The Effect of Lean or Obese Mouse Adipocyte on the Mesenchymal-to-Epithelial Transition of MDA-MB-231 Cells in 3-Dimensional Culture.

By

©Emmanuel Asante, BSc. (Hons.)

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Department of Biochemistry, Faculty of Science

Memorial University of Newfoundland

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ABSTRACT

Obese breast cancer (BC) patients have at least 30% increased risk of death from BC compared to non-obese BC patients. This is because obesity is associated with larger tumor sizes and increased rates of metastasis. Obese BC patients respond poorly to treatment compared to non-obese patients, especially when diagnosed with the triple negative BC (TNBC) subtype. The reasons behind the poor treatment outcomes associated with obesity are not fully understood. The Christian Laboratory had previously established a novel 3-dimensional co-culture system that permitted the co-culture of adipocytes and TNBC cells in a manner that mimics an *in vivo* milieu. Using this system, I demonstrated that lean and obese mice white adipose tissue could induce a mesenchymal-to-epithelial-transition (MET)-like effect in TNBC cells, thus furnishing the BC cells with properties that enable them to colonize and establish tumors in secondary organs. I have also shown that pro-MET factors are systemically secreted. Taken together this study suggests that adipose tissue has the potential to promote secondary tumor formation in lean and obese women. Further work is needed to determine if targeting the MET-like effect induced adipose tissue could reduce metastasis.

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2D	2-dimensional
3D	3-dimensional
BAT	Brown adipose tissue
BC	Breast cancer
BMA	Bone marrow adipocytes
BMI	Body mass index
BMSC	Bone marrow mesenchymal stem cells
CAF	Cancer associated fibroblasts
CM	Conditioned media
CTC	Circulating tumor cells
DCIS	Ductal carcinoma in situ
DIO	Diet induce obesity
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
GSK	Glycogen synthase kinase
HER	Human epidermal growth factor
HFD	High fat diet
HIF	Hypoxia-inducible factor

IF	Immunofluorescence
IL	Interleukin
IntDen	Integrated density
LCIS	Lobular carcinoma in situ
LFD	Low fat diet
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metalloprotease
mRNA	Messenger ribonucleic acid
MUN	Memorial University of Newfoundland
NF-κB	Nuclear factor kappa B
PBS	Phosphate buffered saline
PR	Progesterone receptor
RPMI	Roswell Park Memorial Institute
TGF-β	Transforming growth factor beta
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

CHAPTER 1: INTRODUCTION

1.1 Breast Cancer

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer related mortality among women globally. In 2018, about 2.1 million new cases were recorded worldwide and over 600,000 deaths occurred [1]. BC is ranked second to lung cancer as the leading cause of all cancer deaths [2]. BC survival rates vary geographically. About 80% of BC patients who reside in high income countries have a 5-year relative survival, compared to less than 40% of patients who live in poor or low-income countries [3]. This disparity can be attributed to lack of adequate early detection and treatment programs in low-income countries [4].

The pathoetiology of BC is not fully understood as a significant proportion of the disease develops sporadically [5]. However, several risk factors have been positively associated with the pathogenesis of the disease, include gender [6], age [7], race [8, 9], family history of the disease [10], body weight [11], genetic mutations [12], hormone replacement therapy [13], alcohol consumption [14], radiation exposure [15], and reproductive history [16]. The pathogenesis of BC can be understood by studying the anatomy of the female breast.

The female breast is made up of the mammary gland, adipose tissue, and loose fibrous connective tissue [17]. The adipose tissue and the connective tissue make up the stroma that supports the mammary gland. The mammary gland is composed of lobules, the milk

producing unit, and the lactiferous ducts that drain milk to the nipple. Each lobule is comprised of alveoli, which are hollow cavities of luminal epithelial cells. A layer of supportive myoepithelial cells surrounds the luminal epithelial cells. The lactiferous ducts are also lined with epithelial cells that are supported by myoepithelial cells. BC arises when the cells of the lobule or the duct undergo a neoplastic transformation which may become invasive and metastatic or benign [18]. Cancers arising from the luminal epithelial cells are known as luminal cancers while those arising from the basal cells are termed basal cancers.

There is an intermediate stage of hyperplasia prior to the complete neoplastic transformation of normal cells of the mammary gland [18]. The intermediate stage can be atypical or usual lobular or ductal hyperplasia. Atypical hyperplasia is characterized by abnormalities in number, shape size, appearance, and growth pattern of the cells of the duct or lobule [19]. However, usual hyperplasia is marked by an increase in the number of benign cells [19]. Hyperplastic lesions may progress to ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS) [18]. In carcinoma *in situ*, the hyperplastic cells of the lobule and ducts are non-invasive and are separated from the normal microenvironment by the basement membrane and the myoepithelial cell layer [20]. The hyperplastic cells eventually become cancerous, and breach the integrity of the basement membrane and the myoepithelial cell layer, consequently enabling the cancer cells to invade into surrounding tissues where they further migrate to distal site via the lymphatic and blood streams thus leading to tumor dissemination (Fig 1.1) [20].

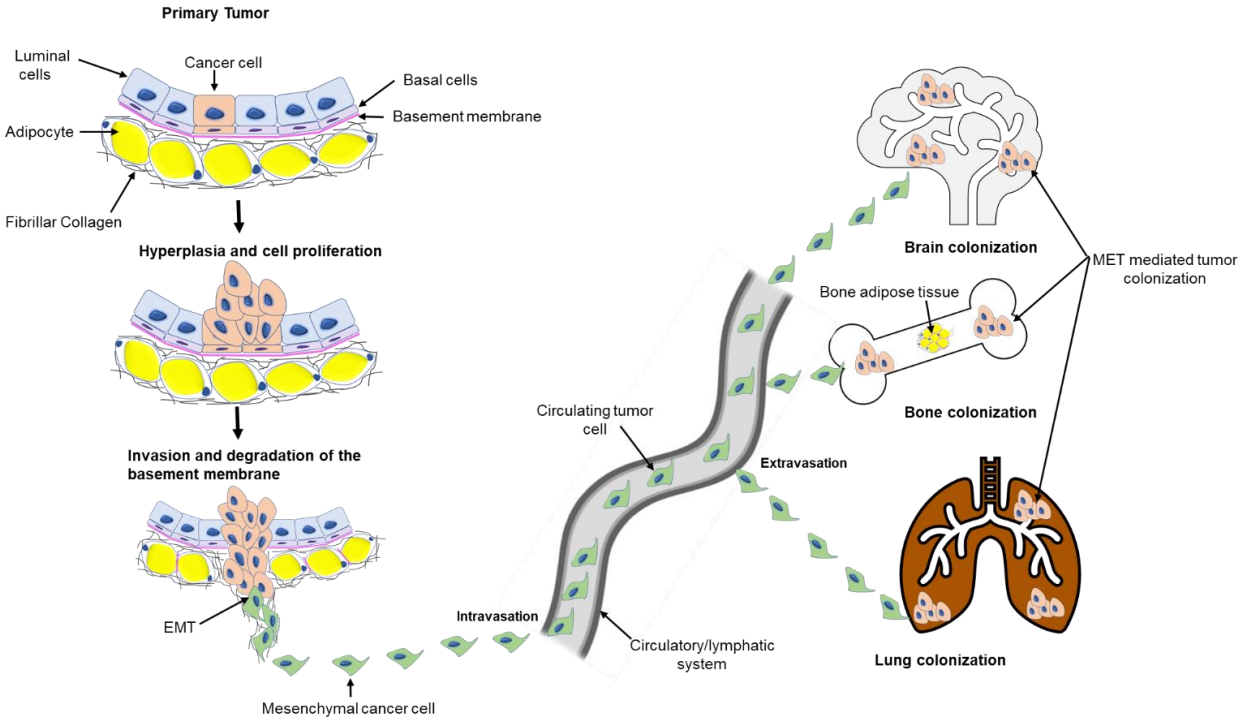


Figure 1.1: A schematic representation of breast cancer initiation and metastatic cascade

Genetic and epigenetic changes in the luminal and basal cells lead to the oncogenic transformation of cells thus making them hyperplastic and proliferative. The cancerous cells undergo epithelial-to-mesenchymal transition (EMT) and degrades the extracellular matrix (ECM) and basement membrane causing cancer cells to invade into the stroma. Consequently, the cancer cells migrate towards the nearest blood or lymphatic vessel where they intravasate into the vessel and are transported as circulating tumor cells to distal organs. Cancer cells extravasates from the blood vessel and undergo mesenchymal-to-epithelial transition (MET) to colonize the brain, lungs and bone. Information was sourced from [21]. The figure was generated with Microsoft Powerpoint 365.

1.2 Subtypes of Breast Cancer

BC is a heterogenous disease with different subtypes exhibiting distinct biological behaviours and clinical outcomes [22]. Broadly, the disease can be classified based on its histological or molecular profile [23]. The histological based classification is dependent on the histological features or morphology of the tumor and it categorizes the disease into two broad subtypes: *in situ* carcinoma and invasive carcinoma [19]. Depending on the growth pattern and the cytological phenotypes of the tumor, breast carcinoma *in situ* can be further subdivided into DCIS or LCIS [19]. The DCIS subtype also presents with distinct histological features and so far, five sub-groupings have been identified. These sub-groupings include the comedo, cribriform, micropapillary, papillary, and solid types [24]. The invasive carcinoma displays a high degree of heterogeneity, and as such, breast carcinomas which fall under this group are further sub-categorized into infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas [19]. Infiltrating ductal carcinoma is the most common carcinoma in this sub-group, accounting for 70-80% of all invasive carcinoma [23]. Infiltrating ductal carcinoma can be sub-stratified into well, moderately or poorly differentiated subtypes depending on factors such as nuclear pleomorphism, glandular/tubule formation and mitotic rate [25].

Although the histological classification system can be used in tumor staging and disease prognosing, it is not robust enough to predict patients' response to targeted therapies [26]. This flaw was addressed by the adaptation of a molecular based classification in conjunction with the histological classification system. The molecular classification system stratifies BC based on the presence or absence of the expression of

progesterone receptor (PR), estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2), and ki67 proliferation marker by the tumor [27]. The receptors are drug targets and its presence or absence on tumor cells can be used to predict patients who are likely to benefit from targeted therapy. The advent of microarray technologies for gene expression analysis and profiling has led to the discovery of additional unique BC subtypes, however these additional molecular subtypes do not have actionable drug targets at this time [23].

BC subtypes under the current molecular classification scheme include the luminal-like, HER2 enriched/positive, basal-like/triple negative breast cancer, and normal-like subtypes [27]. The luminal-like subtype has two variants: luminal A and luminal B [28]. About 40% of BC are of the luminal A subtype and they are characterized by the expression of ER and PR [23]. They have good prognosis and typically respond to the ER antagonist, tamoxofin [29]. The luminal B subtype is ER positive and can be HER2 positive or negative. They accounts for about 20% of the tumors and do not respond well to hormone therapies, thus making them more aggressive than the luminal A subtype [30]. The HER2 enriched class comprises 10-15% of breast tumors [23]. It mostly affects younger women and can respond to the anti-HER2 drug, trastuzumab [31]. The triple negative breast cancer (TNBC) lacks the expression of ER, PR, and HER-2 and mostly affects young and premenopausal women. It accounts for about 15-20% of BCs. TNBC is highly metastatic and has no targeted therapy hence, it has the grimmest prognosis of all the molecular breast cancer subtype [32].

One of the major advantages of the molecular based classification of BC is that it paints a more accurate picture of the disease prognosis by identifying patients who are most likely to respond to targeted therapy and those who are better off receiving a more aggressive chemotherapeutic treatment. The table below summaries the molecular profile of the various breast cancer subtypes (Tab. 1.1).

Table 1.1: Molecular subtypes of breast cancer and available therapies

Molecular Subtype	Frequency/%	Receptor Expression	Ki-67 Status	Available Therapy
Luminal A	40	ER +/PR+ /HER2 -	low	Hormonal therapy
Luminal B	20	ER+/ PR +/HER2 -/+	high	Hormonal therapy, anti-HER2 & chemotherapy
HER-2 Enriched	10-15	ER- /PR - /HER2 +	high	Anti-HER2 & chemotherapy
Triple Negative (Basal)	15-20	ER- /PR - /HER2 -	high	Chemotherapy
Normal-like	10	ER+ /PR + /HER2 -	Low/intermediate	Chemotherapy

PR = progesterone receptor, ER = estrogen receptor, HER2 = human epidermal growth factor 2, "+" = positive and "-" = negative. Adapted and modified from [33, 34]

1.3 Tumour Heterogeneity of Breast Cancer

Tumor heterogeneity relates to the idea that no two BCs are the same. It is the main cause of the variable therapeutic response and clinical outcome among BC patients. The two kinds of tumor heterogeneity include inter-tumor heterogeneity and intra-tumor heterogeneity [35]. Inter-tumor heterogeneity describes the variation in tumors of a given origin (e.g. breast) between patients, considering their histological features, molecular marker expression, biological behavior, and treatment outcomes [36]. Clear evidence of inter- tumoral heterogeneity in BC lies in the myriad of BC subtypes discussed in section 1.2. Intra-tumor heterogeneity on the other hand describes the presence of a heterogeneous cell population within a particular tumor [37]. This kind of heterogeneity can be seen at the histological, biomarker, genetic, and epigenetic levels [37]. Some BCs can show up to two distinct morphologies upon histopathological examination (e.g. IDC and mucinous carcinoma [23]) while others may show ambiguous morphological features, for instance, an IDC tumor may exhibit lobular characteristics.

Variations in breast tumor histology have been associated with specific genetic alterations [38-40]. Variation in ER/PR or HER2 expression within cells of a given BC biopsy is a common phenomenon [41, 42]. A few models have been proposed to explain the origin of intra-tumor heterogeneity including clonal evolution and cancer stem cell. The clonal evolution model proposes that an entire tumor mass is formed from an evolving expansion of monoclonal or polyclonal population of the tumor cells [36]. The cancer stem cell model proposes that the different cell population with a tumor arose from a single precursor cell [36]. A notable challenge posed by tumor heterogeneity is therapeutic

resistance because tumors typically have subpopulations of unique cells which respond differently to a given chemotherapeutic. This varied response can lead to disease relapse after treatment as not all cells are effectively killed [43].

1.4 Breast Cancer Metastasis

Tumor metastasis is a highly complex process that enables the dissemination of the tumor cells from its origin to distal parts of the body where they can establish secondary tumors [44]. Tumor metastasis accounts for about 90% of breast cancer related morbidity and deaths [45]. The most common sites of BC metastasis include the bone, brain, lungs and the liver [46]. Rarely, the tumor can also spread to the extraocular muscle [47] and to the urinogenital regions of the body [48-50].

Tumor metastasis is thought to be a multistep event which must be executed sequentially for tumor cells to successfully spread to distal sites. Broadly, the metastatic cascade can be broken down into four main processes: invasion, intravasation, extravasation, and colonization [21] (Fig 1.1). First, tumor cells dissociate from the primary tumor mass and undergoes an epithelial-to-mesenchymal transition (EMT), a process which enable them to invade the stroma and intravasate into blood or lymphatic vessels that then allows their transportation to distal organs [51]. Upon arriving at distal organs, the circulating tumor cells (CTC) then extravasate and undergo mesenchymal-to-epithelial transition (MET) to colonize distant organs [21].

1.4.1 Epithelial-to-Mesenchymal Transition

EMT is a biological process whereby polarized and adherent epithelial cells undergo biochemical and morphological changes that result in the loss of their baso-apical

characteristics and in the acquisition of mesenchymal cell phenotypes such as migratory capability, invasiveness, increased secretion on ECM components, and resistance to apoptosis [52]. EMT occurs in different biological contexts and is classified into type 1, type 2, and type 3. Type 1 EMT occurs in developmental processes such as implantation, embryogenesis, and organogenesis [53, 54]. Type 2 EMT is activated in response to tissue injury or trauma and is therefore associated with wound healing, fibrosis, and tissue regeneration [55, 56]. Type 3 EMT on the other hand, is associated with tumor progression, and metastasis [57]. Essentially, cancers hijack the EMT process to aid their metastatic effort. The remainder of this section shall expatiate on the type 3 EMT and BC metastasis.

The journey of a tumor cell from its origin to distal sites begins with its ability to invade into nearby tissues and blood vessels. It is hypothesized that this process is initiated when BC cells on the tumor margin undergo EMT to dedifferentiate from epithelial cells to mesenchymal cells [58]. This process is accompanied by the concomitant downregulation of E-cadherin, a protein that regulates cell-cell adhesion, and the upregulation of the expression of N-cadherin and vimentin [59]. The EMT process is under the regulation of the three main pathways: transforming growth factor beta (TGF-β), Notch and Wnt, although it can also be mediated by hypoxic condition in the tumor microenvironment (TME) [60]. The activation of these pathways leads to the upregulation of SNAIL, Zeb, and Twist [58]. The expression of these transcription factors results in the repression of E-cadherin [61]. Hypoxic TME induces EMT by causing changes in mitochondrial function which results in the activation of hypoxia-inducible factor 1 (HIF1) and expression of Zeb1 [58]. Additionally, previous studies have shown that hypoxic

conditions can induce EMT by blocking the activity of glycogen synthase kinase 3 beta (GSK3β) [60].

Upon the acquisition of invasive and migratory properties via EMT, metastatic BC cells then invade into surrounding tissues by degrading the basement membrane. The basement membrane serves as a physical barrier to restrict metastatic BC cells from invading into surrounding tissues [62]. EMT is accompanied by a surge in the production of matrix metalloproteinases (MMPs) by the tumor cells that degrade the basement membrane [63]. Following this, the tumor cells intravasate into the lymphatic and circulatory system where they migrate as CTC, either as single cells or as cluster of mesenchymal cells. The activation of membrane type-1 (MT1)-MMP and MT2-MMP enables the tumor cells to establish contact with endothelial cells thus aiding intravasation [64]. In addition, increased expression of vascular endothelial growth factor (VEGF) and MMPs in tumor cells enables them to breach the integrity of blood and lymphatic vessels during intravasation and extravasation [65].

1.4.2 Mesenchymal-to-Epithelial Transition

MET is the reverse process of EMT. During MET, epithelial cells that had acquired mesenchymal-like migratory characteristic lose this phenotype to regain their original epithelial characteristics [66]. Just like EMT, MET equally plays an important role during developmental processes such as implantation, kidney organogenesis, and somitogenesis [67-69]. However, in cancers it has been hypothesized that MET may play a critical role in the formation of secondary tumors, albeit the exact mechanisms are yet to be defined [66].

While very few studies have demonstrated MET in *in vivo* conditions [70], the re-expression of E-cadherin in secondary tumors cells has been proposed as a marker for detecting MET. This is because some secondary tumor cells tend to re-express E-cadherin following tumor establishment [71]. Yate and co-workers co-cultured mesenchymal human prostate cancer cells with hepatocytes to mimic liver metastasis [72]. They found that the expression of E-cadherin was upregulated in the prostate cancer cells upon contact with hepatocytes, thereby, indicating that the mesenchymal prostate cancer cells had acquired epithelial properties [72]. Yate and co-workers postulated that re-expressed E-cadherin facilitated cell-cell adhesion, a property which is instrumental in the establishment of secondary tumor [72]. In addition, Aokage *et al.*, immunoassayed EMT and MET biomarkers such as E-cadherin, β-catenin, and germinin in surgically resected specimens of metastatic tumors which were en route to the lungs via the lymphatic vessels [73]. The expression of E-cadherin, β-catenin, and germinin was upregulated in the epithelial state and downregulated in mesenchymal state [73]. Aokage and colleagues found that tumor cells in the lymphatic vessels had weak expression of E-cadherin, β-catenin, and germinin hence, indicating that the tumor cells were in a mesenchymal state [73]. However, the expression of the epithelial biomarkers were elevated in tumor cells in the lung parenchymal, therefore, suggesting the tumor cells had transitioned from a mesenchymal to epithelial state [73].

1.5 Breast Tumour Microenvironment

The TME can be described as the milieu around a proliferating tumor. The TME is heterogeneous and it comprises the stroma, blood vessels, endothelial cells, cancer

associated fibroblasts (CAF), immune cells, infiltrating inflammatory cells, adipocytes, signalling molecules, and ECM components [74]. The TME is dynamic and undergoes changes as the tumor progresses. Under physiological conditions, the ductal system of the mammary gland is typically comprised of luminal epithelial cells, myoepithelial, fibroblasts, and an intact basement membrane/ECM. However, as tumorigenesis ensues, the ductal environment becomes infiltrated with immune cells, CAF, as well as bone marrow derived macrophages [75]. In addition, the basement membrane/ECM loses its integrity through the action of molecules such MMPs which are released in the stoma thereby permitting tumor cells to invade into the surrounding tissue [76].

Evidence shows that the stromal cells and tumors cells are intimately connected. Stromal cells can influence tumor cells behaviour via the secretion of ECM proteins, chemokines, cytokines and growth factors, consequently promoting the survival, proliferation, invasion and of tumor cells [77]. The different stromal cells interact with and affect BC cells differently. For instance, CAFs have been shown to secrete growth factors such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF), TGF-β and stromal cell-derived factor-1 (SDF-1) which aid tumor cell proliferation [78-80]. In addition, CAF can promote proliferation, angiogenesis, invasiveness and tumor growth by secreting a variety of cytokines [81-83]. Some studies have also implicated CAF in the induction of EMT via paracrine TGF-β1 signaling [84, 85].

Adipocytes are prominent members of the TME and have also been implicated for their role in BC progression. Adiposity, in general, has been strongly correlated with poor clinical outcomes among BC patients. BC cells co-cultured with cancer associated

adipocytes have exhibited increased invasion and migration in *in vitro* and *in vivo* conditions [86]. Evidence also shows that mature adipocytes can induce EMT-like changes in BC cells thus promoting tumor metastasis [87]. Adiponectin secreted by adipocytes has also been shown to enhance the growth and survival of breast tumors [88].

The ECM can also influence tumor behaviour. The main components of the ECM include fibronectin, collagens, laminins, glycoproteins, and polysaccharides [89, 90]. Tumor progression is accompanied by an increase in ECM collagen, causing the ECM to undergo remodeling to become stiffer [91]. A collagen-rich and stiff TME has been shown to trigger the upregulation of oncogenic mRNAs and signaling pathways that promote tumor proliferation and invasion [92-94]. Angiogenesis, immune cell infiltration, and tumor growth have all been linked to ECM rigidity [95]. In addition, one study found that BC patients with collagen-rich stiff tumors are less likely to respond to neoadjuvant chemotherapy than patients with softer tumors [96]. Together, these data show a stiffer ECM can enhance breast tumor progression.

1.6 Obesity and Breast Cancer Progression

Obesity, crudely defined as body mass index (BMI) > 30 kg/m^2, is a major public health issue in different countries around the globe [97]. A 2013 study that assessed the global prevalence of obesity showed that about 36% of men, 38% of women, and 23% of children were either overweight or obese [98]. The global incidence of obesity is expected to reach approximately 20% by the year 2025 if preventive measures are not properly enforced [99].

Obesity is strongly associated with different pathological conditions such as hypertension, type 2 diabetes mellitus, cardiovascular disorders, and cancer [100]. With

respect to cancer, more than 40% of cancer patients are either overweight or obese at the time of diagnosis [101, 102]. In addition, obesity has been identified as an independent risk factor for BC [103]. Women who are obese have a 30% increased risk of death from BC compared to non-obese women [104]. Of note, post-menopausal obese women are at an increased risk of death from ER or PR positive BC while obese pre-menopausal women are most likely to die from triple negative breast cancer [105]. Obesity is associated with larger tumor sizes and increased rates of metastasis [106, 107]. Furthermore, obese breast cancer patients have poor treatment outcomes as they do not respond well to treatment compared to non-obese patients, especially those with TNBC [105, 108].

The connecting piece between BC progression and obesity is the adipose tissue. Adipose tissue can be described as a multifunctional and metabolically active endocrine organ. It stores excess lipids as triglycerides and secrete a variety of adipokines, cytokines, chemokines and hormone-like factors that regulate different physiological processes such as glucose and lipid metabolism, inflammatory response, insulin sensitivity and vascular endothelial function [109]. There are two types of adipose tissue in the body, namely brown adipose tissue (BAT) and white adipose tissue (WAT). BAT is involved in thermogenesis and thermoregulation particularly in neonates [110]. It accumulates around the kidney and adrenal glands and small amounts can be found on the scapular. The amount of BAT typically decreases within the first week of birth [110]. WAT depots are widely distributed around the body and can be categorized into subcutaneous WAT and visceral WAT [109]. Subcutaneous WAT is found underneath the skin where it guards against dermal infections, acts as an insulator to prevent body heat loss, and acts as a cushion to absorb external

mechanical stress [111]. Visceral WAT resides in the abdominal, thoracic and pelvic cavities and surround vital organs such as the heart, omentum, kidney, and gonads [111].

The WAT secretes a number of bioactive molecules. In the lean state they secrete anti-inflammatory cytokines such as interleukin (IL) - 4, IL-10, IL-13, and TGF-β1, and adiponectin [112]. However, in the obese state, WAT suffers system-wide chronic inflammation secondary to excessive deposition of lipids and expansion of the adipocyte [111]. This results in the activation of the nuclear factor kappa B (NF-κB) pathway and elevated secretion of leptin, as well as pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8, tumor necrosis factor alpha (TNF-α) and VEGF [113]. Furthermore, elevated levels of pro-inflammatory cytokines are strongly associated with increased cancer risk and tumor progression [112]. In particular, elevated levels of IL-6 can promote tumor proliferation, angiogenesis, EMT, and drug resistance in breast cancer [114]. Of note, obesity is linked to visceral metastases [115] and adipocytes from visceral WAT have enhanced effects on EMT of BC cells compared to those from subcutaneous WAT [116]. In summary, obese WAT creates a pro-oncogenic atmosphere which also contribute to poor prognosis in BC patients.

1.7 Model Systems

1.7.1 Mouse Model of Obesity

Animal models of obesity can be broadly categorized into genetic models and diet-induced obesity (DIO) models [117]. The most common genetic models feature rodents (rats and mice) with a spontaneous monogenic mutation or genetically engineered mutation in the leptin pathway or leptin receptor [118]. The first monogenic model to be described

was the *ob/ob* mouse which was serendipitously discovered by the Jackson Laboratory in 1949 [119]. The *ob/ob* mouse had a spontaneous single base deletion on the leptin gene and were unable to produce leptin [120]. Leptin is secreted by adipose tissue and it plays a critical role in appetite regulation. Mice which lack leptin have an insatiable appetite and become morbidly obese from hyperphagia [121]. *Ob/ob* mice suffer obesity related co-morbidities such as impaired glucose intolerance, insulin resistance, and hyperglycemia [122]. Other genetic models which relate to the leptin pathway are those that have mutations on the leptin receptor. Although leptin is secreted, these models do not respond to it thereby resulting in hyperphagia and consequently obesity. Examples of these model include *db/db* mouse, Zucker rat, ZDF rat, and Koletsky rat [118].

DIO models are generated by feeding rodents high fat diet (HFD), often *ad libitum*, causing them to gradually gain weight until they become morbidly obese. There is a school of thought that DIO models closely mirror obesity in humans therefore making them more preferable for obesity related studies than genetically induced obesity models [117, 123]. Although Sprague Dawley rats, Long Evans rats, Sand mice, spiny mice, and AKR mice strains are susceptible to DIO, the most commonly used animal is the C57BL/6N mouse. Evidence shows that C57BL/6N mice on high fat and carbohydrate diet present with metabolic abnormalities that parallel metabolic syndrome in humans [124]. In addition to obesity, the mice develop hyperinsulinemia, hyperglycemia, and hypertension [124]. It usually takes 16-20 weeks for C57BL/6N mice on a HFD to become overtly obese such that their body weight is 20-30% higher than control chow-fed C57BL/6N mice [125]. One notable disadvantage of DIO model is that there is no single standardized HFD, although

some studies use 45 or 60 kcal% fat diets from Research Diets Inc [126-129]. Pure fat diet supplement with regular chow is an alternative but a less commonly used diet for DIO models [117].

1.7.2 Three-Dimensional Cell Culture Model

Cell cultures have become an indispensable tool for studying different physiological and pathological processes *in vitro*. A number of ground-breaking discoveries in biosciences would not be possible without cell culture. There are two main kinds of cell culturing techniques: the conventional 2-dimensional (2D) monolayer culture and 3-dimensional (3D) culture. In 2D culture, cells grow in monolayer on a flat rigid substrate, while in 3D culture, cells grow in aggregates or spheroids on a scaffold or matrix. The main advantage of 3D culture over 2D culture is that cells grown in 3D exhibit features that are similar to the complex *in vivo* environment [130]. The unique characteristics of 3D grown cells are attributed to the scaffold/matrix on which they grow [130]. The scaffold/matrix can be biologically or synthetically derived. The most common commercially available matrices of biological origin include BD Matrigel™ basement membrane matrix (BD Biociences), Cultrex® basement membrane extract (BME; Trevigen), and hyaluronic acid [131]. Synthetically derived scaffold includes polyethylene glycol, polyvinyl alcohol, polylactide-co-glycolide, and polycaprolactone [131]. These scaffolds and matrices are enriched with substances such as laminin, fibronectin, and collagen, which make them mimic an *in vivo* extracellular matrix (ECM), thereby providing a more realistic and biologically active milieu for cells to interact with each other, proliferate, differentiate and carry processes which may not be observed in a 2D culture [130].

Due to its *in vivo* semblance, 3D culture models have been applied in different research fronts such as drug testing and pharmacological screening, gene and protein expression studies and cancer research [132-135]. In cancer research, 3D models have been employed in studying tumor cell signalling, tumor cell-ECM interaction, invasion, metastasis and angiogenesis [130]. In addition, features of tumor spheroids grown in 3D such as apicobasal polarization of cells, and acini-like structure formation have been shown to parallel that of *in vivo* tumors [136].

The utility of 3D culture models in the study of breast cancer progression have been explored [137-139]. One of the earliest studies that employed 3D culture models in breast cancer research was performed by Kenny and co-workers [140]. In this study, they compared the morphological characteristics of different breast cancer cell lines cultured in 3D, in the presence of an extracellular matrix, to those cultured in conventional 2D. The study showed that 3D cultured breast cancer cells can form one of four distinct colony morphologies: stellate, grape-like, round or mass-like structures [140]. These colony morphologies are dependent on the state of differentiation of the BC cells. For instance, mesenchymal breast cancer cell lines such as MDA-MB-231 cells formed stellate colonies with migratory spiky terminals in 3D culture, while epithelial cell lines such as MCF-7 formed robust and stable round/mass-like colonies [140, 141]. They also found that the gene and protein expression profiles of the 3D cultured cells differed from those cultured in 2D and correlated with the respective colony morphologies. Importantly, EMT and MET processes are more accurately modeled in 3D culture systems in comparison to 2D cultures [142]. Of note, the Christian laboratory established a novel 3D co-culture model which can

be used to co-culture breast cancer cells and mature adipocytes thus using a laminin rich ECM [143]. This model can be used to study how breast cancer cells interact with the ECM as wells as adipocytes, which are essential components of TME.

1.8 Rationale and Hypotheses

Numerous studies have established a clear link between obesity and breast cancer progression [144-147]. Obese premenopausal breast cancer patients tend to have a more aggressive disease course and may develop resistance to therapy especially when diagnosed with TNBC [105, 148]. The mechanisms underlying obesity-associated tumor progression remain largely elusive. Adipocytes are abundant in the breast TME and have been shown to fuel tumor growth and metastasis in multiple ways, including promoting EMT, as well as secreting altered levels of signalling molecules such as proinflammatory cytokines, adipokines, proangiogenic factors, and ECM proteins [149, 150]. Moreover, data from the Christian laboratory show that mesenchymal MDA-MB-231 TNBC cell line acquires epithelial phenotypes when co-cultured in 3D culture with mature adipocytes, in the presence of laminin-rich Matrigel [143]. In addition, evidence shows that this process can be, at least partially, mediated by factors secreted by adipocytes. This, therefore, suggests that adipocytes may play a role in MET-mediated secondary tumor establishment. Taken together, it can be surmised that the key to elucidating the mechanisms of obesity-induced tumor aggression and metastasis lies in understanding how adipocytes, especially in the obese state, interact with tumor cells.

I hypothesize that:

1. Adipocytes from obese mice, via the secretion of a pro-MET factor(s), will enhance MET-like changes in MDA-MB-231 TNBC cells, with potential differences between visceral and subcutaneous WAT, and bone marrow adipose tissue depots, when compared to adipocytes from lean mice.
2. Pro-MET factors are systemically secreted and serum from obese mice will promote MET-like changes in MDA-MB-231 TNBC cells more strongly than serum from lean mice.

Research Objectives

1. Determine the effect of lean or obese mouse subcutaneous or visceral WAT on the MET of MDA-MB-231 cells in 3D culture.
2. Determine the effect of lean or obese mouse bone marrow adipocytes on the MET of MDA-MB-231 cells in 3D culture.
3. Determine the effect of serum from lean or obese mouse on the MET of MDA-MB-231 cells in 3D culture.

CHAPTER 2: MATERIALS AND METHOD

2.1 Cell Lines

MDA-MB-231 cells were obtained from American Type Culture Collection (ATCC), (Maryland, USA) and maintained in complete media (Roswell Park Memorial Institute (RPMI) 1640 Medium (Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Gibco - Life Technologies) and 1% penicillin-streptomycin (Life Technologies)). Cells were confirmed to be mycoplasma-free using the MycoAlert™ Plus Mycoplasma Detection Kit from Lonza (Basel, Switzerland). The cells were authenticated by Short Tandem Repeat (STR) profiling by The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, Canada). Cells were used up to 15 passages after initial thawing.

2.2 Animal Care and Experimentation

All animal procedures were approved by the Institutional Animal Use and Care Committee of the Memorial University of Newfoundland (MUN) (Protocol ID: 17-02-SC). Animal handling and experimentations were carried out in strict accordance with the guidance and recommendations of the Canadian Council on Animal Care.

Five-week-old female C57BL/6N mice were obtained from Charles River Laboratories (QC, Canada). Mice were housed in the animal care facility at MUN under ambient conditions of temperature, relative humidity, and 12 hours light/dark cycle. Mice had access to standard rodent pellet diet and water *ad libitum* for a one-week acclimatization period. After this time, mice were randomly paired and stratified into two groups: one group was placed on low-fat diet (LFD) (10% fat by kCal, D12450J, with

matching sucrose to D12492; Research Diets, Inc., New Brunswick, NJ) and the other on high-fat diet (HFD) (60% fat by kCal, D12492; Research Diets, Inc., New Brunswick, NJ). Mice were maintained on these diets *ad libitum* for 10 weeks and their body weight were measured weekly. At week 10, the body weight of mice on HFD was significantly higher than that on LFD (Fig 2.1).

On the tenth week, mice were anesthetized using isoflurane and approximately 1 ml of blood was collected by cardiac puncture [151]. Serum was separated from whole blood after incubating the sample at 37°C for 1 h followed by centrifuging the clotted blood at 10,000 x *g* for 10 min at 4°C [152]. Collected serum was aliquoted and stored at -80 °C for long-term use. Mice were euthanized by cervical dislocation and peri-uterine (visceral) and inguinal (subcutaneous) white adipose tissue (WAT), tibia and femur were isolated. WATs were weighed and placed in transport buffer (1x phosphate buffered saline [PBS] (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and pH 7.4), 5.5 mM glucose, 1% penicillin-streptomycin) at room temperature while the tibia and femur were placed in 1x PBS on ice. The specimens were transported immediately to the biosafety cabinet for further processing.

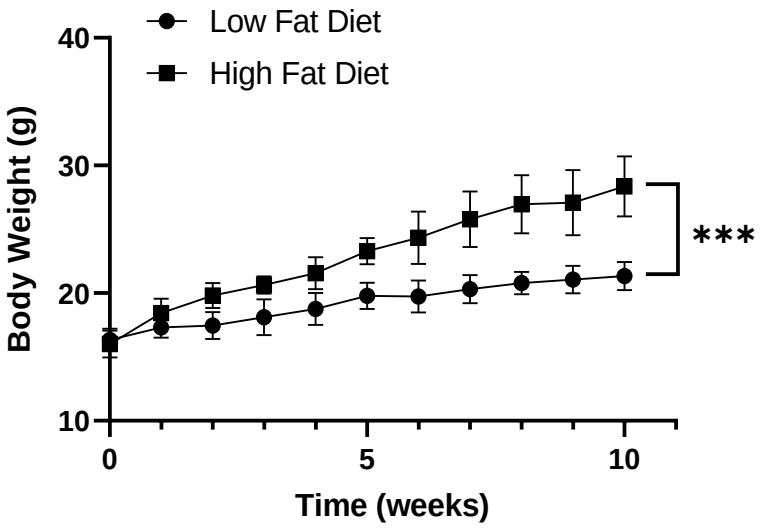


Figure 2.1: Female C57BL/6N mice becomes obese after 10 weeks on high fat diet.

Body weight of C57BL/6N mice fed low fat diet or high fat diet for 10 weeks. Values are means ± SD. n = 6. Differences between means were determined by repeated measure one-way ANOVA. P<0.0001.

2.3 Organotypic Culture of White Adipose Tissue

Peri-uterine and inguinal WAT (200 mg, 400 mg, or 800 mg) were washed briefly in a sterile dish with sterile 1x PBS containing 1% penicillin-streptomycin. Tissue samples were minced into smaller fragment using a sterile razor and washed several times with 1x PBS over a 70 µm nylon mesh. Then, 80 µl of Matrigel (basement membrane matrix; growth factor reduced, phenol red free, Cat# 356231, 9.1 mg/ml, BD Bioscience, New Jersey, USA), which was cooled on ice, was pipetted into a 24-well plate and incubated at 37℃ for 30 min. The tissue fragments were then embedded in the semi-solid Matrigel and incubated for an additional 15 minutes at 37 ℃. This procedure was used to prevent the tissue fragments from floating during culture and provide additional laminin-rich ECM. At this time, 1 ml of mammary epithelial basal media, (Promo Cell, Heidelberg, Germany) containing epidermal growth factor (10 ng/ml), insulin (5 µg/ml), hydrocortisone (0.5 µg/ml), and bovine pituitary extract (0.4%) (3D culture medium) was added to the wells and incubated at 37℃ and 5% CO_2. The WAT explants were cultured for 48 h after which time conditioned media (CM) was collected. Fresh media was added to the WAT explants and they were cultured for an additional 48 hours.

2.4 Bone Marrow Mesenchymal Stem Cell Isolation and Adipogenic Differentiation

Bone marrow mesenchymal stem cells were isolated as previously described [153]. Briefly, tibia and femur were washed twice with 1x PBS to remove residual soft tissue and blood cells. Both ends of the bones were cut off and the bone marrow was flushed out with complete RPMI 1640 Medium using a 23 G needle fitted on a 5 ml syringe. Cell clumps were broken up by slowly pulling the clumps up and down the syringe. Single-cell

suspensions was made by filtering cells through a 70 µm nylon mesh. Isolated cells were placed in a 10 cm dish containing 10 ml of complete media and incubated for 48 h at 37℃ in 5 % CO_2 incubator. After 48 h, non-adherent hemopoietic cells were removed from the adherent mesenchymal cell layer. The adherent mesenchymal cells were washed twice with 1x PBS and cultured until they were 70-90% confluent, approximately 72 h. Cell were removed from the plate with 0.25% trypsin and 50,000 cells/ml were plated in the wells of a 24-well plate.

Bone marrow stromal cells were maintained in complete media until they reached confluency, after which they were treated with and incubated in preadipocyte differentiation medium from Zenbio™ (Cat# DM-2) (DMEM / Ham's F-12 (1:1, v/v), HEPES pH 7.4, FBS, biotin, pantothenate, human insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), peroxisome proliferator-activated receptor gamma (PPARγ) agonist, penicillin, streptomycin, amphotericin B) for 7 days. Adipocytes were confirmed to be differentiated by visual confirmation of lipid droplet accumulation in > 40% of the cells. Differentiated bone marrow adipocytes (BMA) were then overlaid with 110 µl of Matrigel matrix and incubated for 1 h at 37 ℃. At this time, 1 ml of 3D culture medium with 2% Matrigel was added and the cells was cultured for 4 days with CM collected and replaced every 48 h.

2.5 Oil Red O Staining

On the 7th day of adipogenic differentiation, BMA were stained with 500 ml of 0.36% Oil Red O (Millipore, Massachusetts, USA) in 60% isopropanol for 10 min. Excess Oil Red O stain was removed by washing the wells three times with 1 ml of 1x PBS. Images

were captured at 20x using the Leica DMIL LED inverted microscope and analyzed with Infinity Analyse and Capture Software, version 6.5.

2.6 3-Dimensional (3D) Culture

The 3D culture was performed as previously described with slight modifications [143]. Briefly, 70 µl of Matrigel was placed in an 8-well culture slide (Cat# 354118, Corning-Life Science, New York, USA) and incubated for 1 h at 37°C to allow it to solidify. CM from WAT or BMA were mixed with fresh 3D culture medium containing 2% Matrigel at a ratio of 1:1. MDA-MB-231 cells (6,750 cells total) in 500 µl of the diluted CM or 3D culture medium were then overlaid on the solidified Matrigel in the chambered slide. MDA-MB-231 cells were grown for 48 h to allow colony formation. Diluted CM and 3D culture medium were replenished after 48 h and the cells were fixed on the 5th day with 4% paraformaldehyde for 20 minutes at room temperature.

For 3D culture with serum, 6,750 MDA-MB-231 cells in 500 µl of 3D culture medium containing 2% Matrigel were overlaid on the solidified Matrigel. Varying amounts of serum (2 µl, 5 µl and 25 µl) obtained from lean or obese mice were added to 500 µl of the 3D culture medium. The cells were incubated for two days at which point the media were replenished, and the treatment repeated. Cells were incubated for an additional 2 days and fixed on the 5th day.

2.7 Immunofluorescence (IF) Staining

Following fixation, cells were permeabilized with 1x PBS containing 0.5% Triton X-100 for 10 minutes at 4 °C, then washed 3 times with PBS-glycine (100 mM glycine in 1x PBS) and blocked with 10% donkey serum (Cat# D9663, Sigma-Aldrich, St. Louis, MO,

USA) in IF buffer (0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20, 1x PBS) (blocking buffer) for 1 h at room temperature. Primary antibodies were diluted, as detailed below, in blocking buffer, incubated with cells overnight at 4 °C followed by detection with appropriate secondary antibody. Before and after secondary staining cells were washed three times with 300 µl of IF buffer. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Life technologies) for 15 minutes at room temperature and washed one time with 1x PBS. The chambers were gently separated from the slide according to the manufacturer's protocol and excess 1x PBS was gently wiped off with a Kimwipe. An even bead of silicone sealant (GE, Boston, MA, USA) was applied around the Matrigel layer to avoid compression of co-cultures by coverslips. Prolong Gold (Life Technologies) was used for mounting slides, coverslips applied, and the slides were placed in a dark at room temperature to dry overnight.

Primary antibodies and dilutions used were as follows: vimentin (mouse) (1:200; Cat #V2258; Sigma-Aldrich, St. Louis, MO, USA), E-cadherin (mouse) (1:200; Cat #610181; BD Biosciences, Franklin Lakes, NJ, USA), Claudin7 (rabbit) (1:200; Cat #AB27487; Abcam, Cambridge, UK), CD24 (1:100; Cat #NBP1–46390; Novus Biologicals, Littleton, CO, USA), and Ki67 (mouse) (1:100; Cat #M724029–1; Dako, Denmark). Secondary antibodies used were AlexaFluor-647 anti-rabbit (Cat #711–605-152; Jackson Immunoresearch laboratories, West Grove, PA), and DyLight 594 anti-mouse (NBP1–75617; Novus biologicals). The slides were imaged using the 20X objective with the Nikon A1 confocal microscope with NIS elements imaging software (Nikon Inc., Tokyo, Japan).

2.7.1 Assessment of background staining due to non-specific binding of antibodies

To test for possible background staining that may occur due to non-specific binding of antibodies to endogenous Fc receptors or otherwise non-specifically, MDA-MB-231 cells were incubated with mouse IgG isotype and rabbit IgG isotype antibodies that matched the isotype of the staining antibodies to be used. This was followed by secondary antibody staining with DyLight 594 anti-mouse and AlexaFluor-647 anti-rabbit, respectively. Vimentin is expressed by MDA-MB-231 cells [154]; therefore, as a positive control, MDA-MB-231 cells were stained with anti-vimentin primary antibody followed by DyLight 594 anti-mouse secondary antibody. As expected, signals for vimentin staining in MDA-MB-231 cells were detected (Fig. 3.1). No signal was detected in cells stained with mouse IgG isotype or rabbit IgG isotype antibodies indicating the absence of non-specific background staining (Fig. 2.2).

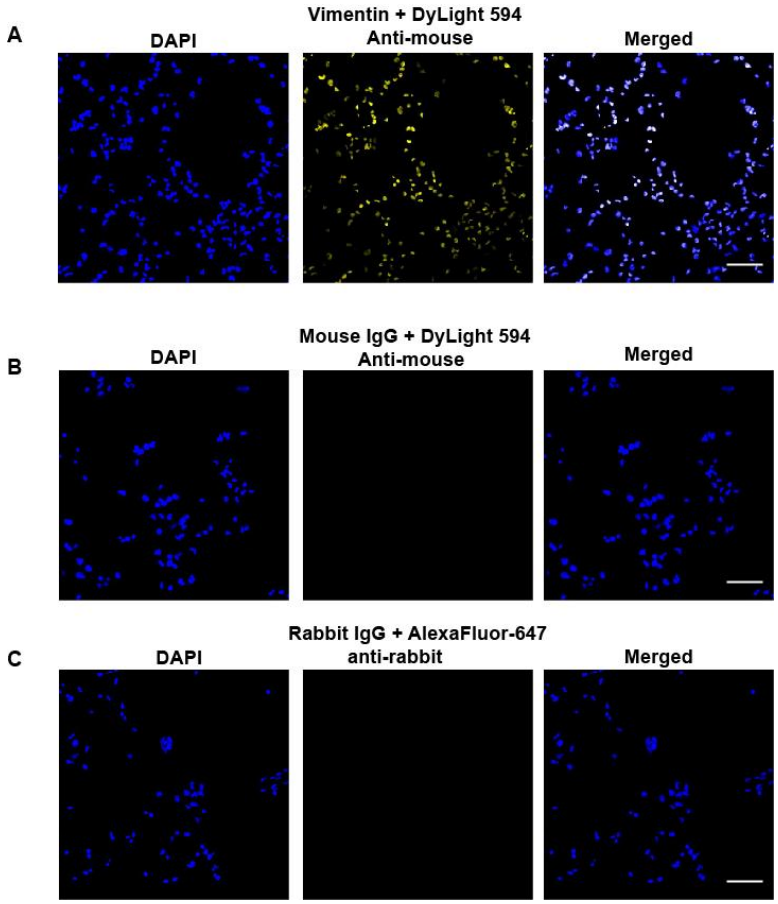


Figure 2.2 Background staining due to non-specific antibody binding was not detected

Representative images of MDA-MB-231 cells stained with: A) anti-vimentin followed by Dylight 594 anti-mouse secondary antibody. B) mouse IgG isotype control followed by Dylight 594 anti-mouse secondary antibody. C) rabbit IgG isotype control followed by Alexafluor 674 anti-rabbit secondary antibody (scale bar = 100 µm).

2.8 Image Analysis

Colony morphologies were assessed by analysis of circularity using ImageJ v.1.52a [155]. Structures present in five images per replicate (minimum 10 structures per replicate) were traced manually and the circularity measurement was obtained. A circularity value of > 0.7 was classified as round/mass-like, 0.7–0.2 was classified as grape-like, and < 0.2 was classified as stellate as reported previously [143]. Protein expression was analyzed using ImageJ by measuring the intensity of fluorescence of the whole image. Integrated density (IntDen) was determined to capture the total fluorescence of each marker. Background was subtracted by taking measurements of selected areas where cells were absent as observed from the respective bright field images. The total fluorescence intensity of each marker was determined by normalizing IntDen of each marker to IntDen of DAPI and then the relative IntDen values of each marker per condition was determined relative to the control.

2.9 Statistical Analysis

Statistical analyse were performed as indicated in figure legends. Differences were considered significant at $p<0.05$. Data were analyzed using GraphPad Prism Version 8.0.1.244 (GraphPad Software, San Diego, CA, USA).

CHAPTER 3: RESULTS

3.1 Mouse white adipose tissue causes MET-Like changes in mesenchymal MDA-MB-231 TNBC cells grown in 3D culture

To assess the effect of mouse WAT on the colony morphology of mesenchymal TNBC cells grown in 3D, 400 mg of peri-uterine (subcutaneous) and inguinal (visceral) WAT were isolated from lean and obese mice and cultured *ex vivo* to generate white adipose tissue-conditioned media (WAT-CM). As expected, MDA-MB-231 cells grown in 3D culture without WAT-CM maintained their characteristic stellate morphology in 3D culture (Fig. 3.1A). In contrast, cells grown with WAT-CM acquired a mixed population of stellate, grape-like and round/mass-like colony morphologies in a manner that was dependent on both depot and obesity (Fig.3.1A). The lean and obese mouse subcutaneous WAT had similar effects on the morphological alteration of MDA-MB-231 cells in 3D culture. Both caused a significant decrease in the proportion of stellate colony in comparison to control, with 60% and 20% of the cell colonies acquiring grape-like and round/mass-like morphologies, respectively. The lean mouse visceral WAT had the least effect on the colony morphology of the MDA-MB-231 cells, with 60% of the colonies retaining their characteristic stellate morphology, and only 30% and 10% of the colonies acquiring grape-like and round/mass-like morphologies, respectively. The obese mouse visceral WAT, on the other hand, had similar effects, as the lean and obese mouse subcutaneous WAT with 29% of MDA-MB-231 cells retaining their stellate colony morphology, while 58% and 13% of the colonies had acquired grape-like and round-like morphologies, respectively.

While both lean and obese mouse subcutaneous and visceral WAT could cause mesenchymal MDA-MB-231 TNBC cells to acquire an epithelial-like morphology in 3D culture, the difference actually lies in the adipose depot. Overall, the subcutaneous WATs (both lean and obese) were most potent in inducing the morphology changes, but the visceral WAT could only induce significant morphology changes in the obese state.

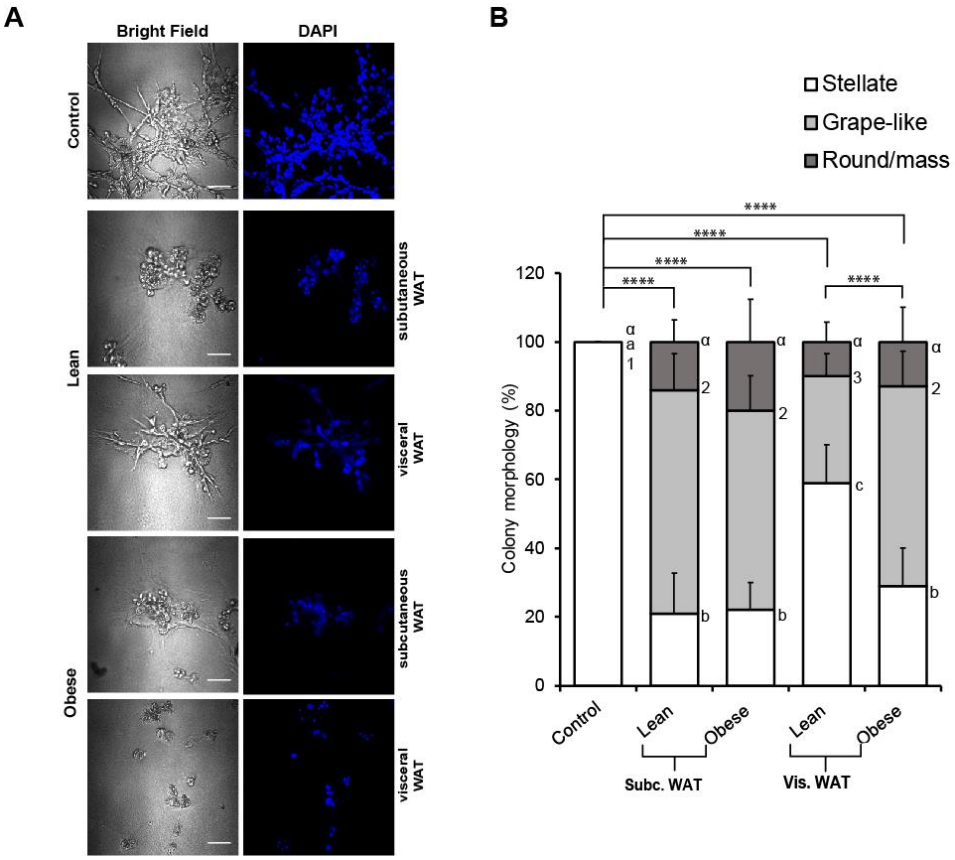


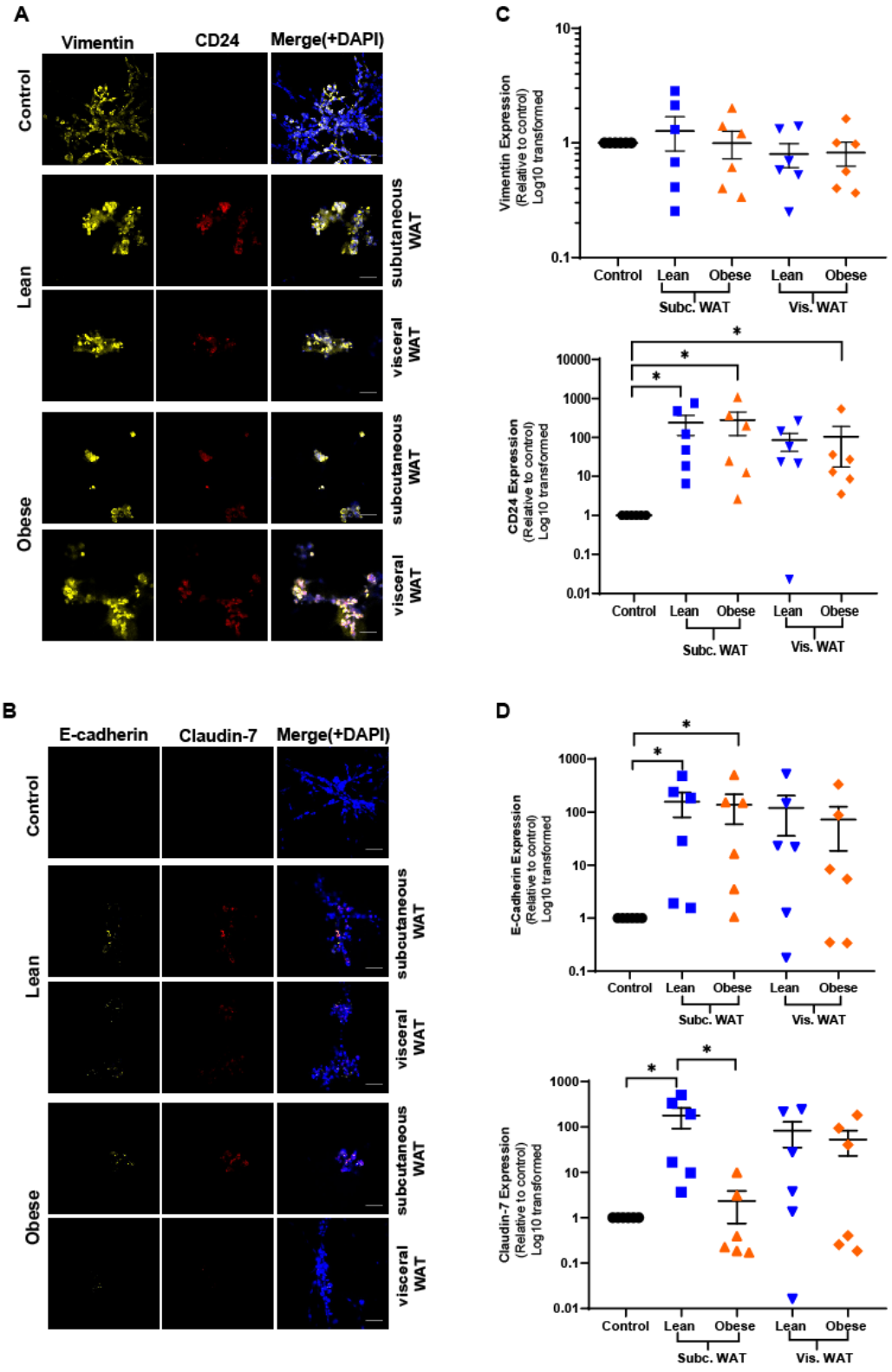
Figure 3.1: White adipose tissue (WAT) conditioned media alters the characteristic morphology of mesenchymal triple negative breast cancer cell line.

(A) Representative images of MDA-MB-231 cells grown in a 3D culture with or without lean or obese subcutaneous or visceral adipose tissue conditioned media (CM). (B) Total percentages of structure shapes are shown as mean ± SEM and were computed from six biological replicates. Overall significance of the proportion of colony shapes between the groups was assessed by χ^2 analysis, ****p<0.0001. Significant differences between groups were determined by one-way ANOVA followed by Tukey HSD post hoc analysis. Different letters or symbols represent statistically different groups: $^{a,b,c}P < 0.05$ for stellate, $^{1,2,3}P < 0.05$ for grape-like, and $^{\alpha,\beta,Y,\theta}P < 0.05$ for round/mass-like.

Having observed that mouse WAT-CM can cause mesenchymal MDA-MB-231 cells to acquire epithelial colony morphologies in 3D culture, I next assessed the effect of WAT-CM on the expression of EMT biomarkers in order to determine if the morphological changes reflected an MET event. Immunofluorescence confocal microscopy was used to detect the expression of EMT protein markers in MDA-MB-231 TNBC cells cultured in 3D with or without mouse WAT-CM. The expression of vimentin (a mesenchymal marker), E-cadherin and claudin-7 (epithelial markers), and CD24 (a stemness and an epithelial maker) was assessed [156].

As expected, MDA-MB-231 cells cultured in 3D without WAT-CM expressed high levels of vimentin but had low or no expression of CD24, claudin-7, or E-cadherin (Fig 3.2). WAT-CM from lean and obese mice caused a significant gain in the expression of CD24, claudin-7, and E-cadherin in MDA-MB-231 cells relative to control but had no effect on the expression of vimentin. There was no statistically significant difference in the expression of the individual biomarkers when comparing lean to obese mice WAT-CM (Fig. 3.2 C-D). Furthermore, while lean and obese mouse WAT-CM induced the expression of claudin-7 in MDA-MB-231 cells, its expression was lower in cells treated with obese mouse subcutaneous WAT-CM (Fig. 3.2D). Overall, these data show that both lean and obese mouse WAT-CM can induce the expression of epithelial biomarkers in mesenchymal MDA-MB-231 cells. A complete loss of the mesenchymal morphology and expression of vimentin coupled with a significant gain in the expression of claudin-7 and E-cadherin would have been an indication of a full MET. However, my results show that the expression of vimentin in MDA-MB-231 cells remained elevated even upon acquiring epithelial

morphology and protein expression, therefore suggesting that lean and obese mouse WAT-CM only induced a MET-like change or partial MET.


A
Vimentin
CD24
Merge(+DAPI)
Control
Lean
Obese
subutaneous WAT
visceral WAT
subcutaneous WAT
visceral WAT
B
E-cadherin
Claudin-7
Merge(+DAPI)
Control
Lean
Obese
subcutaneous WAT
visceral WAT
subcutaneous WAT
visceral WAT
C
Vimentin Expression (Relative to control) Log10 transformed
10
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0.1
Control
Lean
Obese
Lean
Obese
Subc. WAT
Vis. WAT
CD24 Expression (Relative to control) Log10 transformed
10000
1000
100
10
1
0.1
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Control
Lean
Obese
Lean
Obese
Subc. WAT
Vis. WAT
D
E-Cadherin Expression (Relative to control) Log10 transformed
1000
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10
1
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Lean
Obese
Lean
Obese
Subc. WAT
Vis. WAT
Claudin-7 Expression (Relative to control) Log10 transformed
1000
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0.1
0.01
*
*
Control
Lean
Obese
Lean
Obese
Subc. WAT
Vis. WAT

Figure 3.2: Mouse WAT-CM induces the expression of epithelial biomarkers in mesenchymal triple negative breast cancer cell line grown in a 3D culture.

Representative images of MDA-MB-231 cells cultured with or without lean or obese subcutaneous or visceral WAT-CM and co-stained for vimentin, CD24, and DAPI (A), or E-cadherin, Claudin-7 and DAPI (B). (scale bar = 100 μm). C and D are the relative expression of the respective markers, mean ± SEM (n=6 biological replicates). The average protein expression from five images per replicate were normalized to DAPI and analyzed relative to the control cultures. Significance was determined by Friedman test. If significant, Dunn's test was used for further pairwise comparison *$p<0.05$.

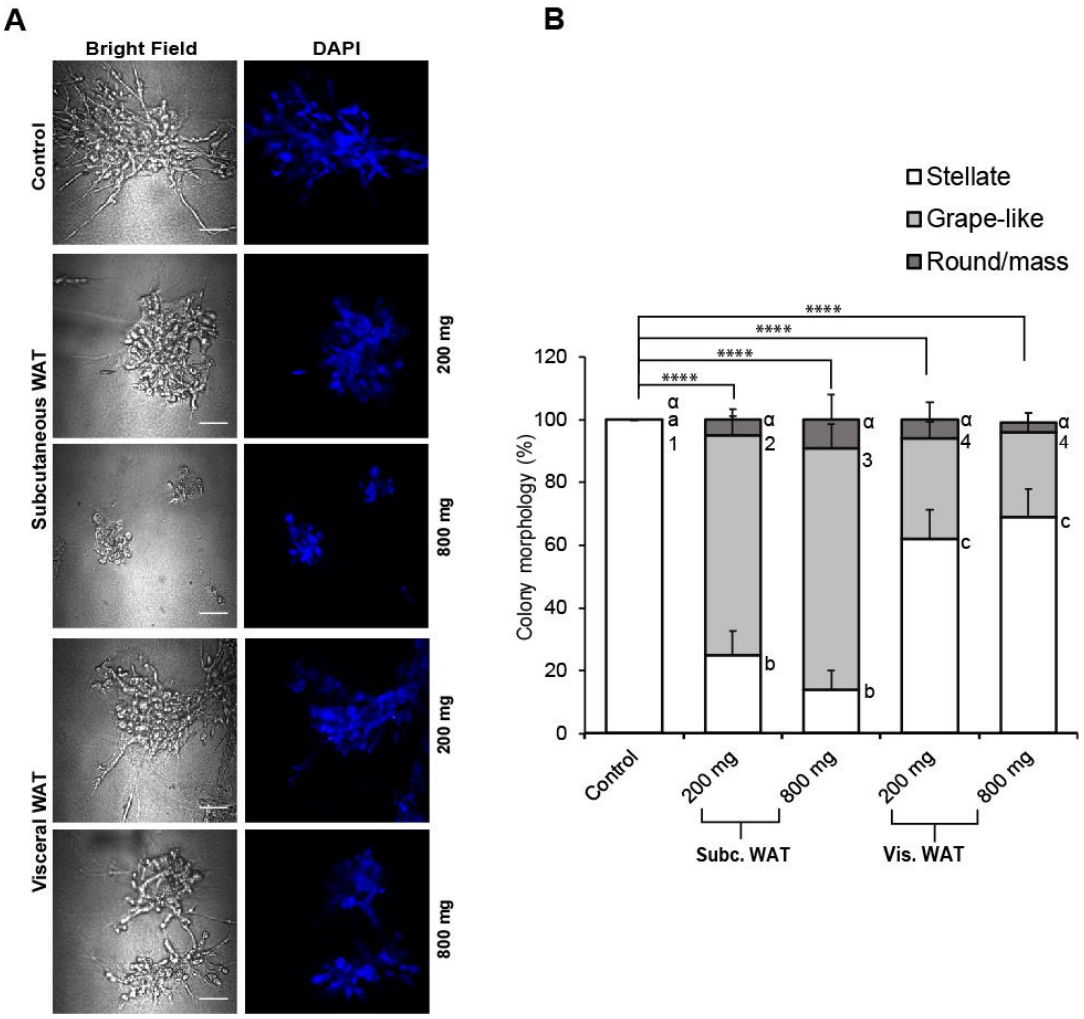
3.2 Mouse WAT-CM-induced MET-like changes in MDA-MB-231 cells is not dependent on the quantity of adipose tissue

One of the phenotypic hallmarks of obesity is the overabundance of adipose tissue [157]. Previous studies have established that obesity favours tumor metastasis relative to the non-obese state [158-160]. Obesity can promote tumor metastasis via different mechanisms such as increased induction of EMT in tumor cells [161], therefore, WAT-induced MET-like changes in 3D cultured MDA-MB-231 cells could be dependent on a qualitative change to WAT in the obese state, as well as the quantity of WAT present. Thus, I hypothesized that the greater the quantity of obese adipose tissue present, the stronger the MET-like effect would be. To test this hypothesis, MDA-MB-231 cells grown in 3D were treated with or without WAT-CM generated from an *ex vivo* culture of 200 mg or 800 mg of obese mouse subcutaneous or visceral WAT. I used obese WAT for this experiment because, approximately 500 mg of WAT can be isolated from the subcutaneous or visceral depots of a lean mouse. Since this quantity of WAT is not enough for the experiment, WATs have to be pooled from multiple lean mice to obtain enough quantity for the experiment.

Similar to what was previously observed, the morphological analysis of the cell colonies formed post WAT-CM treatment revealed a mixed proportion of different colony morphologies (Fig 3.3A). Overall, there was a significant decrease in the proportion of stellate cell colonies and an increase in the proportion of grape-like colonies. For subcutaneous WAT-CM, cells in the 800 mg treatment group formed more grape-like colonies than those in the 200 mg treatment group. However, there was no significant

difference in the proportions of colony morphologies among cells treated with 200 mg or 800 mg of the obese visceral WAT-CM. Of note, cells treated with 200 mg or 800 mg of subcutaneous WAT-CM formed significantly lower proportions of stellate colonies and a higher proportion of grape-like colonies in comparison to cells treated 200 mg or 800 mg of visceral WAT-CM. This suggests that the subcutaneous WAT-CM is more effective than the visceral WAT-CM in altering the characteristic morphology of MDA-MB-231 cells grown in 3D, unlike previous data which showed that the visceral WAT in the obese state was as effective as the subcutaneous WAT. Also, the proportion of round/mass-like structures formed in 200 mg or 800 mg WAT-CM treated cells (Fig. 3.3) was lower than that formed in 400 mg WAT-CM treated cells (Fig 3.1) possibly due to animal related biological variability.

An analysis of the EMT protein marker expression showed that both 200 mg and 800 mg of subcutaneous and visceral WAT-CM can induce the expression of epithelial proteins in 3D cultured MDA-MB-231 cells with no effects on the expression of vimentin, a mesenchymal marker (Fig 3.4). While the expression of all the epithelial biomarkers were generally elevated in all the treatment groups, the expression of epithelial biomarkers were consistently lower in cells treated with 200 mg visceral WAT-CM Overall, there was no significant difference in the expression of the EMT biomarkers among cells cultured with 200 mg or 800 mg of subcutaneous or visceral WAT-CM. Taken together, the data suggest that the adipose tissue induced MET-like change in 3D cultured MDA-MB-231 cells is not dependent on the quantity of obese WAT.


A
Bright Field
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Control
Subcutaneous WAT
Visceral WAT
200 mg
800 mg
200 mg
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B
Stellate
Grape-like
Round/mass
Colony morphology (%)
120
100
80
60
40
20
0
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α
a
1
α
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3
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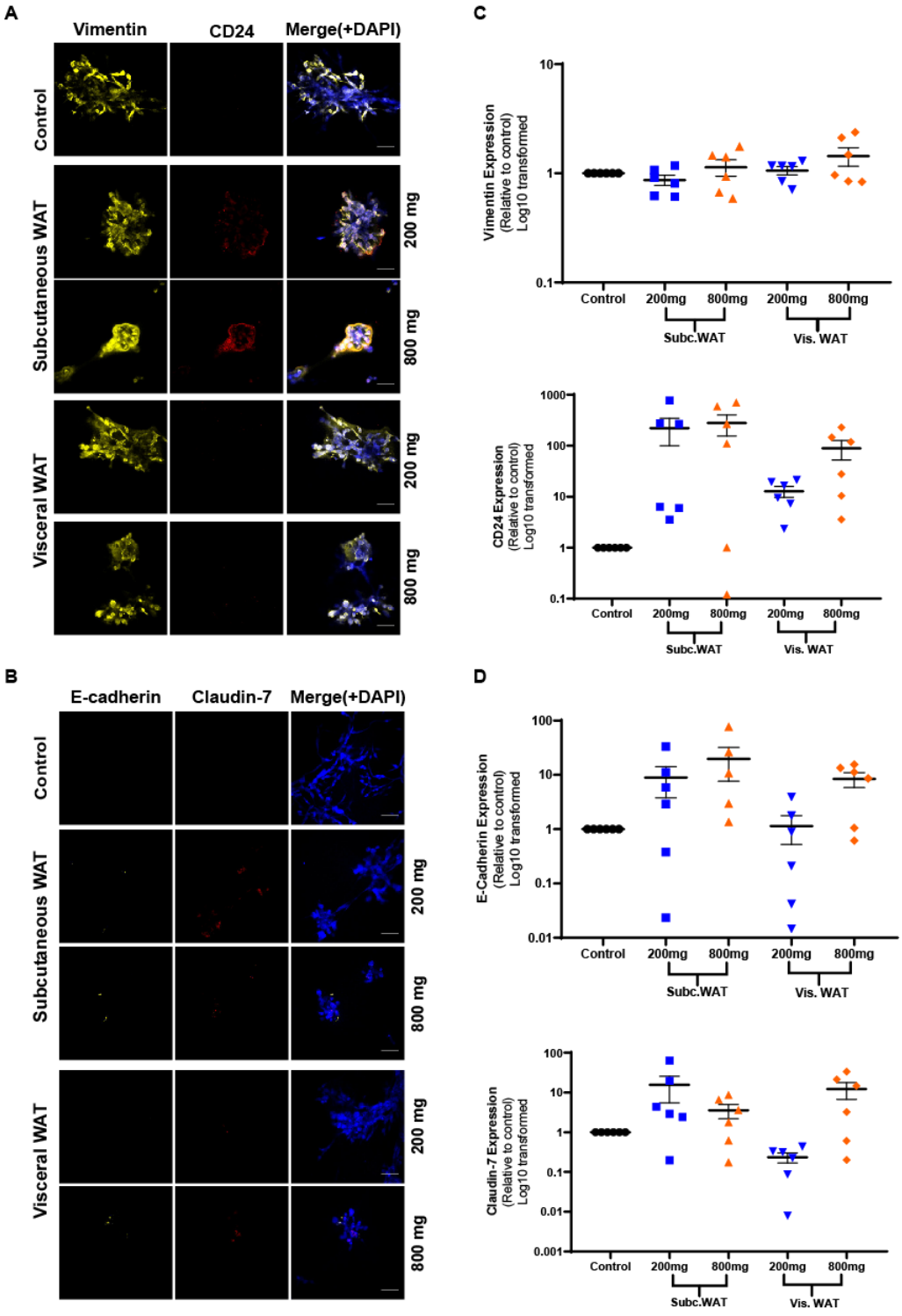
4
c
α
4
c
Control
200 mg
800 mg
200 mg
800 mg
Subc. WAT
Vis. WAT

Figure 3.3: Effect of varied WAT masses on colony morphology changes of MDA-MB-231 cells in 3D culture.

A) Representative images of MDA-MB-231 cells grown in a 3D culture with or without 200 mg or 800 mg of obese moue subcutaneous or visceral adipose tissue conditioned media (CM). B) Total percentages of structure shapes are shown as mean ± SEM and were computed from six biological replicates. Overall significance of the proportion of colony shapes between the groups was assessed by χ^2 analysis, ****$p<0.0001$. Significant differences between groups was determined by one-way ANOVA followed by Tukey HSD post hoc analysis. Different letters or symbols represent statistically different groups: $^{a,b,c}P < 0.05$ for stellate, $^{1,2,3}P < 0.05$ for grape-like, and $^{\alpha,\beta,Y,\theta}P < 0.05$ for round/mass-like.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Subcutaneous WAT
Visceral WAT
200 mg
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B
E-cadherin
Claudin-7
Merge(+DAPI)
C
Vimentin Expression (Relative to control) Log10 transformed
CD24 Expression (Relative to control) Log10 transformed
D
E-Cadherin Expression (Relative to control) Log10 transformed
Claudin-7 Expression (Relative to control) Log10 transformed
Control
200mg
800mg
Subc.WAT
Vis. WAT

Figure 3.4: Effect of varied WAT masses on the EMT biomarker expression of MDA-MB-231 cells in 3D culture.

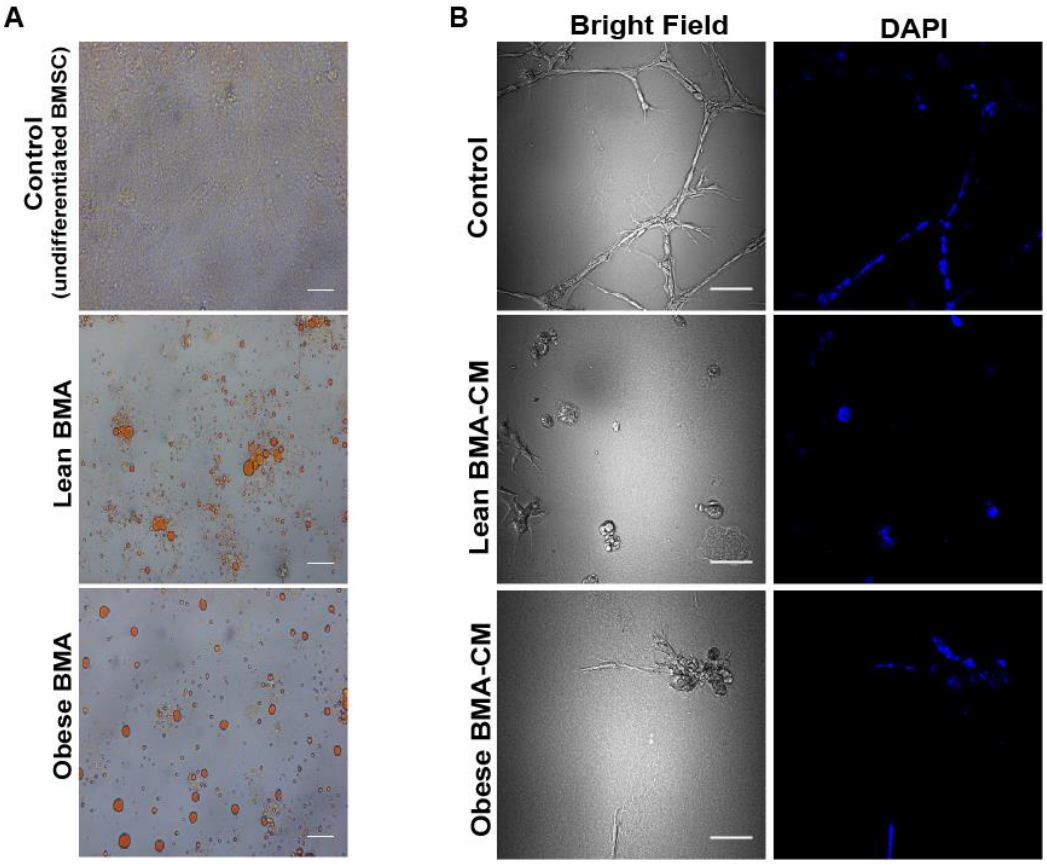
Representative images of MDA-MB-231 cells cultured with or without 200 mg or 800 mg of obese subcutaneous or visceral WAT-CM and co-stained with anti-vimentin, anti-CD24, and DAPI (A), or anti-E-cadherin, anti-Claudin-7 and DAPI (B). (scale bar = 100 μm). C and D are the relative expression of the respective markers, mean ± SEM (n=6 biological replicates). The average protein expression from five images per replicate were normalized to DAPI and analyzed relative to the control cultures. No groups were found to be significantly different by Friedman test.

3.3 Bone marrow-derived adipocytes induced an ambiguous morphology change in 3D cultured MDA-MB-231 cells.

Between 60% and 75% of secondary breast tumors are initially detected in the bone [162], which is associated with a significant decrease in the 5-year survival rate among breast cancer patients [163]. Recent studies have demonstrated the role of bone marrow adipose tissue in the progression of breast cancer [164]. To test the effect of bone marrow-derived adipocyte on MET of MDA-MB-231 cells, bone marrow mesenchymal cells were isolated from lean and obese mice and differentiated to mature adipocytes. During my initial experiments, I found that BMA degraded the basement membrane matrix (Matrigel); this rendered the 3D co-culture of BMA and MDA-MB-231 cells impossible since MDA-MB-231 cells cannot be overlaid on a degraded Matrigel. I therefore employed the CM approach. The differentiated adipocytes were then used to generate bone marrow-derived adipocyte-CM (BMA-CM) which were subsequently used to treat 3D-cultured MDA-MB-231 cells. Adipogenic differentiation of bone marrow mesenchymal stem cells were confirmed initially by visual inspection for lipid droplet accumulation in $\geq 40\%$ of the cells; this was followed by Oil Red O staining of BMA (Fig 3.5 A). In subsequent experiments, successful adipogenic differentiation was confirm only by visual inspection for lipid accumulation.

Results from the first biological replicate of this experiment showed that BMA-CM from both lean and obese mice could alter the characteristic stellate morphology of MDA-MB-231 cells in 3D culture, thus, causing them to adopt a grape-like or mass-like morphology (Fig 3.5 B). In addition, the results showed that BMA-CM had no effect on

the expression of any of the EMT biomarkers tested (Fig. 3.5 C). Specifically, there were no changes in the expression of vimentin and no signal was detected for the expression of epithelial biomarkers. However, results from three addition biological replicates were conflicting. Those experiments showed that BMA-CM does not induce a morphological change of MDA-MB-231 cells in 3D culture nor does it affect EMT biomarker expression (Fig. 3.5 D-E).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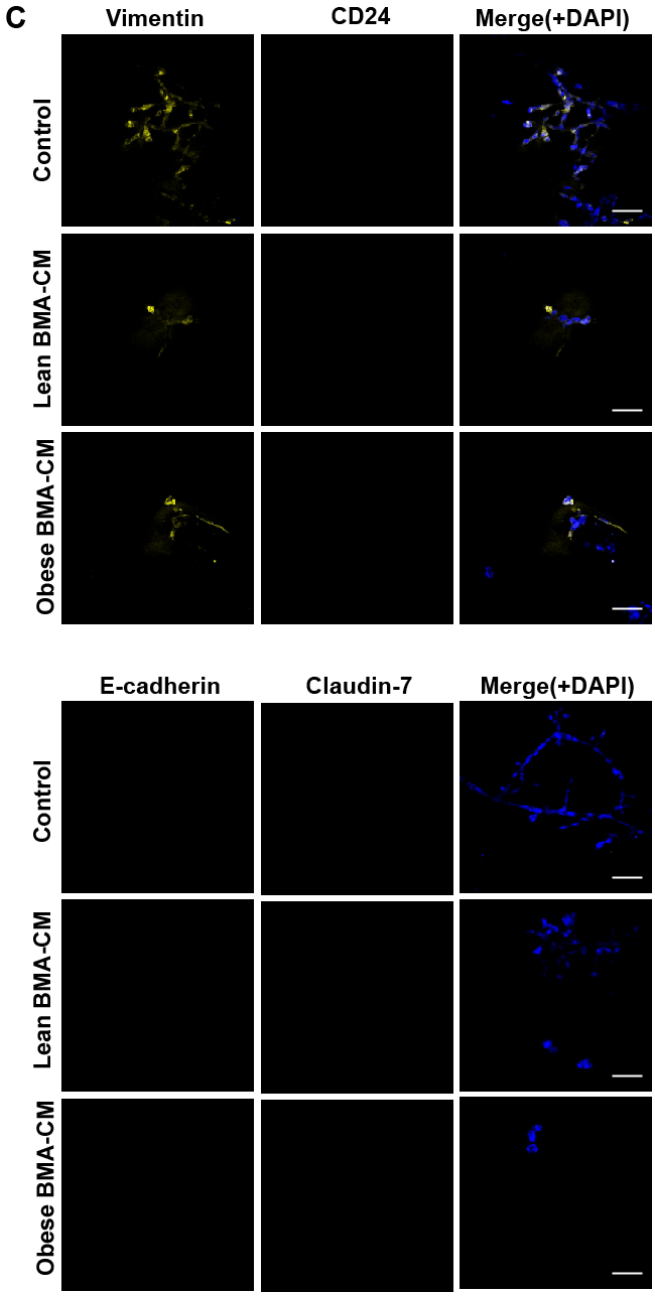
Control
(undifferentiated BMSC)
Lean BMA
Obese BMA
B
Bright Field
DAPI
Control
Lean BMA-CM
Obese BMA-CM


C
Vimentin
CD24
Merge(+DAPI)
Control
Lean BMA-CM
Obese BMA-CM
E-cadherin
Claudin-7
Merge(+DAPI)
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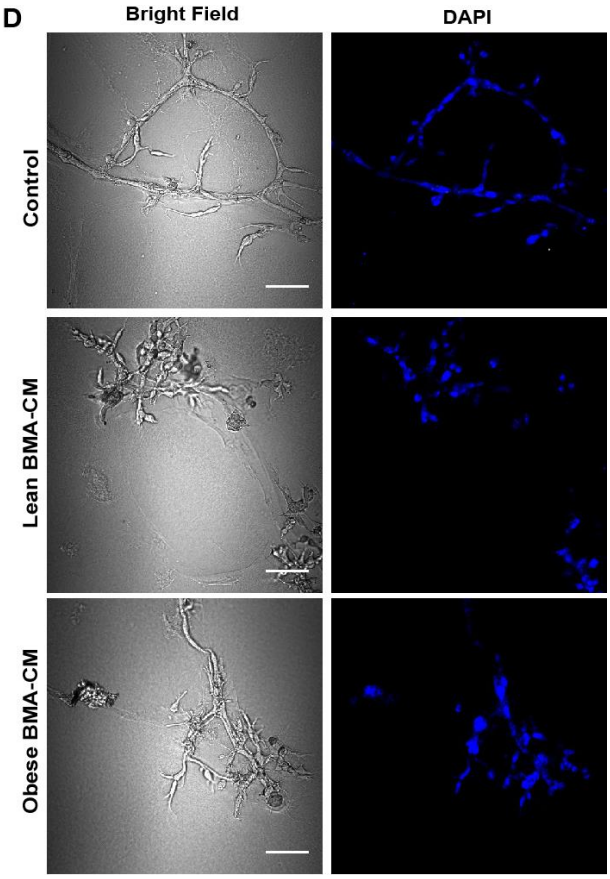
Lean BMA-CM
Obese BMA-CM


D
Bright Field
DAPI
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Lean BMA-CM
Obese BMA-CM

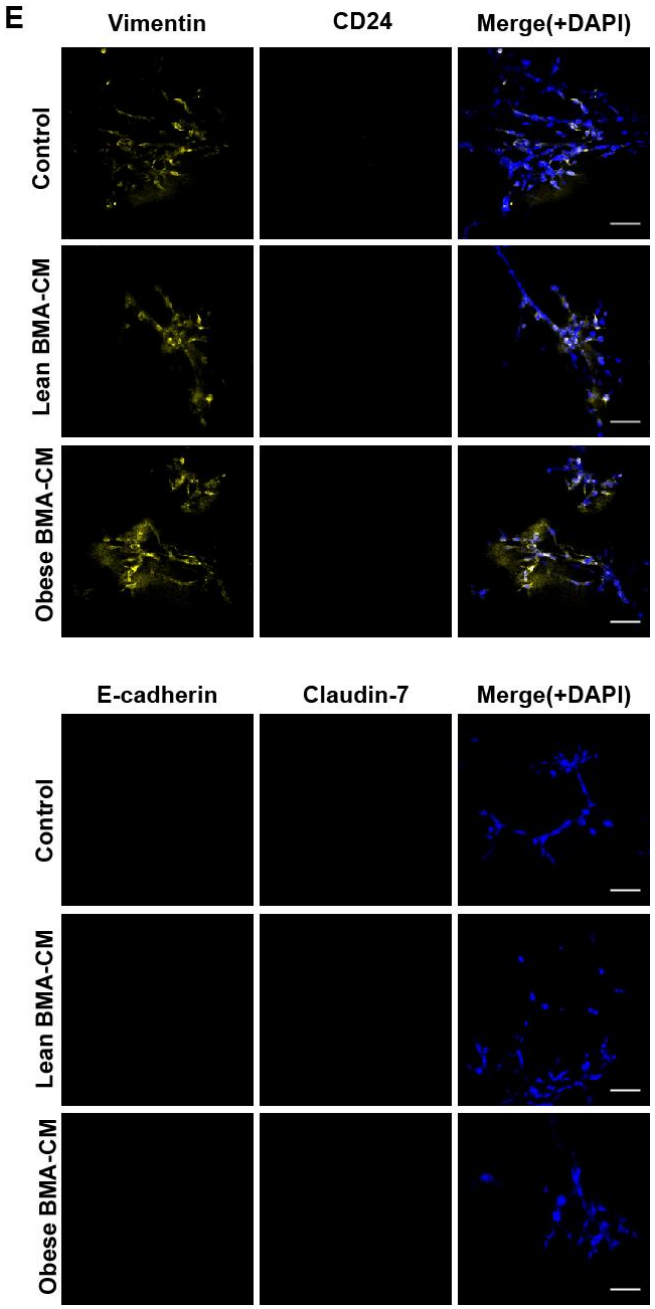

E
Vimentin
CD24
Merge(+DAPI)
Control
Lean BMA-CM
Obese BMA-CM
E-cadherin
Claudin-7
Merge(+DAPI)
Control
Lean BMA-CM
Obese BMA-CM

Figure 3.5: Bone marrow-derived adipocytes (BMA) from lean and obese mice induced ambiguous effects on the morphology of MDA-MB-231 cells in 3D culture.

In the first biological replicate, BMA-CM caused MDA-MB-231 cells to acquire epithelial morphology, however, three additional biological replicates showed no effect. **A)** Representative images of Oil Red O staining of undifferentiated bone marrow mesenchymal stem cells (BMSC), lean, and obese mouse BMA. **B)** Brightfield and DAPI images of MDA-MB-231 cells exposed to normal media (control), or CM from lean or obese mouse BMA. Cells in this experiment acquired epithelial morphology upon CM treatment. **C)** Images of MDA-MB-231 cells cultured with or without BMA-CM and co-stained with anti-vimentin, anti-CD24, and DAPI, or anti-E-cadherin, anti-Claudin-7 and DAPI. (scale bar = 100 μm). Images shown in C are corresponding antibody staining images of B. **D)** Representative images of MDA-MB-231 cells that maintained their characteristic morphology upon BMA-CM treatment (n=3). **E)** Corresponding antibody staining images of D.

3.4 Lean and obese mouse serum alters the characteristic morphology of mesenchymal MDA-MB-231 TNBC cells in 3D in a dose-dependent manner and induces MET-like changes

It was previously shown that adipocytes secrete a factor(s), in the presence of an extracellular matrix, that induces MET-like changes in mesenchymal triple negative breast cells [143]. Another study had also shown that fetal bovine serum can induce EMT in the non-malignant, BEAS-2B lung epithelial cell line, showing that pro-EMT factors may be systemically secreted into the serum [165]. To assess if a pro-MET-like factor(s) is secreted into systemic circulation, MDA-MB-231 cells cultured in 3D were treated with or without 2 µl, 5 µl and 25 µl serum from lean or obese mice, corresponding to 0.4%, 1% and 5% of the culture medium, respectively.

I found that serum from lean and obese mice caused a dose-dependent effect in the formation of cell colony morphologies in 3D culture. Specifically, more MDA-MB-231 cells lost their characteristic 3D stellate morphology and adopted the epithelial grape-like or round-like morphologies as the volume of the serum was increased (Fig 3.6). Overall, both lean and obese mice sera induced colony morphology changes similarly in each treatment group albeit there was no significant difference.0mr Of note, 25 µl of lean or obese mice sera caused almost a complete loss of the stellate colonies with 48.3% and 49.6% of the cells acquiring a grape-like and round/mass-like morphologies, respectively.

Neither lean nor obese mice sera had an effect on the expression of vimentin in 3D-cultured MDA-MB-231 cells, irrespective of the treatment volume (Fig. 3.7). In addition,

25 µl of both sera induced a significant increase in the expression of CD24, and E-cadherin in mesenchymal MDA-MB-231 cells. Even though both lean and obese mice serum induced the expression of claudin-7 in MDA-MB-231 cells, there was no significant difference in the expression of the protein among the different treatment volumes. The expression of E-cadherin in cells treated with 25 µl of lean or obese mice sera was significantly higher than that of the cells treated with 2 µl of lean mouse serum. These data show that sera from both lean and obese mice can induce MET-like changes in 3D-cultured mesenchymal MDA-MB-231 cells. Also, the proportion of epithelial structures formed upon serum treatment is dependent on the amount of serum in the culture system. High amounts of lean or obese mice sera may cause MDA-MB-231 cells to completely lose their mesenchymal phenotype. Taken together, a pro-MET factor(s) may be present in systemic circulation, but it is not clear if the factor(s) is the same as that secreted by the adipose tissue.

The 3D structures formed by mouse serum-treated MDA-MB-231 cells were slightly distinct than those formed by WAT-CM treated cells. This difference is more noticeable among the grape-like and round/mass-like structures. Firstly, in comparison to WAT-CM treated cells, serum treated cells formed more robust and dense structures in which cells appeared to be more interconnected with each other. In addition, the 3D structure of serum treated cells appeared larger than WAT-CM (Fig. 3.8).

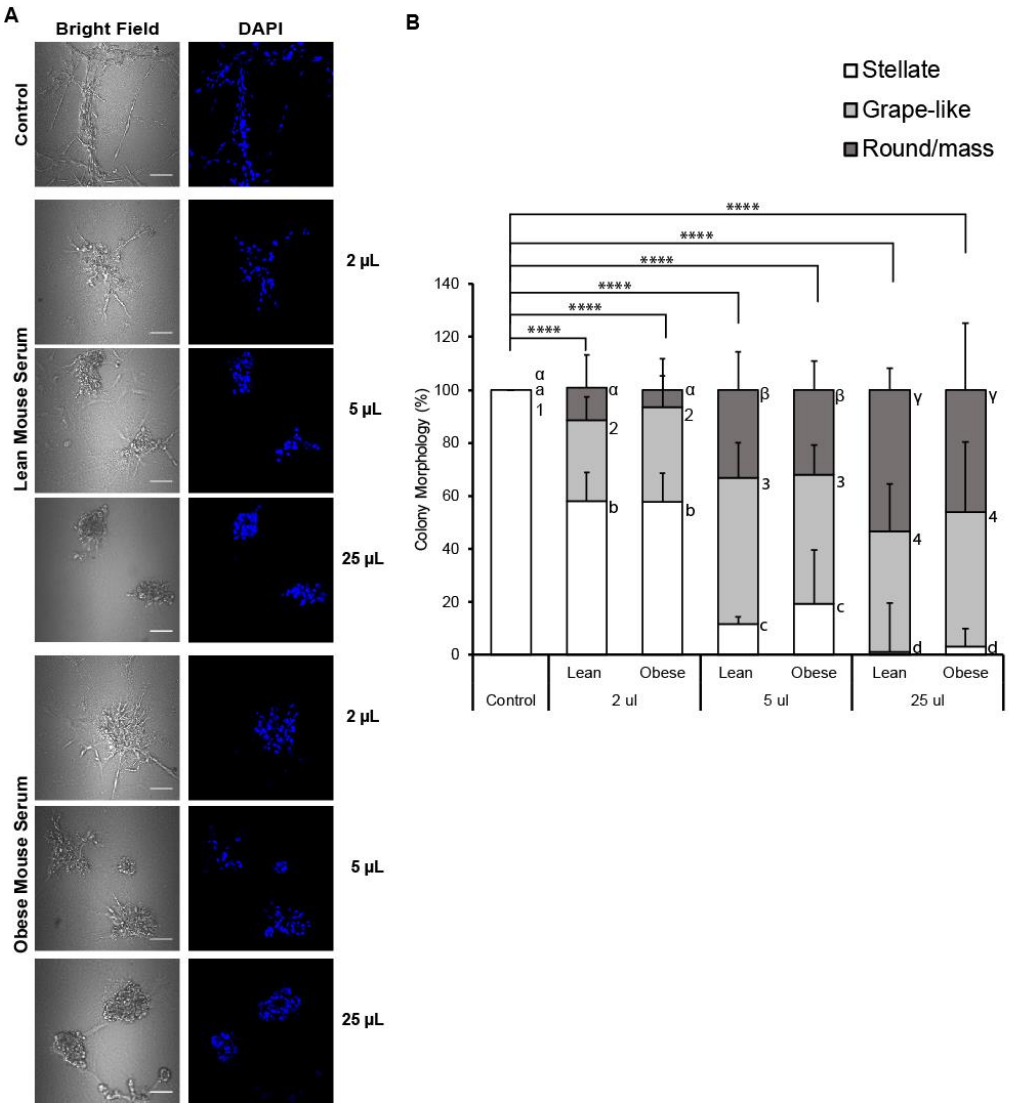
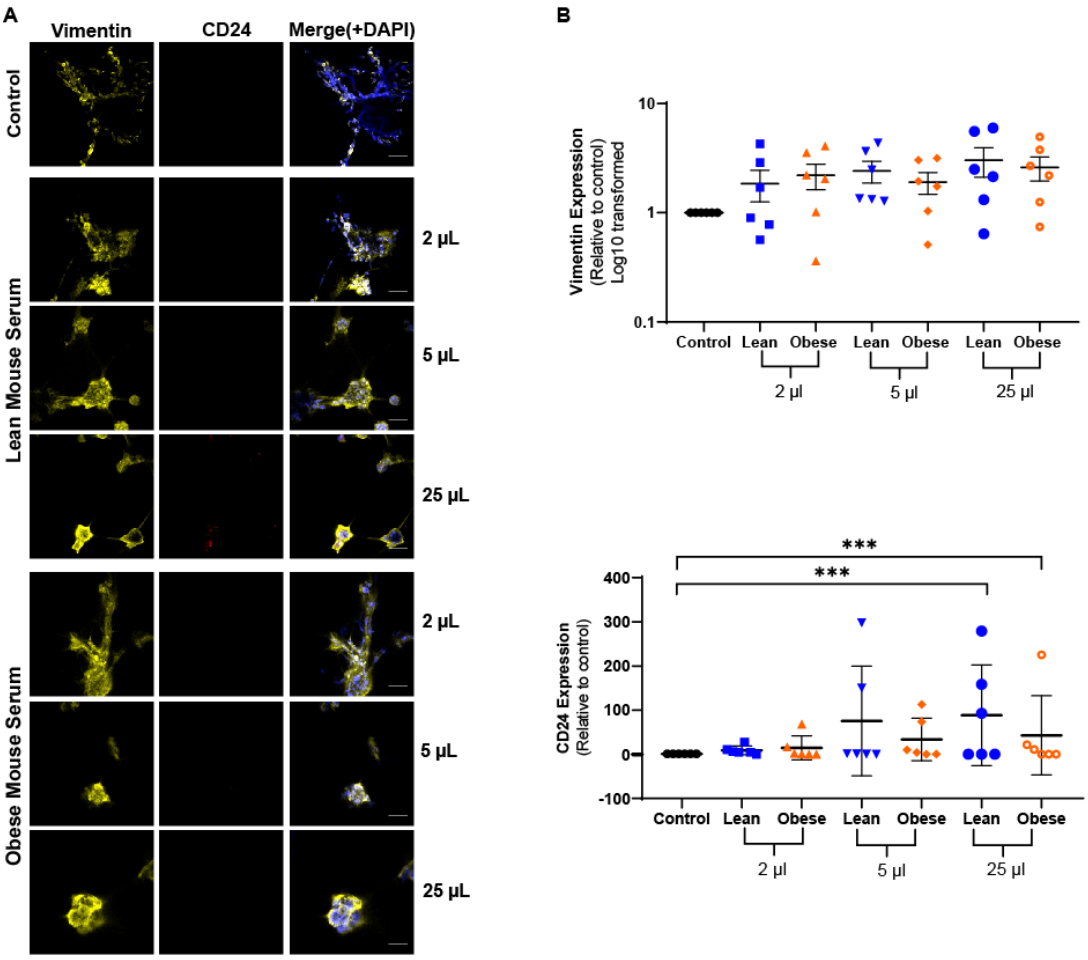

A
Bright Field
DAPI
Control
Lean Mouse Serum
Obese Mouse Serum
2 µL
5 µL
25 µL
2 µL
5 µL
25 µL
B
Stellate
Grape-like
Round/mass
Colony Morphology (%)
****
****
****
****
****
****
Control
Lean
Obese
Lean
Obese
Lean
Obese
2 ul
5 ul
25 ul

Figure 3.6: Lean and obese mouse serum alters characteristic morphology of mesenchymal triple negative breast cancer cell line in a concentration dependent manner.

Representative images of MDA-MB-231 cells grown in a 3D culture with 2 µl, 5 µl and 25 µl of lean or obese mouse serum or no serum controls (scale bar = 100 µm). Total percentages of structure shapes are shown as mean ± SD and were computed from six biological replicates. Overall significance of the proportion of colony shapes between the groups was assessed by χ^2 analysis, ****$p<0.0001$. Significant differences between groups was determined by one-way ANOVA followed by Tukey HSD post hoc analysis. Different letters or symbols represent statistically different groups: $^{a,b,c,d}P < 0.05$ for stellate, $^{1,2,3}P < 0.05$ for grape-like, and $^{\alpha,\beta,Y}P < 0.05$ for round/mass-like.


A
Vimentin
CD24
Merge(+DAPI)
Control
Lean Mouse Serum
Obese Mouse Serum
2 µL
5 µL
25 µL
2 µL
5 µL
25 µL
B
Vimentin Expression
(Relative to control)
Log10 transformed
10
1
0.1
Control
Lean
Obese
Lean
Obese
Lean
Obese
2 µl
5 µl
25 µl
***
***
CD24 Expression
(Relative to control)
400
300
200
100
0
-100
Control
Lean
Obese
Lean
Obese
Lean
Obese
2 µl
5 µl
25 µl

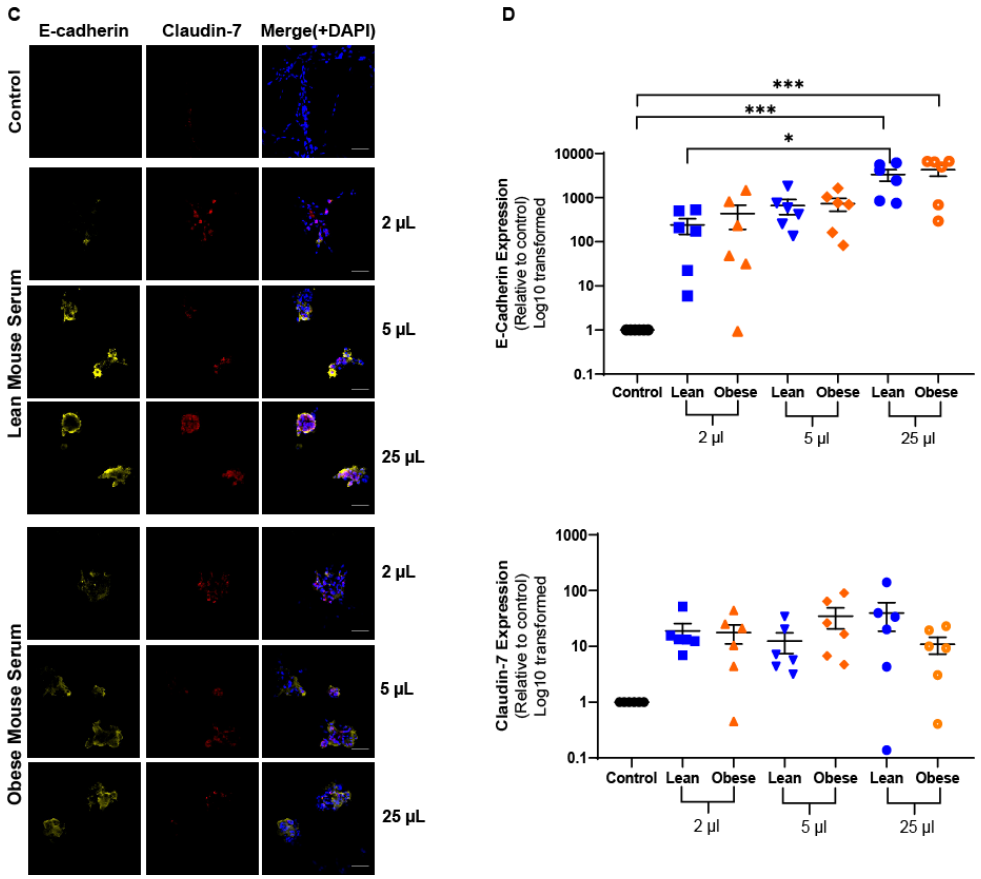


Figure 3.7: Lean and obese mouse serum induces the expression of epithelial biomarkers in mesenchymal triple negative breast cancer cell line grown in a 3D culture.

Representative images of MDA-MB-231 cells cultured with or without lean or obese mouse serum and co-stained with anti-vimentin, anti-CD24, and DAPI (A), or anti-E-cadherin, anti-claudin-7 and DAPI (C). (scale bar = 100 µm). B and D are the average protein expression from five images per replicate which were normalized to DAPI and analyzed relative to the control cultures. Significance was determined by Friedman test. If significant, Dunn's test was used for further pairwise comparison *$p<0.05$.

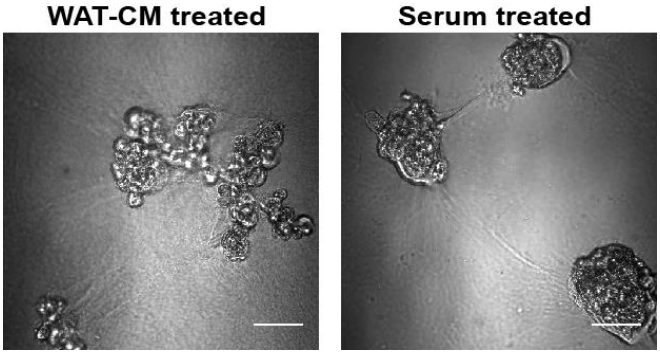


Figure 3.8: Comparison of 3D structures formed by serum or WAT-CM treated MDA-MB-231 cells.

Representative brightfield images of WAT-CM or serum treated MDA-MB-231 cells in 3D culture. Images were selected to illustrate the structural differences between WAT-CM and serum treated MDA-MB-231 cells. (scale bar = 100 µm).

CHAPTER 4: DISCUSSION

4.1 Summary

The purpose of this study was to determine the effects of lean and obese mice adipose tissues, from different depots, and sera on the MET of mesenchymal triple negative breast cancer cells in 3D culture. A previous study from our laboratory had shown that mature adipocytes, in the presence of an ECM, secretes a factor(s) that induces MET-like changes in mesenchymal TNBC cell lines. Given that obesity is associated with tumor progression, I hypothesized that: (1) adipose tissue from obese mice, via the secretion of a pro-MET factor(s), would enhance MET-like changes in MDA-MB-231 TNBC cells, with potential differences between visceral and subcutaneous WAT, when compared to adipose tissue from lean mice, (2) pro-MET factors are secreted into the blood, and (3) obese mouse serum will promote MET-like changes in MDA-MB-231 cells with a greater magnitude than the lean mouse serum. Surprisingly, I found that both lean and obese mouse subcutaneous or visceral WAT cause 3D-cultured MDA-MB-231 cells to acquire epithelial morphology and protein expression whereas the expression of the mesenchymal marker, vimentin, was unaffected. While the induction of epithelial protein expression was not depot dependent, the adipose depots affected morphological alteration differently. Lean and obese mouse subcutaneous WAT, and obese mouse visceral WAT, were more efficacious in inducing MET-like morphology changes in MDA-MB-231 cells, while lean visceral WAT had the least effect on the morphological changes. I have also demonstrated that pro-MET factors secreted into the blood with both lean and obese mice sera promoted MET-like changes in mesenchymal TNBC cells in a similar way.

4.2 Effects of lean and obese mouse WAT on MDA-MB-231 cells grown in 3D culture

The 3D culture model utilized in the previous study from the Christian laboratory was designed such that differentiated 3T3-L1 adipocytes and breast cancer cells sandwiched a basement membrane matrix [143]. The basement membrane provides a physical medium through which the two different cells interact with each other. This approach was unsuccessful in my study as the *in vitro* adipogenic differentiation of mouse preadipocytes, which were isolated by stromal vascular fractionation of WAT, yielded low differentiation. Although WAT depot-specific properties are preserved in *in vitro* differentiated adipocytes, obesity associated characteristics such as increased pro-inflammatory cytokine secretion and macrophage infiltration may be lost to the stromal vascular fractionation of WAT and subsequent induced differentiation [166, 167]. This led to the adaptation of an organotypic culture model in which mouse WAT was cultured *ex vivo* to generate WAT CM. TNBC cells grown in 3D on a basement membrane were then treated with WAT CM. This model permitted an indirect assessment of the effects of the WAT on the TNBC cells via the CM.

Using this modified approach, I found that both lean and obese mouse subcutaneous and visceral WATs caused MET-like changes in mesenchymal MDA-MB-231 cells, as evidenced by their acquisition of epithelial morphology and protein expression. In general, no marked difference was observed in the MET induction capacity of lean and obese mouse WAT, however, the subcutaneous WAT and visceral WAT affected MET-like morphology changes in MDA-MB-231 cells distinctively. Both lean and obese mouse subcutaneous

WAT proportionately increased the “epithelialization” of MDA-MB-231 cells relative to the lean visceral WAT. Meanwhile, in the obese state, mouse visceral WAT induced the same magnitude of effect as the lean and obese mouse subcutaneous WAT. It is worth noting that subcutaneous and visceral WATs are morphologically and functionally distinct [168, 169]. They arose from different progenitor cells, and have a differential gene expression pattern [170, 171]. In addition, the two depots also have a distinct biomolecule and adipokine secretion profiles [168]. Therefore, the depot dependent MET-like morphological changes may be due to differential secretion of pro-MET factor(s) by the two depots, hence, reflecting the fundamental biological differences between subcutaneous and visceral WAT.

In principle, true MET is characterized by the following: a complete acquisition of epithelial morphology, downregulation of mesenchymal proteins, and upregulation of epithelial proteins [172, 173]. In this study, I found that even though the WAT upregulated the expression of epithelial biomarkers in MDA-MB-231 cells, the expression of vimentin, a mesenchymal biomarker, remained unaltered. Thus, MDA-MB-231 cells appeared to be in a hybrid mesenchymal/epithelial state. This was not in keeping with the previous study from the Christian lab which showed that mature adipocytes caused a significant downregulation of the expression of vimentin, while it upregulated the expression of epithelial biomarkers [143]. Of note, in that study, mature adipocytes were co-cultured with MDA-MB-231 cells in 3D for 5 days. This differs from the CM approach I adopted, which exposed MDA-MB-231 cells to fresh CM twice within 5 days. It is possible that the other cell types present in WAT are influencing the ability of adipocytes to induce an MET-like

change. Alternatively, a constant and uninterrupted supply of the pro-MET factor(s) in the culture system is crucial for initiating enough signal to downregulate the expression of the mesenchymal biomarker, which is lacking in a CM approach.

A state of phenotypic duality in cancer cells have been previously described, particularly in cells undergoing EMT [178, 179]. Evidence shows that carcinoma cells can successfully metastasize to other organs without completely undergoing EMT [174, 175]. Tumor cells in the hybrid epithelial/mesenchymal state possess migratory and cell-cell adhesion properties tend to migrate as multicellular aggregates in the bloodstream of cancer patients [176-178]. These tumor cells have superior survival advantages than cells that had undergone complete EMT and are migrating as single cells [176, 179]. Cells migrating in clusters are more likely to survive anoikis en route to distal organs [180, 181]. In addition, they have been shown to be immuno- and chemo-resistant [179]. Thus, a hybrid tumor state can contribute to increased tumor aggression. The hybrid phenotype I found in my study was different from the previously reported phenotype, since MDA-MB-231 cells were originally mesenchymal and do not express epithelial biomarkers. They only acquired an epithelial phenotype after exposure to WAT CM and were potentially on course to a full MET. Gunasinghe and colleagues used an MDA-MB-468 xenograft model to demonstrate that BC cells undergoing local lymphovascular invasion could transition from a metastable state to an epithelial state [173]. They showed that tumor emboli in the lymphovasculature which co-expressed vimentin and E-cadherin gradually lost the expression of vimentin to predominantly express E-cadherin. This evidence supports the idea that the hybrid phenotype observed in my study could be a transitionary phase which might be followed

by the downregulation of vimentin possibly upon the amplification a MET activation signal.

Although accumulating evidence indicates that the MET process plays as critical role in secondary tumor establishment, the underlying mechanism and regulatory factors remains largely unknown [70, 71, 173, 182]. The common denominator among evidence that supports MET mediated secondary tumor formation is the re-expression of E-cadherin in migrating tumor cells [71, 173]. E-cadherin belongs to a family of Ca^{2+}-dependent cell adhesion molecules which mediate cell-cell adhesion and plays an important role in maintaining tissue integrity [21]. The expression of E-cadherin is reduced or lost prior to the initiation of tumor invasion and metastasis [21]. E-cadherin repression in tumor cells consequently triggers the activation of EMT induction machinery that promotes invasive and migratory phenotypes [21]. Hypermethylation of the E-cadherin promoter [183, 184], and hypoxia [185] are among the factors that repress the expression of E-cadherin in tumors. Transcription factors such as SNAIL, Slug, ZEB1, and ZEB2 can also repress E-cadherin expression by binding to the E-box of the E-cadherin promotor [186]. WAT-induced MET-like changes and E-cadherin re-expression in MDA-MB-231 cells could possibly be due to the inactivation of SNAIL, Slug, ZEB1, or ZEB2. Demethylation of the E-cadherin promoter could also be plausible mechanism. Further studies are needed to elucidate the exact mechanisms.

One of the main goals of cancer therapeutics is to inhibit metastatic dissemination of cancer cells to distal organs since this is the major cause of mortality among patients [187]. Targeting key processes in the tumor metastasis cascade such as EMT/MET

represents a viable strategy for achieving this goal. In theory, targeting EMT could abrogate the migratory and invasive potential of the tumor thereby preventing the metastatic dissemination of tumor cells from their primary origin. Inhibiting MET could prevent tumor colonization in secondary organs. WAT derived pro-MET factor(s) could be a potential therapeutic target for inhibiting metastasis formation in both lean and obese TNBC patients. This therapeutic strategy could be combined with other established treatment regimens, such as chemotherapy, to help ameliorate the disease progression.

4.3 Effects of lean and obese mice BMA on MDA-MB-231 cells grown in 3D culture

The bone is the most frequent site of metastasis in breast cancer patients [175]. Bone metastasis generally worsens the disease prognosis and presents with excruciating pain, bone fracture, hypercalcemia, and immobility [177]. Factors which promote bone metastasis remain elusive. For decades, Stephen Paget's "seeds" and "soil" theory has been touted as a possible mechanism for bone metastasis [188]. This theory proposes that metastatic tumor cells ("seed") only migrate to organs ("soil") that provide a fertile milieu to support their growth and survival. The bone hosts a variety of cells such as mesenchymal stem cells, osteoclasts, osteoblasts, immune cells, and bone marrow adipocytes, which have all been implicated for their role in driving bone metastasis [189]. Recently, the bone marrow adipose tissue is gaining the spotlight as a key contributor to bone metastasis [189]. Herroon *et al.,* showed that bone marrow adipocytes promoted prostate cancer growth via the secretion of fatty acids and activation of hypoxia inducible factor-1a [190]. Furthermore, bone marrow adipocytes have been shown to promote the growth of leukaemia cells via lipid transfer [191].

Having established that WAT can induce MET-like changes in MDA-MB-231 cells and considering the potential role of MET in secondary tumor colonization, I investigated the effects of mice BMAs on the MET of MDA-MB-231 cells. The first replicate of this experiment showed that both lean and obese mice BMA could cause MET-like morphological changes in MDA-MB-231 cells with no effect on the EMT biomarker expression. However, results from three additional biological replicates showed that BMAs had no effect on the morphological alteration of MDA-MB-231 cells nor did it affect the expression of EMT biomarker. The reasons behind these conflicting findings are not clear. Of note, this experiment had some limitations. I generated CM from BMAs that were obtained from the adipogenic differentiation of bone marrow mesenchymal stem cells because it is technically challenging to isolate whole bone marrow adipose tissue or adipocytes from the mouse bone marrow. Although Oil Red O staining of the differentiated BMAs showed visible lipid droplets in all replicates, the proportion of lipid droplets varied highly with each experiment, possibly due to the heterogeneity of the bone marrow stroma and the variable population of isolatable mesenchymal cells in the bone marrow [192]. It is not clear if the proportion lipid droplets formed following adipogenic differentiation would correlate with the magnitude of MET-like effect in this instance. But logically, low adipogenic differentiation corresponds to low secretion of adipocyte-derived pro-MET factors into the CM. The conflicting findings may be resolved by exploring efficient models which utilizes intact bone marrow adipose tissue.

4.4 Effects of lean and obese mice sera on MDA-MB-231 cells grown in 3D culture

Different types of cells in the body, including adipocytes, actively secrete biomolecules such as hormones, lipids, and free proteins into the systemic circulation, which are often needed for a specific biological function. Since the blood serum is rich in these secreted biomolecules, I hypothesized that pro-MET factors are secreted into the blood and secondly, that obese mouse serum would induce MET changes in MDA-MB-231 cells more strongly than the lean mouse serum. My results showed that pro-MET factor(s) are systemically secreted into lean and obese mice sera as both sera induced MET-like changes in MDA-MB-231 cells. Interestingly, both lean and obese mice sera induced MET-like changes in a similar manner with dose-dependent MET-like changes in MDA-MB-231 cells. The dose-dependent effect was more apparent in the morphological alteration of the cell colonies and in the induction of CD24 and E-cadherin expression.

It is unclear if the serum-derived pro-MET factor(s) is the same as that secreted by the adipose tissue. A visual comparison of 3D structures formed by serum or WAT-CM treated MDA-MB-231 cells revealed a few morphological differences. Serum-treated MDA-MB-231 cells formed larger and more dense cell colonies compared to WAT-CM treated cells. The large, dense cell colonies formed by serum-treated cells may be due to increased cell proliferation. The blood serum is rich in growth factors such as platelet derived growth factors and epidermal growth factors which have been previously shown to stimulate tumor proliferation and growth [193-195]. To the best of my knowledge, no study has linked a serum derived factor or biomolecule to the induction of MET in tumor cells. So far only one study has linked the serum to EMT where Malm *et al.*, showed that fetal

bovine serum can induce EMT in the non-malignant, BEAS-2B lung epithelial cell line [165]. More work needs to be done to identify and characterize serum derived pro-MET factors.

Systemically secreted pro-MET factors could have dire implications for metastatic breast cancer patients. This is because bloodborne pro-MET factors could interact with CTC and also perfuse distal organs like the brain, lung, and liver to prime the organs as well as the tumor cells for a MET-mediated metastasis. However, the identification of this factor opens a window of opportunity for therapeutic interventions that inhibit the activity of pro-MET factors to prevent secondary tumor formation. This is however contingent upon the identification the pro-MET factor and its mechanism of action.

4.5 Conceptual Model for Adipose Tissue-Driven Secondary Breast Tumor Metastasis

Obesity is characterized by the excessive deposition of adipose tissues in the body [196]. Numerous studies have established a clear link between obesity and breast cancer progression [144-147]. Adipocytes, being the predominant cell type in the breast tumor microenvironment, have been shown to fuel tumor growth and metastasis by secreting altered levels of signaling molecules such as proinflammatory cytokines, adipokines, proangiogenic factors and extracellular matrix constituents [149, 150]. Evidence also suggest that adipocytes can induce EMT, cause tumor invasion, and migration. My study and previous work done in the Christian lab [143] have consistently demonstrated that adipocytes can induce MET-like changes in mesenchymal TNBC cells via the secretion of an unknown factor(s). Thus far, our study sheds light on a previously unknown role of

adipocytes in the tumor progression cascade. Here, I will attempt to paint a broader picture of how adipocytes could be promoting breast tumor metastasis at all stages in the metastatic cascade (Fig. 4.1).

First, adipocytes interact with breast cancer cells to promote EMT in the tumor cells [161]. This endows the tumor cells with invasive and migratory phenotypes, therefore, enabling the tumor cells to invade the tumor microenvironment and migrate to distal organs via the lymphatic system and blood stream. As the mesenchymal CTCs migrate toward distal organs, they interact with systemically secreted pro-MET factors that then activate the MET cascade. This would include the re-expression of E-cadherin in the CTCs but would not affect the mesenchymal phenotype as this phenotype is required for the continued migration of the cells to distal organs. Upon arrival at a distal organ, the tumor cells could then adhere to the organ via E-cadherin mediated cell-cell adhesion, leading to secondary tumor establishment and colonization. This model may help explain MET-mediated tumor colonization in organs such as the brain or lungs, which are distal to WATs. In instances where target organs are proximal to WAT, for example the liver and the bone, locally secreted pro-MET factors would also interact with tumor cells to facilitate tumor colonization at the organ. The interaction activates the MET pathway, thereby making the tumor cells more receptive for a MET dependent secondary tumor formation.

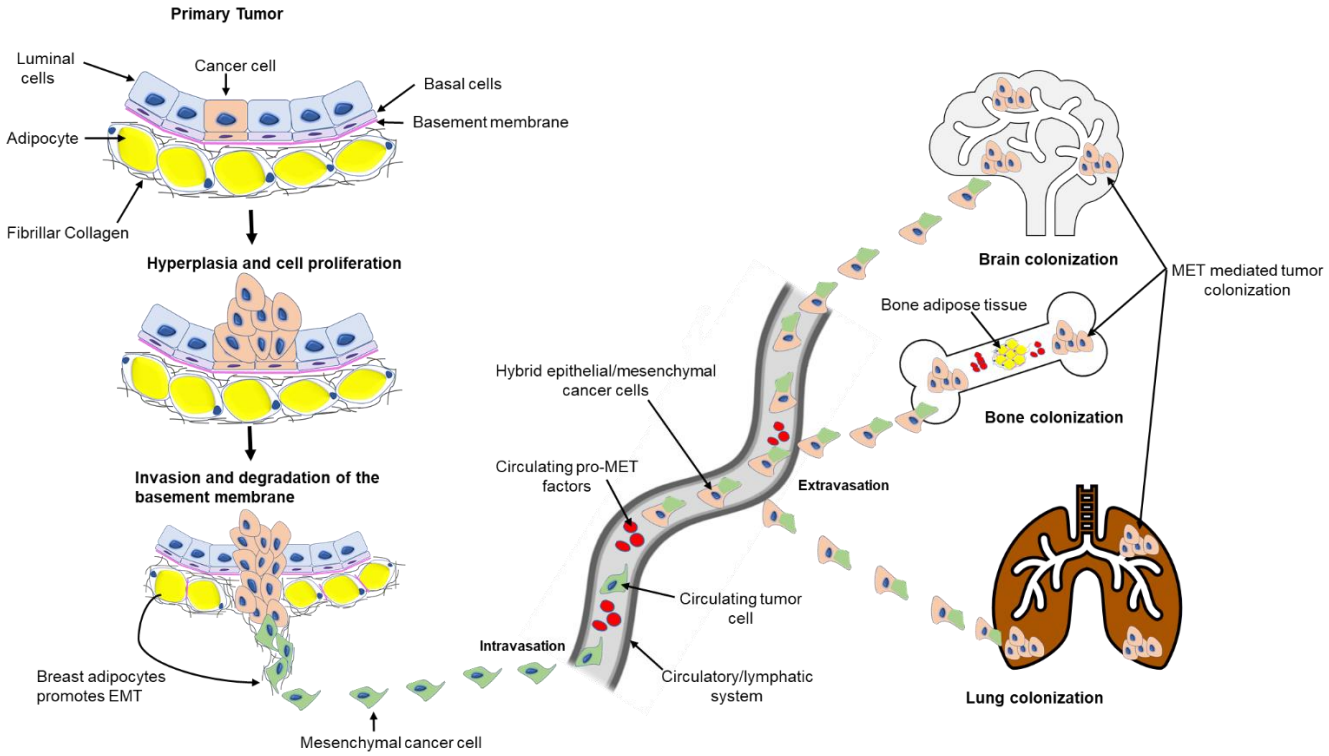


Figure 4.1: Schematic representation of a conceptual model for adipose tissue-driven secondary breast tumor metastasis

Breast adipocytes interacts with pre-metastatic epithelial tumor cells to promote EMT in the tumor cells. EMT of tumor cells results in their acquisition of mesenchymal-like properties due to the upregulation of mesenchymal proteins such as vimentin and N-cadherin and the downregulation of epithelial proteins such as E-cadherin. Mesenchymal cancer cells intravasates into the circulatory/lymphatic system where they interact with bloodborne pro-MET. This interaction leads to the activation of the MET pathway but does not affect epithelial protein expression therefore, resulting in the concomitant expression of mesenchymal/epithelial phenotypes (hybrid phenotype) by the circulating tumor cells. The hybrid mesenchymal/epithelial cancer cells homes to their target organs where they extravasate and undergo full MET to establish a secondary tumor.

4.6 Conclusion and Future Direction

Overall, this study revealed that lean and obese mouse WAT can cause MET-like changes in mesenchymal TNBC cells via the secretion of an unknown pro-MET factor(s). Contrary to my original hypothesis, the MET induction capacity of lean and obese mice WAT was not significantly different. However, adipose tissue from the subcutaneous and visceral depots affected MET-like changes in TNBC cells differently. The lean and obese mouse subcutaneous WAT cause a much stronger MET-like effect in MDA-MB-231 cells compared to the lean visceral WAT. However, in the obese state, the visceral WAT induces a comparable MET-like effect to the lean and obese subcutaneous WAT. Unfortunately, the effects of BMA on the MET of TNBC cells could not be ascertained because the methodology that is needed to study this effect requires further optimization. Lastly, this study demonstrated that pro-MET factors are systemically secreted in both lean and obese states. Serum derived pro-MET factor(s) promote MET-like changes in TNBC cells in the same manner, irrespective of the state of adiposity.

Taken together, findings from this study suggest that WAT derived pro-MET factor(s) could potentially play a role in MET dependent in secondary tumor colonization in TNBC patients, irrespective of their state of obesity. This means that therapeutic inhibition of the factor(s) could be a viable strategy for preventing tumor spread in both lean and obese TNBC patients. Future work should focus on the identification and characterization of serum and adipose tissue derived pro-MET factor(s) possibly via a proteomics or gene expression analysis. In addition, it would be beneficial to explore the

mechanisms underlying adipocyte-induced MET-like changes in TNBC cells. Identification of the pro-MET factor(s) and its implicated signaling pathways would help identify the best approach for therapeutic interventions that would inhibit tumor metastasis. Furthermore, future studies may explore efficient models for studying the effect of BMA on the MET of mesenchymal TNBC. This will provide insights on the role of BMA in metastasis.

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