% FBS and 1X penicillin/streptomycin.

C.2.2 Co-culture experiments

2×10^4 cells/cm^2 of LX1 cells were seeded in 24-well plates and cultured for an overnight in complete DMEM+10% FBS medium. HMEC-1 cells were labeled with 10μM CMTPX CellTracker (Molecular Probes) for 45 minutes prior to co-culture with LX1 cells. 2×10^3

cells/cm^2 RFP-labeled cancer cells or CMTPX-labeled normal cells were seeded onto LX1 culture in serum-free hepatocyte maintenance medium (HMM). Breast cancer or normal cells cultured in HMM and RPMI/MEGM complete media were used as negative and positive control, respectively. The samples were imaged for RFP signals after 4-6 hours of co-culture (day 0). On day 2, the media were exchanged and 20μM of EdU (Invitrogen) were added to each sample. On day 4, the samples were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and imaged for RFP signals at the same spots of day 0. RFP percent (RFP %) area was quantified using ImageJ and normalized to day 0 for growth fold change. EdU staining was performed according to manufacturer's protocol. Samples were randomly imaged for RFP, EdU-Alexa Fluor 488 (Invitrogen) and DAPI (as a counterstain). The number of EdU positive cells was counted using ImageJ and plotted as a percentage to the total RFP cells.

C.2.3 Transwell assay and CXCR2 neutralization

LX1 cells were seeded in transwell inserts and cultured overnight. The next day, RFP-labeled cancer cells were seeded in 24-well plates and cultured with LX1 transwell insert in HMM. 1μg/ml CXCR2 (R&D) was added into additional transwell samples for CXCR2 neutralization. RFP% area and EdU incorporation were imaged and analyzed as previously described.

C.2.4 Cell culture on polyacrylamide (PA) gels

PA gels were prepared according to a published protocol (Dingal et al, 2015). Briefly, top glass coverslips and bottom glass coverslips were sterilized with boiling ethanol for 10 minutes. The bottom coverslips were rinsed in distilled water, ethanol, chloroform and silanized with

chloroform +0.1% triethylamine (Sigma) and 0.1% allyltrichlorosilane (Sigma) for 30 minutes at room temperature. The coverslips were then rinsed in chloroform, ethanol, distilled water and stored in a desiccator. 0.3kPa polyacrylamide solution was prepared with 2.6% acrylamide (Bio-Rad) and 0.07% bis-acrylamide (Sigma) whereas 40kPa solution was prepared with 10% acrylamide and 0.3% bis-acrylamide in distilled water. 0.1% ammonium persulfate (Sigma) and 0.1% tetramethylethylenediamine (Sigma) were added to the solutions to initiate polymerization. 7μl/cm2 PA solution was pipetted onto the silanized bottom coverslips and sandwiched with top coverslips and incubated at room temperature for 30 minutes for polymerization. Sandwiched PA gels were rinsed with phosphate-buffered solution (PBS) and stored at 4^{o}C in distilled water. To prepare the gels for cell culture, the top coverslip was removed and the gel was rinsed with PBS and distilled water. 5mg/ml of sulfo-SANPAH (Thermo Fisher) was prepared in 50mM HEPES pH8 and pipetted onto the gels. The cross-linker was activated with 365nm ultra-violet light for ten minutes. The activated PA gels were rinsed with PBS and distilled water and coated with 0.1mg/ml laminin (Corning) for an overnight at 4^{o}C. The coated gels were rinsed twice with sterile PBS and sterilized with UV light in the culture hood for at least two hours. LX2 cells were cultured on the gels for about 24-48 hours. The activation state of the LX2 cells was verified with α-SMA, F-actin and Lamin A/C staining. Cell area was quantified based on F-actin staining using ImageJ. The fluorescence intensity for Lamin A/C was quantified using a published protocol with ImageJ (McCloy et al, 2014). To assess the induction of cell proliferation, the media in both 0.3kPa and 40kPa gels were changed to DMEM+0.2% dialyzed FBS. The condition media were harvested after 24 hours and applied to MCF7 and MDA-MB231 culture to determine the level of EdU incorporation as previously described.

C.2.5 Protein array

LX1, MCF7, MDA-MB231 cells were seeded and cultured for an overnight. The following day, the cells were washed once and the media were changed to HMM. After 24 hours, the supernatants were collected and the numbers of cells were counted. Protein array membranes (RayBiotech Human Cytokine Array C5) were prepared according to manufacturer's protocol prior to supernatants collection. The supernatants were incubated with the membranes for an overnight at 4°C. Antibodies incubations were performed at room temperature for two hours and the signals for the cytokines were detected using chemiluminescence detection on film. The intensity of selected spots was quantified using ImageJ.

C.2.6 3D *Ex vivo* Liver MPS

The 3D *ex vivo* liver MPS was assembled according to manufacturer's recommendation (CN Bio Innovations Ltd, Oxford, UK). Polystyrene scaffolds were coated with 1% rat tail collagen I (BD Biosciences) for an hour at room temperature prior to cell seeding. The assembled MPS was passivated with 1% BSA for one hour at 37°C. Primary human hepatocytes were obtained from the laboratory of Dr. David Geller through the NIDDK-funded Liver Tissue and Cell Distribution System (LTCDS) at the University of Pittsburgh. The isolated primary hepatocytes were seeded at 6×105 cells per scaffold in William's E medium (Gibco) supplemented with the Hepatocytes Thawing and Plating Supplement Pack (Life Technologies). The medium was exchanged to in-house physiologic medium (PHM) the next day and after every 48 hours and supernatants were collected and stored at -80°C for downstream assays. Physiologic medium was prepared by diluting phenol-red free William E medium (Gibco) to 5.5mM glucose and

supplemented with 6.25μg/ml Transferrin (Lonza), 6.25ng/ml sodium selenite (Sigma), 550nM hydrocortisone, 800pM Insulin and 25mg/ml human serum albumin. The experimental workflow is summarized in supplementary figure 1. Briefly, tissue formation was estimated to be completed after 3 days and 1×103 RFP-labeled MDA-MB231 cancer cells were introduced into the MPS. Cells were treated with 1μM doxorubicin for 72 hours starting on day 7. On day 13 to day 19, the cells were treated with 250ng/ml of IL-8 (R&D Systems) or stimulated with 20nM mouse EGF (Sigma) and 1μg/ml lipopolysaccharides (Sigma) as the positive controls. Unstimulated doxorubicin-treated cells were used as the negative controls. 10μM of EdU (Life Technologies) were added to the physiologic medium for 96 hours to detect proliferating cells. Finally, the scaffolds were fixed with 2% cold-paraformaldehyde for 1 hour in 4^{o}C and stained for EdU according to manufacturer's protocol. RFP % area for the whole scaffolds were imaged with wide-field 2X objective lens on Olympus Provis microscope and Olympus Fluoview 1000 confocal microscope was used to image for EdU incorporation in all RFP-labeled cells.

On day 17, a control scaffold was rinsed with 0.4ml of a cocktail of CYP substrates followed by an hour of incubation with 1.6ml of the CYP substrates. The supernatants were collected and kept in -80^{o}C prior to analysis.

For Luminex assay, isolated human NPC fraction was further purified using 25%:50% Percoll-gradient centrifugation method. 6×105 of human primary hepatocytes were cultured with or without 6×105 of primary NPC in supplemented William's E medium. Media were changed to fresh William's E medium on day 3 and every 48 hours thereafter. Supernatants were collected for Luminex analyses.

C.2.7 Luminex multiplex immunoassays

Luminex analyses were performed as previously published (Wheeler et al, 2014). Briefly, the level of 55 analytes were determined using human group 1 27-plex, cancer panels 1 and 2 according to the manufacturer's recommendations (BioRad Laboratories, Inc., Hercules CA). Samples and antibodies were diluted by 2-fold and a total volume of 50µl diluted sample was analyzed per multiplex panel. Prepared arrays were assessed by the Bio-Plex 3D Suspension Array System (BioRad Laboratories, Inc.) utilizing xMAP technology licensed by Luminex. Data were collected with xPONENT for FLEXMAP 3D software, version 4.2 (Luminex Corporation, Austin TX).

C.2.8 Flow cytometry for MCF7 growth curve

2×10^3 cells/cm^2 RFP-labeled MCF7 cells were seeded together with 2×10^4 cells/cm^2 of stromal cells in HMM. MCF7 cells in HMM and RPMI complete media were used as negative and positive control, respectively. 6 hours post-seeding, the cells were trypsinized and centrifuged. The pellets were re-suspended in 300µl of PBS+0.2% FBS with 10µl of CountBrightTM Absolute counting beads (Thermo Fisher) and run on FACS Calibur (BD Biosciences). On day 2, the media were exchanged and 20µM of EdU (Invitrogen) were added to each sample. On day 4, the cells were harvested and run on FACS Calibur. The absolute cell number was determined using the formula provided with the beads. Kruskal-Wallis one-way ANOVA was performed to determine the statistical significance for all conditions and uncorrected Dunn's multiple comparison tests was performed to determine the significance of each treatment compared to the negative controls. n= at least 3.

C.2.9 Quantitative reverse-transcription PCR (qRT-PCR)

LX1 cells were seeded in DMEM+10% FBS and cultured for an overnight. The following day, the medium was change to HMM alone, HMM with 2.5ng/mL TGFβ1 (R&D) or HMM with 10ng/mL IL-10 (R&D). After 24 hours of treatment, the samples were washed with PBS (Gibco). RNA was harvested using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Reverse transcription was performed using Quantitect Reverse Transcription kit (Qiagen) and qRT-PCR was performed using Fast SyBr Green master mix (Invitrogen) on MX3000P Stratagene machine (Agilent Genomics). The primers used were as follows: 5'-GAGTCAACGGATTTGGTCGT-3' (GAPDH forward), 5'-TTGATTTTGGAGGGATCTCG-3' (GAPDH reverse), 5'-ATGAGAAATTCCACCACAAGATG-3' (HSP47 forward), 5'-GATCTTCAGCTGCTCTTTGGTTA-3' (HSP47 reverse), 5'-AACATGACCAAAAACCAAAAGTG-3' (α1(I) procollagen forward) and 5'-CATTGTTTCCTGTGTCTTCTGG-3' (α1(I) procollagen reverse). Gene expression fold-change was normalized against GAPDH and calculated using ΔΔCt method.

C.2.10 *In vitro* chemokine treatments

RFP-labeled MCF7 breast cancer cells were seeded in serum free RPMI medium supplemented with 1.0μg/ml IL-8 (R&D), 20nM human EGF (Sigma), 0.5μM SCH772984 (Cayman Chemicals) or 0.25μg/ml MCP-1 (R&D). 0.25μg/ml IL-8 (R&D) was used for MDA-MB231 cells. 4 to 6-hour post-seeding, the samples were imaged for RFP signals (day 0). On day 2, the media were changed to fresh media with appropriate chemokines/growth factors treatments plus

20μM of EdU (Invitrogen). On day 4, samples were fixed and imaged for RFP signals. The quantification and analysis for RFP % area and EdU incorporation were performed as described.

C.2.11 Immunoblotting for ERK activation

Cancer cells were seeded and cultured in complete medium overnight. Cells were then washed and serum-starved for 24 hours and followed with 1.0μg/ml of IL-8 treatment (0.25μg /mL for MDA-MB231) with or without 0.5μM SCH772984 under serum-free condition for 15 minutes. RPMI complete medium or 20nM EGF was used as positive controls while serum-free samples served as the negative controls. The samples were washed with ice-cold PBS and lysed with RIPA buffer. Proteins were separated using SDS-PAGE and the samples were probed for phospho-ERK (Cell Signaling), total ERK (Cell Signaling) and β-tubulin (Santa Cruz Biotechnology).

C.2.12 Immunofluorescence

Cells were plated on glass coverslip in DMEM complete medium and cultured overnight. The cells were then fixed with cold 4% paraformaldehyde, permeabilized with 0.5% Triton-X 100 (Sigma) and blocked with 5% bovine serum albumin (Sigma). Cells were incubated with the primary antibodies at 4^{o}C overnight. After washing, the cells were incubated with Alexa Fluor 488 or 594 secondary antibodies (Invitrogen) for one hour at room temperature. DAPI was used to counterstain the cells. Cells were imaged using Olympus BX40 or Olympus Fluoview-1000. The following antibodies were used for immunofluorescence staining: mouse anti-human alpha smooth muscle actin (αSMA) (Abcam), mouse anti-human glial fibrillary acidic protein (GFAP)

(Cell Signaling), rabbit anti-human Von Williebrand factor (vWF) (Santa Cruz), goat anti-human CD31 (Santa Cruz), Lamin A/C (Cell Signaling) and rhodamine-phalloidin (Invitrogen).

C.2.13 Clinical chemistry and Cytochrome P450 assays

Clinical assays for glucose (GLU), blood urea nitrogen (BUN), aspartate transaminase (AST) and alanine aminotransferase (ALT) were performed in certified clinical laboratories in the University of Pittsburgh Medical Center (Pittsburgh, PA). Cytochrome P450 enzyme activities were measured using LC-MS/MS to analyze an established CYP cocktail substrates that were prepared according to FDA guidelines for *in vitro* CYP P450 analysis (Pillai et al, 2013).

C.2.14 Data analysis and statistics

Image analysis was performed using ImageJ 1.51k (NIH Bethesda, MD, USA). Graphs were generated using GraphPad Prism 7 for Windows (GraphPad Software, Inc La Jolla, CA, USA). Statistical tests were computed using GraphPad Prism 7 for Windows and Microsoft Excel (2016).

Appendix D

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