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METABOLICALLY ACTIVATED MACROPHAGES DRIVEN BY OBESITY PROMOTE TNBC PROGRESSION

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ABBREVIATIONS:

Adipose tissue macrophages (ATMs)

Androgen receptor (AR)

Body mass index (BMI)

Bone marrow-derived macrophages (BMDMs)

Cancer stem-cells (CSCs)

Cardiotrophin-1 (CT-1)

Cardiotrophin-like cytokine (CLC)

Ciliary neurotrophic factor (CNTF)

Conditioned media (CM)

C reactive protein (CRP)

Diet-induced obesity (DIO)

Epidermal growth factor (EGF)

Epithelial-mesenchymal transition (EMT)

Florescent in-situ hybridization (FISH)

Free fatty acids (FFAs)

Glycoprotein 130 (GP130)

Herceptin Receptor (HR)

High-fat diet (HFD)

Human monocyte-derived macrophages (HMDMs)

Immunomodulatory (IM)

Interleukin-1β (IL-1β)

Interleukin-6 (IL-6)

Interleukin-8 (IL-8)

Interleukin-27 (IL-27)

Interleukin-31 (IL-31)

Leukemia inhibitory factor (LIF)

Low-fat diet (LFD)

Luminal androgen receptor (LAR)

Colony stimulating factor (CSF)

Mesenchymal (M)

Mesenchymal stem-like (MSL)

Metabolically activated macrophages (MMe(s))

NADPH oxidase 2 (NOX2)

Nox2-deficient mice (mNox2-/-)

Obesity-associated Inflammation (OAI)

Oncostatin-M (OSM)

Quantitative real-time polymerase chain reaction (qRT-PCR)

Serum amyloid-A (SAA)

Short hairpin RNA (shRNA)

Stromal vascular cells (SVCs)

Triple-negative breast cancer (TNBC)

Tumor necrosis factor (TNF)

Vector control (VC)

White adipose tissue (WAT)

Wt. (wild type)

ABSTRACT

Obesity is associated with increased incidence and severity of triple-negative breast cancer (TNBC); however, mechanisms underlying this relationship are incompletely understood. Here, we show that obesity reprograms mammary adipose tissue macrophages to a pro-inflammatory metabolically-activated phenotype (MMe) that alters the niche to support tumor formation. Unlike pro-inflammatory M1 macrophages that antagonize tumorigenesis, MMe macrophages are pro-tumorigenic and represent the dominant macrophage phenotype in mammary adipose tissue of obese humans and mice. MMe macrophages release cytokines in an NADPH oxidase 2 (NOX2)-dependent manner that signal through glycoprotein 130 (GP130) on TNBC cells to promote stem-like properties including tumor formation. Deleting Nox2 in myeloid cells or depleting GP130 in TNBC cells attenuates obesity-augmented TNBC stemness. Moreover, weight loss reverses the effects of obesity on MMe macrophage inflammation and TNBC tumor formation. Our studies implicate MMe macrophage accumulation in mammary adipose tissue as a mechanism for promoting TNBC stemness and tumorigenesis during obesity.

CHAPTER 1. BACKGROUND AND SIGNIFICANCE

Triple-negative breast cancer (TNBC)

TNBC is a heterogeneous group of breast cancers, defined by lack of expression of estrogen, progesterone and Her2 receptor and do not respond to hormonal therapies. About 1 in 8 U.S. women (about 12.4%) will develop breast cancer over the course of her lifetime. According to Breastcancer.org, as of January 2018, there are more than 3.1 million women with a history of breast cancer in the U.S. In 2018, an estimated 266,120 new cases of invasive breast cancer are expected to be diagnosed, along with 63,960 new cases of non-invasive (in situ) breast cancer. About 40,920 women in the U.S. are expected to die in 2018 from breast cancer. TNBC accounts for about 20 percent of all breast cancers. About ~10% of breast cancers can be linked to inherited gene mutations (BRCA1/2); however, 85% of cases occur in women who have no family history.

Triple-negative breast cancer has extremely poor prognosis due to 1). Higher grade at the time of diagnosis, 2). More likely to metastasize to distant organs such as lungs, and brain, 3). Higher recurrence rate after treatment, and 4). Lack of targeted drug therapies because they will not respond to hormonal therapies and drugs targeting Her2. All these factors contribute to lower five-year survival rates for TNBC compared to other types of breast cancer. For instance, 77% of women with TNBC survived at least five years, versus 93% of women with HR+ and/or Her2+ breast cancer (Breast cancer.org, last accessed July 27, 2018). However, this difference in survival disappear after five years, and TNBC patients do better since HR+ cancer relapse after decades.

The primary challenge in treating TNBC is the use of chemotherapy as the first-line adjuvant therapy due to the lack of targeted therapy, which results in 1. Lack of less toxic treatment that can be used for the long-term to keep micro-metastases under control after the primary tumor has been surgically removed, and 2. Lack of salvage therapy when tumor relapse occurs years after. The reason for lack of targeted therapy is because TNBC is not one disease; the negative definition of TNBC is capturing several clinical entities. Thus, TNBC subtyping is necessary to identify molecular-based therapies.

Based on the gene expression data, a research group classified TNBC into six subtypes, including two basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem-like (MSL), and a luminal androgen receptor (LAR) subtype(Lehmann et al., 2011). BL1 and BL2 subtypes had higher expression of cell cycle and DNA damage response genes, M and MSL subtypes were enriched in gene expression for epithelial-mesenchymal transition (EMT) and growth factor pathways, and the LAR subtype was characterized by androgen receptor (AR) signaling (Lehmann et al., 2011). Another group (Prat et al., 2013) classified TNBC tumors as luminal A, luminal B, HER2E, basal-like, and normal-like TNBC tumors. Basal-like is the most common with features similar to those of the outer basal cells surrounding the mammary ducts. As expected, TN/luminal tumors showed high expression of estrogen-related genes, TN/Basal-like tumors showed high expression of cell cycle-related genes such as KI67 and aurora kinase B, and TN/HER2E tumors showed an overall intermediate gene expression, except for high expression of genes involved in oxidation reduction-related biological processes (Prat et al., 2013). There is an overlap between these two classifications, where the vast majority of the PAM50-defined HER2E and luminal tumors highly expressing the

LAR cluster, the PAM50-defined basal-like tumors were split into three main groups based on the expression of the immune-related genes, the stromal-related genes, and the basal genes.

Obesity, a pathological condition present in 30% of TNBC patients and is linked with poor prognosis in TNBC, might help us in identifying a subgroup of TNBC associated with specific molecular characterization for two reasons. First, an obese environment may put a selective pressure resulting in the fitness of specific oncogenic mutations. Second, tumors that evolve within an obese microenvironment might be driven by hyper-nutrition and inflammatory cytokines and may exhibit a dependency on certain obesity associated physiological conditions (Olson et al., 2017). Thus, understanding the mechanistic link between obesity and TNBC is clinically relevant.

Link between Obesity and Cancer

Obesity is clinically defined by a higher body mass index (BMI). People with BMI range between 18-24 are considered lean, 25 to 29 overweight and BMI above 30 are considered as obese. It has become an epidemic in the US and continues to grow despite growing recognition of the problem. The latest estimates are that approximately 34% of adults and 15–20% of children and adolescents in the U.S. are obese (Mitchell et al., 2011, 2015). Obesity is a risk factor for many diseases such as diabetes, atherosclerosis as well as cancer (Osborn and Olefsky, 2012).

Obesity is responsible for an estimated ~20% of cancer-related deaths in adults. It competes with smoking as the leading preventable risk factor for cancer incidence and mortality (Calle et

al., 2003). Several types of cancer have been linked to obesity such as pancreatic cancer, colon cancer, ovarian cancer, hormone positive breast cancer, including TNBC.

TNBC tumors occur more often in younger women and Black/African-American women (Kohler et al., 2015; Partridge et al., 2016; Sineshaw et al., 2014) (American Cancer Society, 2016). They are also common among Hispanic women compared to white/non-Hispanic white women (Howlader et al., 2014). Both Black/ African-American and Hispanic race and ethnicity tend to be more overweight or obese compared to white women. Therefore, obesity is also considered a risk factor for TNBC. Obese women have a 36-50% higher risk for developing TNBC than women with the lowest BMI (Calle et al., 2003; Picon-Ruiz et al., 2017). Obesity is also an independent prognostic factor for breast cancer patients both pre and post-menopausal (Chen et al., 2016; Matthews and Thompson, 2016). The risk of developing distant metastasis increases by 46% and breast cancer-related death by 38% in obese patients compared to non-obese patients (Picon-Ruiz et al., 2017). These epidemiological studies suggest the role of obesity in TNBC initiation, progression, and metastasis. However, the mechanism by which obesity promotes TNBC progression is not very clear.

Role of inflammation in obesity-associated cancer

Obesity causes many health conditions including hyper nutrition, hyperinsulinemia, hypercholesterolemia, and chronic inflammation. Although all these health conditions may play a role in cancer progression, inflammation, in particular, has been casually linked with cancer initiation and progression.

OAI (obesity-associated inflammation) lowers the mutational and epigenetic barriers for the transformation of a premalignant cell. For example, in the colon, epigenetic changes observed in normal epithelial cells during obesity are similar to those found in cancer cells (Li et al., 2014a), thus lowering the number of mutational events required for malignant transformation (Bordonaro and Lazarova, 2015). OAI dramatically alters the tissue composition and thus creating a pro-tumorigenic niche that supports tumor growth and metastasis. For example, in both breast (Seo et al., 2015) and pancreas (Incio et al., 2016), OAI is associated with altered extracellular matrix mechanical properties caused primarily by fibroblast accumulation and promotes tumor growth and metastasis. Similarly, OAI fosters neutrophilia in the lungs, which then promotes breast cancer metastasis to the lungs (Quail et al., 2017). OAI can also contribute to cancer progression at the primary tumor site by tumor-infiltrating myeloid cells producing interleukin-1β (IL-1β), an inflammatory cytokine that promotes tumor angiogenesis by stimulating the production of vascular endothelial growth factor–A by adipocytes (Kolb et al., 2016). In the pancreas, obesity is associated with an increase in IL-1β production by adipocytes which promote the recruitment of immunosuppressive neutrophils to the tumor and accelerate tumor growth (Incio et al., 2016). Collectively, these studies demonstrate that obesity-associated systemic inflammation disturbs the homeostatic communication between different organs, normal immune and non-immune cells and primary tumor microenvironment, to mediate enhanced malignancy. Thus, understanding the relationship between TNBC and obesity-associated inflammation is critical.

During obesity, metabolic dysregulation of white adipose tissue (WAT) that stores lipids, recruits macrophages and contributes to chronic, systemic inflammation and metabolic syndrome (Osborn and Olefsky, 2012; Tam et al., 2014). Interestingly, ~20% of lean adults display WAT inflammation, and insulin resistance, classified as metabolic obesity and ~50% of obese

individuals remain metabolically healthy (Stefan et al., 2018). Thus, BMI alone cannot accurately capture immune and metabolic dysfunction in an individual. Therefore, preclinical studies that define the causal relationships between WAT inflammation, and cancer will potentially have clinical relevance for patients across all weight categories. Macrophages are an important causal link between obesity and inflammation, and mechanistic understanding of obesity-associated adipose tissue macrophage inflammation will provide potential therapeutic targets.

Role of adipose tissue macrophages in obesity-associated inflammation and cancer

Macrophages are mononuclear phagocytic myeloid immune cells that are present in all tissues in mammals and play diverse functions ranging from tissue development, homeostasis, and repair by scavenging debris, pathogens, and apoptotic or necrotic cells (Wynn et al., 2013). Circulating monocytes differentiate into macrophages once they reach the tissues including spleen, liver (Kupffer cells), lung (alveolar macrophages), brain (microglia), bone (osteoclasts and marrow macrophages), lymph nodes, intestines, and fat (adipose tissue macrophages, ATMs). Each of these tissue macrophages has a specialized function to maintain the local tissue microenvironment and inflammatory tone. Based on their inflammatory states, macrophages are classified into two categories. These include the classically activated macrophages (M1) and alternatively activated macrophages (M2), defined by responses to the cytokines IFNγ and activation of TLRs and IL4/IL13 respectively. While this is a useful classification it cannot represent the complex *in vivo* environment for most macrophage types where numerous cytokines and growth factors interact to define the final differentiated state of macrophages. Indeed transcriptional profiling of resident macrophages by the "Immunological Genome

Project" find that these populations show great transcriptional diversity with minimal overlap suggesting many unique classes (Gautier et al., 2012). Therefore, macrophages are an extremely diverse set of plastic cells constantly shifting their functional state in response to changes in tissue physiology or environmental challenges. This phenotypic diversity enables macrophage to play diverse roles in tissue and unfortunately are often subverted to contribute to many diseases such as obesity and cancer.

Tumors are abundantly populated by macrophages, which are classified as tumor-associated macrophages (TAMs) (Williams et al., 2016). Tumors educate the TAMs to adopt an immunosuppressive phenotype (M2) that promotes tumor initiation, angiogenesis, and metastasis by secreting several cytokines such as CSF-1, granulocyte-macrophage (GM)-CSF, IL-3, and chemokines such as CCL-2 and CCL-5 (Qian and Pollard, 2010). Role of TAMs in breast cancer is widely studied and reported. However, the role of adipose tissue macrophages in breast cancer progression is unclear. During obesity, macrophages accumulate in the adipose tissue of mice and humans (Weisberg et al., 2003; Xu et al., 2003) and are key contributors to inflammation (Chawla et al., 2011; Lumeng and Saltiel, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2005). Since both macrophages and inflammation are linked with cancer initiation and progression, the obesity-associated mammary ATM inflammation is a potential mechanistic link between obesity and TNBC.

Based on gene expression upon exposure to TLR/IFNγ signals or Th2 cytokines, macrophages are categorized as being classically activated (M1) or alternatively-activated (M2) macrophages, respectively (Gordon and Taylor, 2005). M1 macrophages are pro-inflammatory and anti-tumorigenic, and M2 macrophages are anti-inflammatory and pro-tumorigenic (Gordon

and Taylor, 2005). The breast adipose tissue of obese women is highly enriched with macrophages. These macrophages surround the dying adipocytes and form inflammatory foci (Choi et al., 2016; Quail et al., 2017; Vona-Davis et al., 2008). These obesity associated macrophages play a significant role in tumor progression. Depletion of macrophages diminished the effects of obesity on breast cancer growth and metastasis (Quail et al., 2017). It is reported that during obesity, M2 macrophage phenotype switches to M1 macrophage phenotype (Castoldi et al., 2016). Then how could obesity promote breast cancer if it elicits an M1, anti-tumor macrophage phenotype? Our lab has recently shown that obesity does not promote M1 phenotype but instead metabolically activate the visceral ATMs (Kratz et al., 2014).

Metabolically-active Macrophages (MMe(s))

Using a combination of proteomics, immunology, and cell biology we have previously identified a novel pro-inflammatory macrophage phenotype produced by exposure to high levels of insulin, glucose, and palmitate ('metabolically activated'; MMe), conditions characteristic of obesity and diabetes. Importantly, this pro-inflammatory phenotype is mechanistically distinct from classically activated M1 macrophages. Furthermore, pro-inflammatory adipose tissue macrophages express MMe markers (ABCA-1, PLN2, CD36) and not M1 markers (CD38 and CD319) in obese humans/mice in visceral adipose tissue (Kratz et al., 2014). Unlike during the classical activation of macrophages that happens when LPS binds to toll-like receptor-4 (TLR4), metabolic activation of macrophages has two phases. First, FFA secreted by adipocytes during obesity binds the TLR2 receptor. It causes MYD88/NFkB activation resulting in the expression of pro-inflammatory cytokines. Inflammatory cytokines cause insulin resistance in adipocytes and signal them to release more FFA. Adipose tissue macrophages then uptake the excess FFA

and dampen the expression of proinflammatory cytokines via P62 causing insulin sensitivity in adipocytes that cause adipocytes to store more fat and reduce FFA secretion. This cycle goes on, and therefore, unlike M1-like macrophages, which are associated with high-grade inflammation, MMe macrophages leads to chronic low-grade inflammation, characteristic of obesity.

Although we have identified the presence of MMe macrophages in visceral adipose tissue, its presence in mammary adipose-tissue is yet to be determined. This is important because adipose tissue depots are very different and may exhibit distinct metabolic phenotype during obesity. Here, we will investigate whether obesity induces metabolic activation of macrophages in the mammary adipose tissue of human and mice and its role in breast cancer development and progression.

Role of NADPH Oxidase 2 (NOX2) in metabolic activation of macrophages

NADPH oxidase (NOX) is a family of enzymes with a primary function to produce ROS by the generation of superoxide and/or hydrogen peroxide via the transfer of electrons from NADPH to molecular oxygen. They have flavin adenine dinucleotide (FAD)-binding and NADPH-binding sites on the enzymes' C-terminal tail. The NOX family is comprised of seven members; namely NOX1 through 5 and DUOX1 & 2. Importantly, these isoforms are functionally and structurally-related but differ in their tissue distribution, level of expression, the nature of ROS produced, and control by distinct signaling modulators. Of these, Nox2, which is found in phagocytes (e.g., eosinophils, macrophages, and neutrophils) (Segal et al., 1981) and dendritic cells (Elsen et al., 2004; Graham et al., 2015) was the first to be discovered.

NOX2 is essential for antimicrobial host defense through the production of reactive oxygen species (ROS), activation of granular proteases and generation of neutrophil extracellular traps (NETs). Genetic disorders of NOX2 cause Chronic granulomatous disease (CGD) characterized by severe life-threatening bacterial and fungal infections and by excessive inflammation, including Crohn's-like inflammatory bowel disease (IBD). NOX2 limits inflammation and injury by modulation of critical signaling pathways that affect neutrophil accumulation and clearance. NOX2 also plays a role in antigen presentation and regulation of adaptive immunity. Specific NOX2-activated pathways such as nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor that induces antioxidative and cytoprotective responses, may be important therapeutic targets for CGD and, more broadly, diseases associated with excessive inflammation and injury.

Role of NOX2 in neutrophil-mediated host defense is well studied. However, the role of NOX2 in macrophages is less established. The most substantial evidence for the role of NOX2 in macrophage-induced host defense is from the finding that mutations in gp91phox that selectively affect macrophages lead to increased susceptibility to mycobacterial diseases (Bustamante et al., 2011). In our recent work, we identified NOX2 as a key regulator of the inflammatory cytokine expression and lysosomal exocytosis in MMe macrophages (Coats et al., 2017). We found that ablating Nox2 (Gp91 subunit) attenuated Il6 and Il1β levels in MMe macrophages and this effect was specific to MMe macrophages since cytokine expression in M1 macrophages was unaffected.

We use this information to our advantage to study the role of MMe macrophages-induced pro-inflammatory cytokine in TNBC progression. We fed myeloid cell-specific Nox2-deficient

mice (mNox2-/-) and littermate controls (wt.) an HFD and test if depleting Nox2 from myeloid cells can prevent the effect of obesity on TNBC growth and progression.

Inflammation and Cancer stem cells (CSCs)

Cancer cells are not all the same. Within a tumor, there are rare cancer stem cells also called tumor-initiating cells. These terms are derived from the ability of a specific population of cells being able to propagate a tumor *in vivo* upon transplantation into an animal model. Like normal stem cells, these cells can both self-renew by dividing and give rise to many cell types that constitute a tumor, and can, therefore, form tumors. Such cells have been found in various types of human tumors. However, these cancer stem cells or cancer-initiating cells are not necessarily the cell of origin of the initial primary tumor. It has been shown that cancer cells can de-differentiate into CSCs under certain circumstances (Chaffer et al., 2011; Hanahan and Weinberg, 2011). This could be the first step in the process for cancer cells to become CSCs followed by progression through the EMT and migration to secondary sites. They can also act as a reservoir of cancer cells that may cause relapse after surgery, radiation or chemotherapy has eliminated all observable signs of cancer.

Studies on identification and characterization of CSCs from hematologic malignancies have been done extensively based on decades of knowledge on hematopoiesis. However, studies of solid tumor CSCs has been limited due to a lack of understanding of normal adult tissue stem cells. CSC markers such as CD44+ CD24low were identified by their ability to generate tumors in immunodeficient mice (Al-Hajj et al., 2003). Similarly, CD133 was identified for brain tumors (Singh et al., 2003). Another more commonly used marker is increased aldehyde dehydrogenase activity (ALDH) activity (Ginestier et al., 2007). However, the importance of these markers

remains unclear and vary between different mouse models and cancer cell lines. The more unifying and robust way is to identify them qualitatively using gene expression of embryonic stem cells markers such as SOX2, OCT4, and NANOG (Ben-Porath et al., 2008; LING et al., 2012). Besides, functional assays can help us in identifying the presence of these rare stem cells. For instance, *in vitro* tumorsphere assay is used to test the potential of cancer cells to form colonies from single cells in a non-adherent condition, which is a property of cancer stem cells. *In vivo* limiting dilution assay test the minimum number of cancer cells required to form a tumor, which is again the property of cancer stem cells since studies have shown that depleting cancer stem cells from tumor abrogates the potential of cancer cells to form a tumor. Therefore, using these functional assays and gene expression of stem markers, cancer stem-cell-like properties of cancer cells can be robustly tested.

In 1863, Rudolf Virchow (Virchow, 1863) proposed a functional relationship between inflammation and cancer. He hypothesized that the origin of disease was at sites of chronic inflammation. It is now clear from the existing clinical evidence linking inflammatory states and cancer development. For instance, epidemiologic studies have demonstrated associations between ulcerative colitis, Hepatitis C and chronic pancreatitis to the development of cancers of the colon, liver, and pancreas. In fact, it is clear now that inflammation is fundamental to cancer growth and progression. For example, genetic polymorphism in genes of inflammatory cytokines IL-1β, IL-6 and IL-8 predisposes to several cancer types including breast cancer (Huang et al., 2013; Niu et al., 2014; Zhang et al., 2016). The level of chronic inflammation as assessed by serum C reactive protein (CRP) or serum amyloid-A (SAA) are correlated with risk of breast cancer recurrence after primary therapy and poor survival (Allin et al., 2011; Pierce et al., 2009). Furthermore, the Stat3 and NF-κB pathways play a critical role in inducing and maintaining a

pro-carcinogenic inflammatory microenvironment at the initiation of malignant transformation and tumor progression These inflammatory cytokines including IL-1, IL-6 and IL-8 may influence tumor growth by regulating cancer stem cells populations (Bromberg and Wang, 2009; Grivennikov et al., 2009, 2010; Korkaya et al., 2011; Liu et al., 2010).

Inflammation results in increased stemness-associated gene expressions as well as functional properties, leading cancer cells to adopt a CSC phenotype. For instance, cancer cells exposed to IL-6 possessed stem-like properties, such as an enhanced capacity for tumorsphere formation and increased expression of stem-markers. Once IL-6 binds to its receptor (IL-6R and GP130), the JAK/STAT3 pathway is activated and has been shown to be important in converting non-CSCs into CSCs (2013). Also, IL-6 promotes Notch3/Jagged1 signaling pathway which promotes breast cancer cells self-renewal, hypoxia survival, and invasiveness potential, characteristics of CSCs (Sansone et al., 2007). IL-6 driven epigenetic changes have been associated with CSC features (expression markers and functional phenotype) in breast cancer cells (D'Anello et al., 2010).

GP130 and CSCs

Glycoprotein 130 (gp130) is a shared receptor utilized by several cytokines. These cytokines belong to IL-6 family and comprises of nine secreted soluble ligands; IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), interleukin-27 (IL-27) and interleukin-31 (IL-31). Each ligand interacts with a specific non-catalytic transmembrane receptor, which then binds to the transmembrane protein glycoprotein-130 beta-subunit (GP130, also known as IL6ST or CD130) to activate the downstream signaling JAK/STAT3. Gp130

cytokines are casually linked with the development of inflammation by assisting in the recruitment of leukocytes, regulating B-cell responses at the wound site. Gp130 also mediates the balance between inflammation and cancer and that the dysregulated Gp130 signaling contributes to cancer. For example, gastric cancer and colorectal cancer are associated with aberrant inflammation of the mucosa, and gp130 and its ligands IL-11 and IL-6 has a well-characterized role in for mediating disease progression through maintenance and enrichment of cancer stem cells (Silver and Hunter, 2010). Like IL-6 several of these cytokines have been shown or predicted to be involved in promoting CSC-like properties in cancer cells. For example, LIF plays an essential role in embryonic implantation and maintaining pluripotential of murine stem cells (Edwards et al., 2017; Freudenberg et al., 2012; Ohtsuka et al., 2015) as well as in regulating glioma stemness (Edwards et al., 2017). LIF also promotes breast cancer tumorigenesis and metastasis via the PI3K/AKT pathway (Li et al., 2014b). Another IL-6 family cytokine, OSM, has been shown to promote breast CSC phenotype in non-CSCs by activating STAT-3 in cooperation with TGFβ/SMAD3 signaling. A study showed that OSM promotes phenotypic changes associated with mesenchymal and stem cell-like differentiation in breast cancer via the PI3K/AKT pathway (West et al., 2014). Together, these studies suggest that dysregulation of GP130 and its ligands, and increased levels of STAT3 activation would contribute to the development of cancer in multiple neoplasias, including breast cancer. Since obesity is linked with increased expression of inflammatory cytokines such as IL-6, it is important to understand the expression and role of other IL-6 family cytokines in inducing CSC-phenotype in TNBC.

Summary

Obesity is a major modifiable risk factor for breast cancer and is responsible for approximately 20% of cancer deaths (Calle et al., 2003). In addition to its role in breast cancer pathogenesis, obesity is recognized as a marker of poor prognosis in pre- and post-menopausal women with breast cancer (Chan and Norat, 2015). Epidemiological studies have linked obesity with increased risk of developing different subtypes of breast cancer, including triple-negative breast cancer (TNBC)(Pierobon and Frankenfeld, 2013; Trivers et al., 2009; Vona-Davis et al., 2008), a particularly aggressive form of breast cancer with poor outcome and few therapeutic options. Among TNBC patients, progression and disease-free survival (DFS) are strongly correlated with excessive weight and obesity (Choi et al., 2016). However, mechanisms by which obesity leads to worse breast cancer prognosis are incompletely understood.

One clue to its action is that obesity causes chronic inflammation. A recent study showed that obesity-induced neutrophil accumulation in the lung could promote breast cancer metastasis (Quail et al., 2017). In addition to inflammation at metastatic sites, obesity also promotes local inflammation in adipose tissue which is mediated by macrophage infiltration and activation(Lumeng and Saltiel, 2011; Xu et al., 2003). Obesity-induced inflammation in mammary adipose tissue (Howe et al., 2013; Vaysse et al., 2017) may be of particular significance because breast cancers form in this niche and inflammation promotes stem-like properties in cancer cells and an increased propensity to form tumors (Grivennikov et al., 2009). Thus, pro-inflammatory macrophage accumulation in mammary fat may augment TNBC tumor formation during obesity.

Macrophages are heterogeneous and have been broadly classified as either classically (M1) or alternatively (M2) activated (Gordon and Taylor, 2005). Th2 mediators (e.g. IL-4) drive the M2 phenotype, which scavenges debris, and promotes angiogenesis and tumor growth (Noy and Pollard, 2014). In contrast, the M1 phenotype is promoted by Th1 mediators (e.g. LPS and IFNγ) and is characterized by the production of pro-inflammatory cytokines and anti-tumor activity (Pyonteck et al., 2013). Earlier studies showed that obesity promotes an M1-like ATM phenotype (Lumeng et al., 2007), which would be expected to oppose tumor formation. However, more recent studies from multiple labs have challenged the notion that obesity supports an M1 phenotype (Kratz et al., 2014; Xu et al., 2003).

Studies from our group showed that obesity produces a pro-inflammatory 'metabolically activated' (MMe) ATM phenotype that is both mechanistically and functionally distinct from the M1 phenotype (Coats et al., 2017; Kratz et al., 2014). Although we showed that MMe macrophages accumulate in visceral and subcutaneous adipose tissue of obese humans and mice, their presence in mammary fat, and their ability to promote TNBC tumor formation have not been explored.

Here, we show that MMe macrophages accumulate in mammary fat of obese mice and humans. We demonstrate that these MMe macrophages secrete cytokines in an NADPH oxidase 2 (NOX) dependent manner, that signal through GP130 on murine and human TNBC cells to promote stem-like properties and tumor formation during obesity. These findings reveal an important mechanism by which obesity enhances TNBC tumorigenesis.

Significance

Obesity is a chronic low-grade inflammatory disease. Tumors evolving in inflamed obese milieu might be addicted to inflammatory cytokines and their associated signaling pathways and presents a window of opportunity for the treatment. Here we identify metabolically activated macrophages in obese mammary adipose tissue as an important source of inflammation that fuels triple-negative breast cancer (TNBC) stemness and tumorigenesis. We found that these metabolically active mammary adipose tissue macrophages (ATMs) secrete pro-inflammatory cytokines that signal through GP130 on TNBC cells to promote their cancer stem cell (CSC) like phenotype. Targeting mammary ATM inflammation or GP130 signaling in cancer cells, attenuate the effect of obesity on the tumor-forming potential of TNBC cells and CSC-like phenotype. Moreover, weight loss intervention inhibited the effect of obesity on inflammation of metabolically active macrophages and tumor-forming potential of TNBC cells. Since the CSC phenotype is associated with tumor relapse and metastasis, and poor prognosis in TNBC, our mechanistic insights provide potential targets for treating obesity-associated TNBC. We showed that weight-loss, a non-invasive intervention can reverse mATM inflammation and tumor-forming potential of TNBC cells.

CHAPTER 2. METHODS

Regulatory

Animal studies were approved by the University of Chicago IACUC (ACUP #72209 and ACUP #72228). Human studies were approved by the Institutional Review Boards at the University of Chicago (IRB16-0321) and Northwestern University (NU 11B04).

Subject recruitment

Human breast adipose tissue was obtained from women undergoing breast reduction surgery surgeries at Northwestern Memorial Hospital. Exclusion criteria included cancer or any other breast related diseases. Human breast adipose tissue was collected by surgeons and tissue was processed immediately to obtain the stromal vascular cells (SVC) for flow cytometric analyses.

Mice

Wild-type and Nox2-/- (Cybb-/-) female mice on the C57BL/6 background, and C3(1)-TAg mice are from Jackson Labs. Myeloid cell specific Nox2-/- mice (mNox2-/-) were generated by crossing $Cybb^{fl/fl}$ mice (Sag et al., 2017) with LysM-cre knock in mice (Jackson Labs, 004781) to generate LysM-$cre^{+/-}$ $Cybb^{fl/fl}$ and litter mate control $Cybb^{fl/fl}$ mice as previously described (Coats et al., 2017). Mouse genotype was confirmed by PCR (see Table S1 for primers).

DIO studies

Female C57BL/6 or C3(1)-TAg female mice were placed on a low-fat (Harlan) or 60% high-fat diet (D12451, Research Diets Inc.) at 6 weeks of age for up to 12 weeks. Body weight was monitored every week.

Plasma metabolic measurements

Mice were fasted for 3h and blood glucose levels were measured with a One Touch Ultra 2 glucometer (Lifescan) and serum insulin levels were measured by ELISA (Millipore).

TNBC cell lines

The TNBC cells lines used in this study include: E0771 cells, originally isolated from a spontaneous breast adenocarcinoma in C57BL/6 mice (Casey et al., 1951); M6C cells, originally isolated from the C3(1)/SV40 Large T-antigen transgenic mouse model (Holzer et al., 2003); Py8119 cells, derived from a mammary adenocarcinoma that spontaneously arose in a MMTV-PyMT transgenic C57BL/6 female mouse (Gibby et al., 2012); and SUM159PT cells, derived from a primary TNBC tumor from a patient with invasive ductal carcinoma (Neve et al., 2006). Py8119 and SUM159PT cells were obtained from ATTC and cultured according to their guidelines. All other cells were maintained in DMEM supplemented with 10% FCS.

Cancer-cell isolation from tumor

Tumors were digested with collagenase and hyaluronidase (1:3) and 0.1 mg/mL DNase I (Worthington, Lakewood, NJ), filtered through 70-μm mesh, incubated with RBC lysis buffer, filtered through 40-μm mesh, and resuspended in PBS with 1% BSA. To isolate cancer cells for

qRT-PCR analysis, mononuclear cells were depleted by centrifuging using Ficoll-Paque PREMIUM, with a density of 1.077± 0.001 g/mL for 40 minutes at 400 g after RBC lysis. The pellet at the bottom was re-suspended in RLT buffer for RNA isolation.

ATM isolation and analysis

SVC was obtained by digesting human or mouse mammary adipose tissue with collagenase type 2 for 60 minutes and filtering the supernatant with 40 μm filter after red blood cell lysis. Mammary ATMs in the SVC obtained from murine and human mammary fat were interrogated by flow cytometry. Murine and human mammary ATMs were isolated using anti-CD11b antibody coupled to magnetic beads as previously described (Kratz et al., 2014) and purity was assessed by flow cytometry. ATMs were interrogated by qRT-PCR and media was collected for functional assays.

Differentiation and metabolic activation of murine bone marrow-derived macrophages and human monocyte-derived macrophages

Murine bone marrow-derived macrophages (BMDMs) were generated and metabolically activated as previously described (Kratz et al., 2014). Briefly, bone marrow was flushed from the bones of the hind legs and differentiated to macrophages by culturing for 6 days in six-well plate in DMEM with 10% FCS plus conditioned media from L929 cells at a 1:3 ratio. Human peripheral blood monocytes were isolated from healthy donors and differentiated to macrophages in the presence of M-CSF (125 ng/mL). For MMe activation, macrophages were treated with glucose (30mM), insulin (10nM), and palmitate (0.3mM) for 24h.

Collection of mammary ATM conditioned media

Conditioned media (CM) was generated by culturing primary immune cells in regular DMEM serum free media for 24hrs, using .6 mL of media for every 1 million macrophages seeded per well of 24-well tissue culture plate.

Limiting dilution assay

E0771 or PY8119 cells were transplanted into the #4 mammary fat pad. Tumors were considered established when they became palpable for 2 consecutive weeks. Analyses of tumor incidence were confirmed by two independent investigators. To test the effects of ATMs or MMe macrophages on E0771 tumor-initiating potential, we treated them with macrophage conditioned media for 48h in DMEM supplemented with 2.5% FBS, counted the cells, and injected them into the mammary fat pad.

Tumorsphere assay

E0771, M6C, or SUM159PT cells were plated at 500 cells/well of an ultra-low attachment 12-well plate (Corning) in standard mammosphere media comprising DMEM-F12 supplemented with FGF (20ng/mL, Gold Bio.), EGF (20ng/mL, Gold Bio.), Heparin (4μg/mL, Sigma) and B27 supplement (Life Technologies). Tumorspheres were counted following 5-8 days of growth. To study the effects of macrophage media on tumorsphere formation, 2 x 10^5 tumor cells were plated per well of 12-well plate, and were pre-treated with 400ul of macrophage conditioned media diluted 1:2 with DMEM supplemented with 5% serum for 48h in DMEM. At this time, cells were collected, counted, and plated for tumorsphere assays. To study the effects of

macrophage media on tumor formation, tumor cells were serum-starved overnight and pre-treated with macrophage conditioned media for 24h in DMEM supplemented with 2.5% serum.

Intravasation assay

MAD-MB-231 cells were transplanted into the #4 mammary fat pad of athymic nude mice mixed with or without Mo/MMe macrophage at 2:1 dilution. Mice were sacrificed after five weeks when tumors were close to 200cm^3 and blood was collected from the right ventricle in EDTA. Red blood cells were lysed using RBC lysis buffer. After spin down cell pellet was resuspended in triazol and RNA was extracted. Later RT-PCR is performed for human GAPDH and normalized with mouse GAPDH to detect number of human cells in circulation per blood volume.

Invasion assay

5*10^5 MDA-MB-231 cells were plated per well of six-well plate and serum starved for 24 hours. After which these cells were pretreated for 24hrs with macrophage conditioned media diluted 1:2 with DMEM containing no serum. 5*10^4 of these pre-treated MDA-MB-231 cells were then plated in 24-well trans-well inserts with 8um pores (Corning) coated with Matrigel. After incubating at 37 C for 24hrs, inserts were transferred to an empty well and stained with 4ng/ul of Calcein AM (Corning) for one hours. Stained cells were gently wiped with Q-tips to remove cells on the top layer of the insert, then placed in non-enzymatic dissociation solution (Trevigen) using gentle shaking for one hour at 37 C and 150 RPM. Fluorescence measured using a Victor X3 fluorescent plate reader with excitation at 465nm and emission at 535 nm.

Immunoblotting for phosphorylated stat-3

For testing the phosphorylation of stat-3 in cancer cells upon treatment with macrophage conditioned media I performed immunoblotting. 2*10^5 cancer cells seeded on to 12 well plate were serum starved overnight and were treated with macrophages conditioned media diluted 1:2 with serum free DMEM for one hour. After which cells were lysed with RIPA buffer containing protease inhibitor and PMSF (Sigma). For immunoblotting, antibody against phosphorylated stat-3 was purchased from cell signaling (Catalogue# 9131) and western blot assay was performed following the exact protocol on the cell signaling website recommended for this antibody. As an internal control we used tubulin antibody (Fisher).

GP130 knockdown

Short hairpin RNA (shRNA) specific for murine or human GP130 ligated into the lentiviral vector pLKO-1 were purchased from Dhramacon. Virus particles were packaged, E0771 and SUM159PT cells were infected, and infected cells were selected for by treatment with puromycin. Lentiviral pLKO-1 vector without shRNA was used as a control. Knockdown of GP130 was verified by immunoblotting.

Antibodies for flow cytometry

Antibodies for the measurement of CD45, CD11b, CD206, CD14, ABCA1, CD36, CD38, CD45, and CD206, CD319, F480 were purchased from BD Biosciences (San Jose), Beckman Coulter (Danvers), Novus Biologicals (Littleton) BioLegend (San Diego), eBioscience (San Diego), or Miltyeni (Auburn).

Protein Isolation, identification and quantification for proteomics

Macrophage-conditioned medium (1 million cells/600ul) was collected, clarified by centrifugation (5 min at 1000 3 g), supplemented with 0.02% sodium deoxycholate and 20% trichloroacetic acid, and incubated overnight at 4^0 C. Proteins were harvested by centrifugation (15,000x g for 30 min at 4^0 C). The protein pellet was washed twice with ice-cold acetone, reconstituted in digestion buffer (0.1% Rapigest [Waters Corp.], 50 mM Tris-HCl [pH 8.8]), and then reduced, alkylated, and digested overnight at 37^0C with sequencing grade trypsin (1:50, w/w, trypsin/protein; Promega). Tryptic digests were mixed with acetic acid (1:1, v/v) and subjected to solid-phase extraction on a C18 column (HLB, 1 ml; Waters Corp.) according to the manufacturer's protocol. Fractions containing peptides were dried under vacuum and resus-pended in 0.3% acetic acid/5% acetonitrile (1 mg protein/ml) for analysis. MS/MS spectra were searched against the mouse International Protein Index (IPI) database (version 2006/04/18) (Kersey et al., 2004), using the SEQUEST search engine with the following search parameters: unrestricted enzyme specificity, 2.8 amu precursor ion mass tolerance, 1.0 amu fragment ion mass tolerance, fixed Cys alkylation, and variable Met oxidation. SEQUEST results were further validated with PeptideProphet (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003), using an adjusted probability of R 0.90 for peptides and R 0.96 for proteins. When MS/MS spectra could not differentiate between protein isoforms, all isoforms were included in the analysis. Proteins detected by liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS)were quantified by spectral counting (the total number of MS/MS spectra detected for a protein).

Statistics

Statistical significance was assessed using an unpaired, two-tailed, Student's t-test. Replicate numbers for each experiment are indicated in the figure legends.

qRT-PCR

RNA was isolated using Qiagen Midi-prep kits, reverse transcribed with Quantiscript (Qiagen) using random hexamers (Invitrogen), and mRNA levels were measured with specific primers using SYBR green on a One Step Plus system (Applied Biosystems). Relative levels of each target gene were calculated using the ΔΔCt formula, using 18S RNA as a control.

Gene	Forward primer	Reverse primer
Sox2	TTAACGCAAAAACCGTGATG	GAAGCGCCTAACGTACCACT
Pou5f1	CGGAAGAGAAAGCGAACTAGC	ATTGGCGATGTGAGTGATCTG
Nanog	TTGCTTACAAGGGTCTGCTACT	ACTGGTAGAAGAATCAGGGCT
Il-11	TGCTGACAAGGCTTCGAGTAG	ACATCAAGAGCTGTAAACGGC
Lif	ATTGTGCCCTTACTGCTGCTG	GCCAGTTGATTCTTGATCTGGT
Cntf	AGCCTTGACTCAGTGGATGG	TGGAGGTTCTCTTGGAGTCG
Osm	CCCGGCACAATATCCTCGG	TCTGGTGTTGTAGTGGACCGT
Ctf1	CCACCAGACTGACTCCTCAAT	CTCCCTGTTGCTGCACGTA
Il-6	TACTCGGCAAACCTAGTGCG	GTGTCCCAACATTCATATTGTCAG
Tnf-α	CACCACGCTCTTCTGTCTACTG	GCTACAGGCTTGTCACTCGAA
Il-1β	AACTCAACTGTGAAATGCCACC	CATCAGGACAGCCCAGGTC
Plin2	AAGAGCCAGGAGACCATTTC	ACTCCACCCACGAGACATAGA
Cd36	GAACCACTGCTTTCAAAAACTGG	TGCTGTTCTTTGCCACGTCA
Abca1	CATCGTGTCTCGCCTGTTCT	CTTGATCTGCCGTAACATTCTC
Nox2	GTAAATTCACTGTTCTGGGTC	ACATGTTCTTCTCACAGGCTC
LysM-cre	CCCAGAAATGCCAGATTACG	
LysMcom	CTTGGGCTGCCAGAATTTCTC	
LysM-wt	TTACAGTCGGCCAGGCTGAC	
Sox10	CCCACACTACACCGACCAG	GGCCATAATAGGGTCCTGAGG
Snai1	TGTCTGCACGACCTGTGGAAAG	CTTCACATCCGAGTGGGTTTGG
Lin28b	GAGACGGCAGGATTTACTGATGG	CTTTGGCTGAGGAGGTAGACTG
Nr5a2	GGATGGTTACCAGACAAACTCCC	CTCTTGCTGGAGGTAAGCCATG
Foxd3	CAAGAACAGCCTGGTGAAGCCA	ACGGTTGCTGATGAACTCGCAG
Thy1	CCTTACCCTAGCCAACTTCACC	TTATGCCGCCACACTTGACCAG
Aldh1a1	GGAATACCGTGGTTGTCAAGCC	CCAGGGACAATGTTTACCACGC
Abcg1	GACACCGATGTGAACCCGTTTC	GCATGATGCTGAGGAAGGTCCT
Sema6a	AATGGCAGCCTTTTCCTGGAGG	GCAACATAGAGTGAGCCACTCG

Table 1. List of mouse primers

Gene	Forward primer	Reverse primer
NANOG	CCCCAGCCTTTACTCTTCCTA	CCAGGTTGAATTGTTCCAGGTC
SOX2	CCATGCAGGTTGACACCGTTG	TCGGCAGACTGATTCAAATAATACA
POU5F1	GGGCTCTCCCATGCATTCAAAC	CACCTTCCCTCCAACCAGTTGC
LIF	CCAACGTGACGGACTTCCC	TACACGACTATGCGGTACAGC
IL11	CGAGCGGACCTACTGTCCTA	GCCCAGTCAAGTGTCAGGTG
OSM	CACAGACTGGCCGACTTAGAG	AGTCCTCGATGTTCAGCCCA
CNTF	ACAGAGCATTCACCGCTGAC	TCAGGTCTGAACGAATCTTCCTT
CTF-1	GGAGGCCAAGATCCGTCAG	AGCTGCACATATTCCTGGAGC
18s	CCCAACTTCTTAGAGGGACAAG	CATCTAAGGGCATCACAGACC
ABCA1	TGGTCAATGGAAGGTTCAGG	AAATCCTGGACAGGCTTCAG
IL-6	GATGAGTACAAAAGTCCTGATCC	CTGCAGCCACTGGTTCTGT
PLIN2	CTCTCATGGGTAGAGTGGAAAA	GATGTTGGACAGGAGGGTG
CD36	TGCAAAATCCACAGGAAGTG	ATTGGGCTGCAGGAAAGAG
TNF-α	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
IL-1	TCTGTACCTGTCCTGCGTGT	ACTGGGCAGACTCAAATTCC

Table 2. List of human primers

CHAPTER 3. DIET-INDUCED OBESITY PROMOTES TNBC STEMNESS AND TUMOR FORMATION.

Introduction

Obesity is a systemic low-grade chronic inflammatory disease. Inflammation has been causally linked with tumor initiation and formation and CSC-phenotype, including stem marker expression, tumor-initiating potential, and tumorigenesis. Moreover, obesity-associated inflammation has been linked with colon cancer tumorigenesis. Therefore, to understand the role of obesity on TNBC progression, we investigated if obesity plays a role in promoting TNBC tumorigenesis and CSC-like properties of TNBC cells.

Results

Diet-induced obesity (DIO) promotes TNBC tumorigenesis.

To determine if DIO promotes TNBC tumorigenesis, we first studied genetically engineered C3(1)-TAg mice. These mice express SV40 large tumor (T)- antigen under the promoter C3 expressed only in mammary or prostate gland. When expressed large T antigen binds and inhibits the tumor suppressor genes P53 and retinoblastoma protein resulting in the development of spontaneous TNBC tumors in multiple mammary glands (Green et al., 2000). Female C3(1)-TAg mice (on the FVB/N background) were fed a low-fat diet (LFD) or high-fat diet (HFD) for 12 weeks. Although FVB/N mice are somewhat protected from DIO (Montgomery et al., 2013), HFD-fed mice had increased body weight, fasting glucose, and mammary/visceral fat pad weight compared to LFD-fed mice (Figs.1A-D). As previously reported (Mustafi et al., 2017), DIO increased the total tumor burden in C3(1)-TAg mice (Fig.1F), and this increased burden was due, in part, to the presence of more tumors in obese mice (Fig.1G), suggesting that DIO may promote tumor initiation in a genetically engineered mouse model of TNBC.

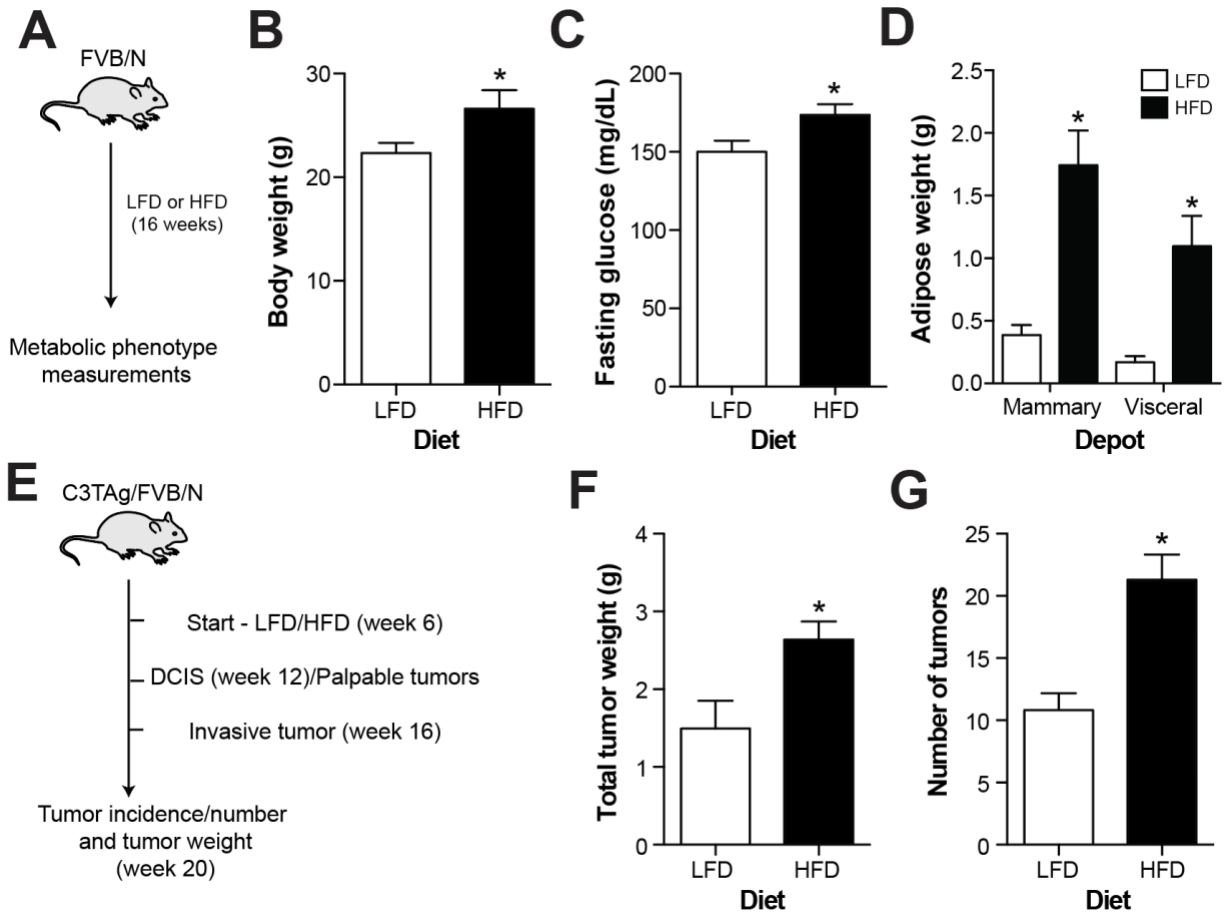


Fig.1. Diet-induced obesity promotes TNBC cell tumor formation. Panels A-D: Female FVB/N mice were fed a low-fat diet (LFD) or high-fat diet (HFD) for 12 weeks. Panel A: Graphical representation. Panel B: Body weight. Panel C: Fasting glucose levels. Panel D: Mammary and visceral adipose tissue weight. Panels E-G: C3(1)-TAg mice were fed a low-fat diet (LFD) or high-fat diet (HFD) starting week 6 and tumors were collected at week 20. Panel E: Graphical representation. Panel F: Total tumor weight measured after collecting all tumors developed per mouse. Panel G: Number of tumors per mouse. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=10 mice/group.

Diet-induced obesity promotes the expression of CSC-markers in cancer cells in a spontaneous TNBC mouse model.

The tumor-initiating capacity of cancer cells has been linked to their stem-like properties (Nguyen et al., 2012). We, therefore, quantified the expression of the stem-associated genes Sox2, Oct4, and Nanog (Klonisch et al., 2008), in cancer cells isolated from tumors of lean and obese C3(1)-TAg mice (Fig.2A). Briefly, tumors were digested using collagenase and hyaluronidase. Cancer cells were enriched using density gradient centrifugation (Ficoll) and were lysed to collect RNA. We found that obesity increased the mRNA expression of Sox2, Oct4, and Nanog in cancer cells (Fig.2B). Next, we investigated the enrichment of CSC population in tumors isolated from obese mice relative to lean mice using flow cytometry. Several markers such as CD133, CD24, CD44, EpCAM, and CD26 have been identified to isolate and characterize CSCs (Al-Hajj et al., 2003; Ghani et al., 2011; Ricci-Vitiani et al., 2007). However, none of these markers or their combinations are universal and therefore, cannot be used to isolate CSCs from all types of cancer. Consequently, we looked for CD90 cell surface expression in cancer cells because it has been used in previous studies to identify CSC populations across multiple cancers and multiple TNBC mouse models (Buishand et al., 2016; Shaikh et al., 2016). We used CD45 (immune cell marker) and CD31 (endothelial cell marker) as a negative gate on flow cytometry to enrich for cancer cell population. Although we did not see enrichment of a unique CD90 positive population, we did see an overall increase in the expression of CD90 in cancer cells isolated from obese tumors (Fig.2C). Together, these findings suggest that obesity may create an environment that enhances the CSC-like phenotype in TNBC cells.

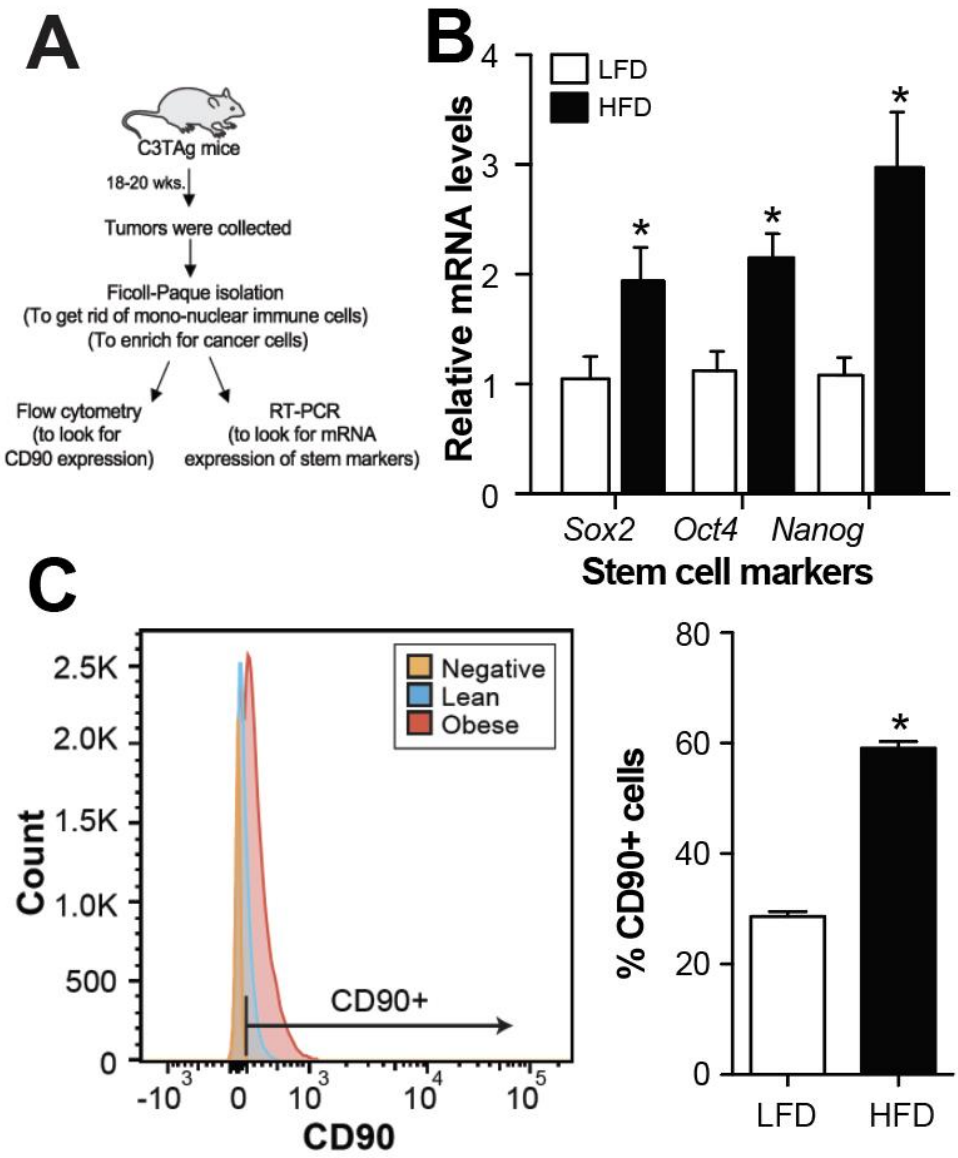
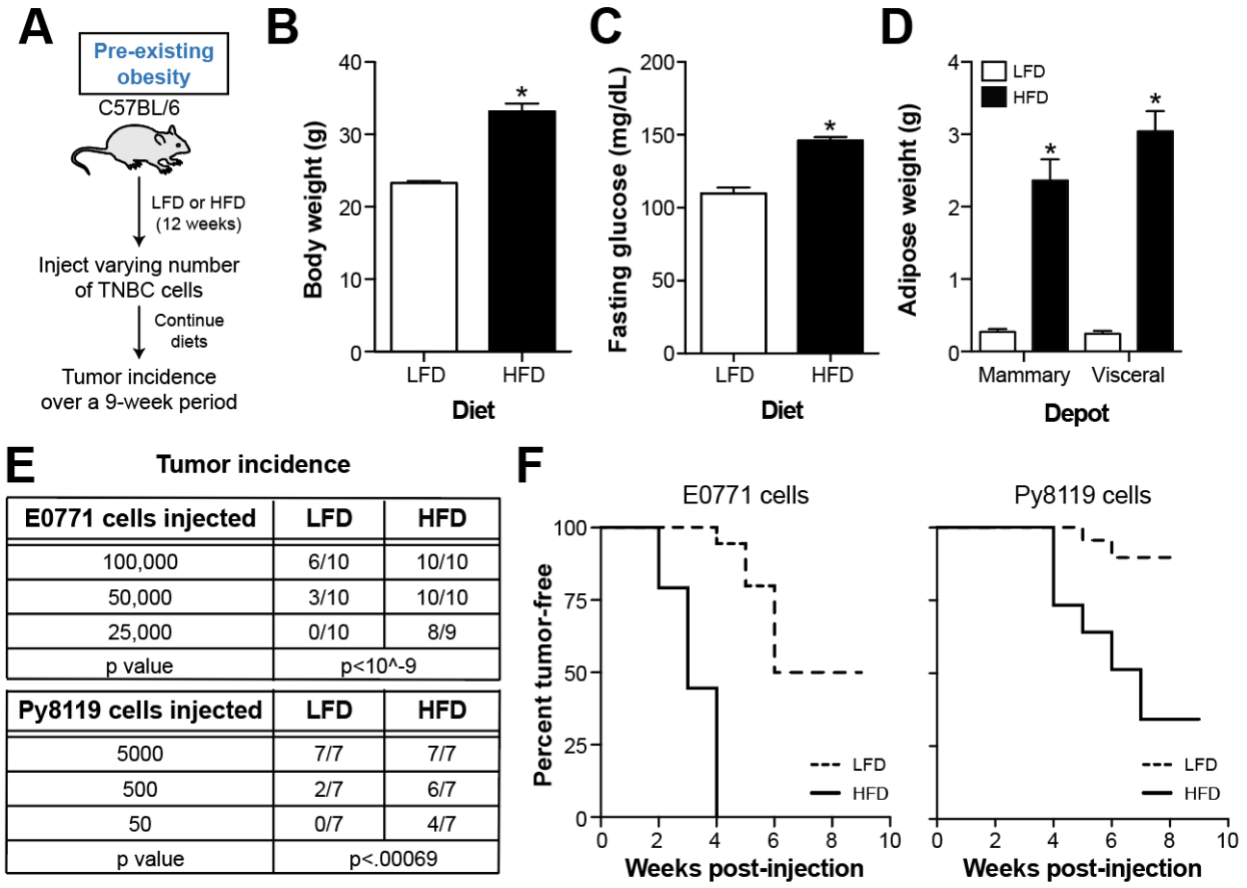


Fig.2. Diet-induced obesity promotes expression of CSC-markers in TNBC tumor. Female C3(1)-TAg mice were fed a low-fat diet (LFD) or high-fat diet (HFD) for 12 weeks. Panel A: Tumors were processed to enrich for cancer cells. Panel B: Stem cell marker expression in isolated cancer cells. Panel C: Flow cytometry to analyze CD90 expression. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5 mice/group.

Diet-induced obesity promotes the tumor-forming potential of TNBC cells.

In the C3Tag transgenic mouse model of TNBC, obesity and cancer progression happen simultaneously, and therefore the effect of pre-existing obesity on cancer progression, which mimics conditions in obese TNBC patients cannot be dissected. To test the possibility that tumors evolving in a pre-existing obese environment have increased CSC-phenotype, we switched to a syngeneic orthotopic transplant model in obesity-prone C57BL/6 mice. This model allowed us to establish an obese environment, and then determine whether this pre-existing environment is more capable of supporting the tumor-initiating potential of TNBC cells, a key function ascribed to cancer stem cells. We fed female C57BL/6 mice a LFD or HFD for 12 weeks to induce obesity, hyperglycemia, and mammary and visceral fat expansion (Figs.3A-D). At this time, murine E0771 or Py8119 TNBC cells were injected at limiting dilutions into mammary fat pads of lean or obese mice, diets were continued, and their tumor-initiating potential was assessed using a limiting dilution assay. We found that DIO decreased the number of E0771 or Py8119 cells required to form tumors (Fig.3E) as well as tumor latency (Fig. 3F), reinforcing the notion that obesity promotes TNBC tumor formation.



E0771 cells injected	LFD	HFD
100,000	6/10	10/10
50,000	3/10	10/10
25,000	0/10	8/9
p value	p<10^-9	

Py8119 cells injected	LFD	HFD
5000	7/7	7/7
500	2/7	6/7
50	0/7	4/7
p value	p<.00069	

Fig.3. Diet-induced obesity promotes TNBC tumor-forming potential. Panel A: Female C57BL/6 mice were fed a LFD or HFD for 12 weeks to create pre-existing obesity. At this point mice were either sacrificed for measuring fat or tumors were injected and HFD was continued. Panel B: Body weight. Panel C: Fasting glucose levels. Panel D: Mammary and visceral adipose tissue weight. Panel E: Limiting dilution assay of E0771 or Py8199 TNBC cells injected into mammary fat of mice. Panel F: Tumor latency. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=7-10 mice/group. Tumor incidence p value is based on extreme limiting dilution analysis (ELDA).

DIO-induced tumor-forming potential is independent of nutrient excess.

The ability of DIO to promote the tumor-initiating potential of TNBC cells could be due to high nutrient levels that support tumor growth, or due to a pathophysiological change induced by chronic high-fat feeding. To differentiate between these possibilities, we fed female C57BL/6 mice for 4 days with a LFD or HFD to create conditions of nutrient excess without changes in body weight, fasting glucose, or mammary/visceral adipose tissue mass (Figs.4A-D). At this time (after 4 days on LFD or HFD), we injected E0771 or Py8119 cells, continued diet feeding, and quantified tumor incidence over a 9-week period. Short-term pre-exposure to HFD did not support increased E0771 and Py8119 tumor formation (Fig.4E), even though mice were maintained on the HFD for 7 weeks following tumor cell injection. These data suggest that the induction of TNBC tumor-initiating potential requires a state of pre-existing obesity, and is not simply reliant on nutrient excess.

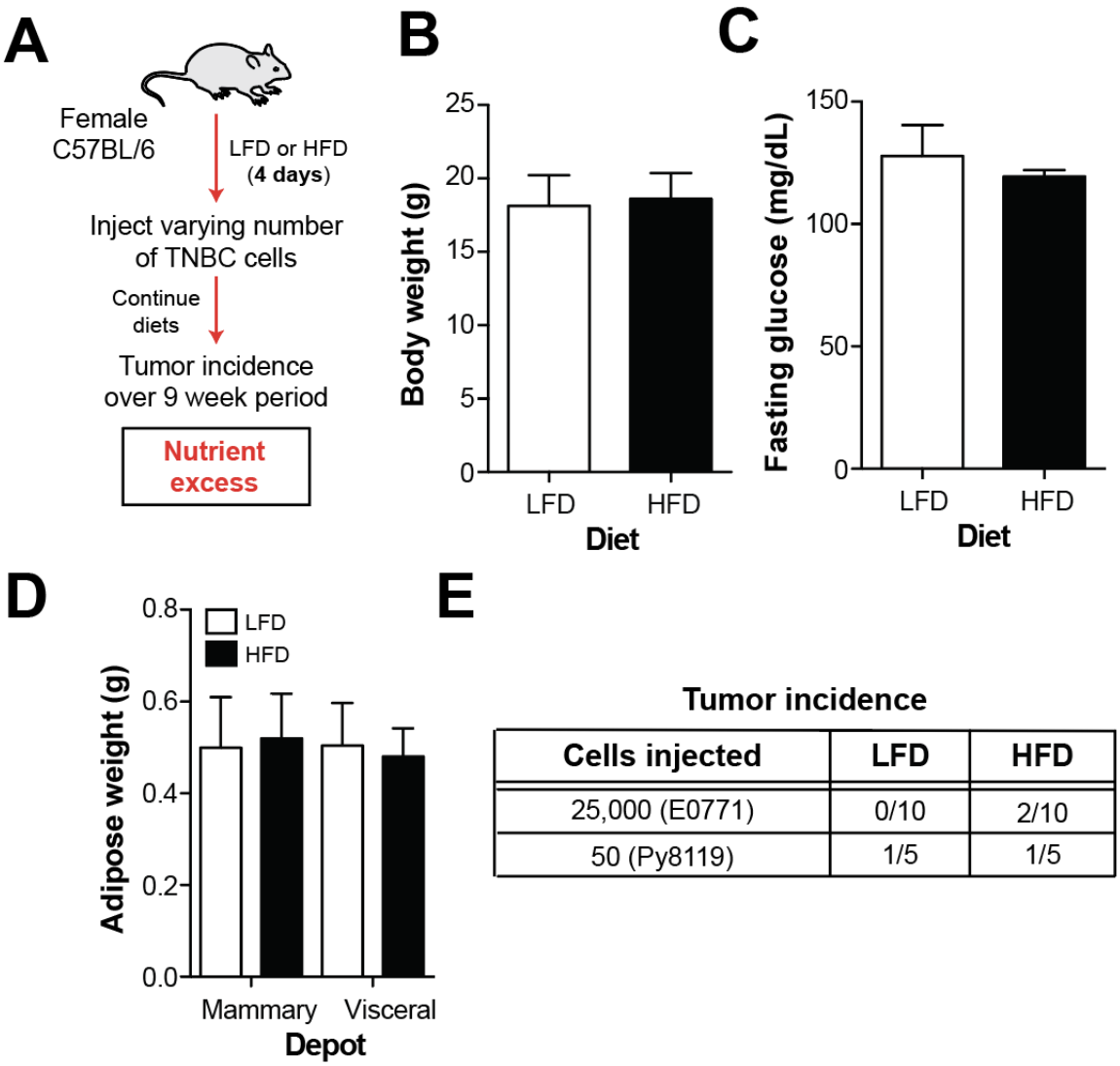


Fig.4. DIO-induced TNBC-tumor initiating potential is independent of nutrient excess. Panel A: Female C57BL/6 mice were fed a LFD or HFD for 4 days to create nutrient excess without pre-existing obesity. At this point mice were either sacrificed for measuring fat or tumors were injected and mice on HFD stayed for 9 weeks. Panels B: Body weight. Panel C: Fasting glucose levels. Panel D: Mammary and visceral adipose tissue weight. Panel E: Limiting dilution assay of E0771 or Py8199 TNBC cells injected into mammary fat of mice (Panel D). Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5-10 mice/group.

Summary and Discussion

Together, these findings suggest that obesity promotes CSC-like phenotype in cancer cells and their tumor-forming potential in a spontaneous and syngeneic TNBC mouse model. This pro-tumorigenic effect of obesity is independent of excess nutrient since HFD in the absence of obesity could not promote TNBC tumor-initiating potential. Thus, suggest that pathophysiological changes to the tumor microenvironment due to chronic obesity promotes the TNBC tumor-initiating potential.

Here, we investigated the effect of 60% saturated high-fat-diet-induced obesity on tumor formation and stemness. However, there is extensive literature on the differential effects of diets with very different fatty acid compositions on weight gain as well as metabolic phenotypes (Lai et al., 2014). Moreover, the type of protein and carbohydrates in the high-fat diet can exert differential effects on insulin sensitivity and weight gain in rodents. Taken together, due to a broad range of dietary interventions confounded by other lifestyle factors modulating physiology and metabolism, it has not been possible to define the 'ideal' HFD that can mimic obesity in human. Therefore, it is important to test the results of this study using various diets to induce obesity in rodents and its effect on tumor formation.

Using C3(1)-TAg mouse model on FVB/n background, we showed that obesity promotes tumor formation in a spontaneous TNBC mouse model. However, in this mouse model both obesity and tumor development are happening simultaneously, and therefore it is difficult to conclude that the effect on tumor-formation is due to obesity and not the high-fat-diet. One approach to test this is to put mice on HFD after week 12 when DCIS is developed, to analyze the effect of excess nutrients after initial tumor development. Another approach is to use ER/PR

negative MMTV-PyMT mouse model on the C57BL/6 background to test the effect of obesity and excess nutrients on tumor development. Due to its C57BL/6 background, this model is more prone to diet-induced obesity and has longer tumor latency. Thus, obesity can be established prior to tumor formation. One limitation to ER-negative mouse models is that spontaneous breast cancer mouse models start as ER-positive, and therefore, the effect of excess estrogen due to excess fat on tumor-formation cannot be completely excluded. Nonetheless, the increase in mRNA expression of stem cell transcription factors (SOX2, OCT4, and NANOG) and protein expression of CSC markers (CD90) in cancer cells isolated from tumors from obese mice relative to lean mice supports the hypothesis that HFD-induced obesity promotes CSC-like phenotype.

CHAPTER 4. OBESE MAMMARY ATMS INDUCE STEM-LIKE PROPERTIES IN TNBC CELLS.

Introduction

Pro-inflammatory cytokines have been shown to enhance the tumor-initiating potential of many types of cancer cells (Nguyen et al., 2012). ATMs are an important source of inflammation in breast adipose tissue and play several physiological roles. For example, it has been shown that during mammary gland development, macrophages are essential for ductal development as well as to support mammary stem cell function (Gyorki et al., 2009; Nguyen et al., 2012). Similarly, during lactation mammary gland macrophages promote CSC-like properties in normal and malignant breast epithelial cells (Celià-Terrassa and Kang, 2016). Since ATMs are an important source of proinflammatory cytokines during obesity, we hypothesized that an increase in pro-inflammatory ATMs in mammary adipose tissue might help to explain how DIO promotes expression of stem markers and TNBC cells tumor-forming potential.

Results

DIO did not increase the percentage of ATMs in the stromal vascular cells of mouse mammary fat pad.

To begin to test the role of mammary ATMs in obesity-associated TNBC, we first determined whether DIO could increase the number of mammary fat ATMs and/or their pro-inflammatory cytokine expression in female C57BL/6 mice fed the HFD for 12 weeks. Although DIO substantially increased mammary adipose tissue mass (see Fig.1D), the percent CD45+ immune cells or ATMs in the stromal vascular cells (SVC) were not increased (Figs.5A-E).

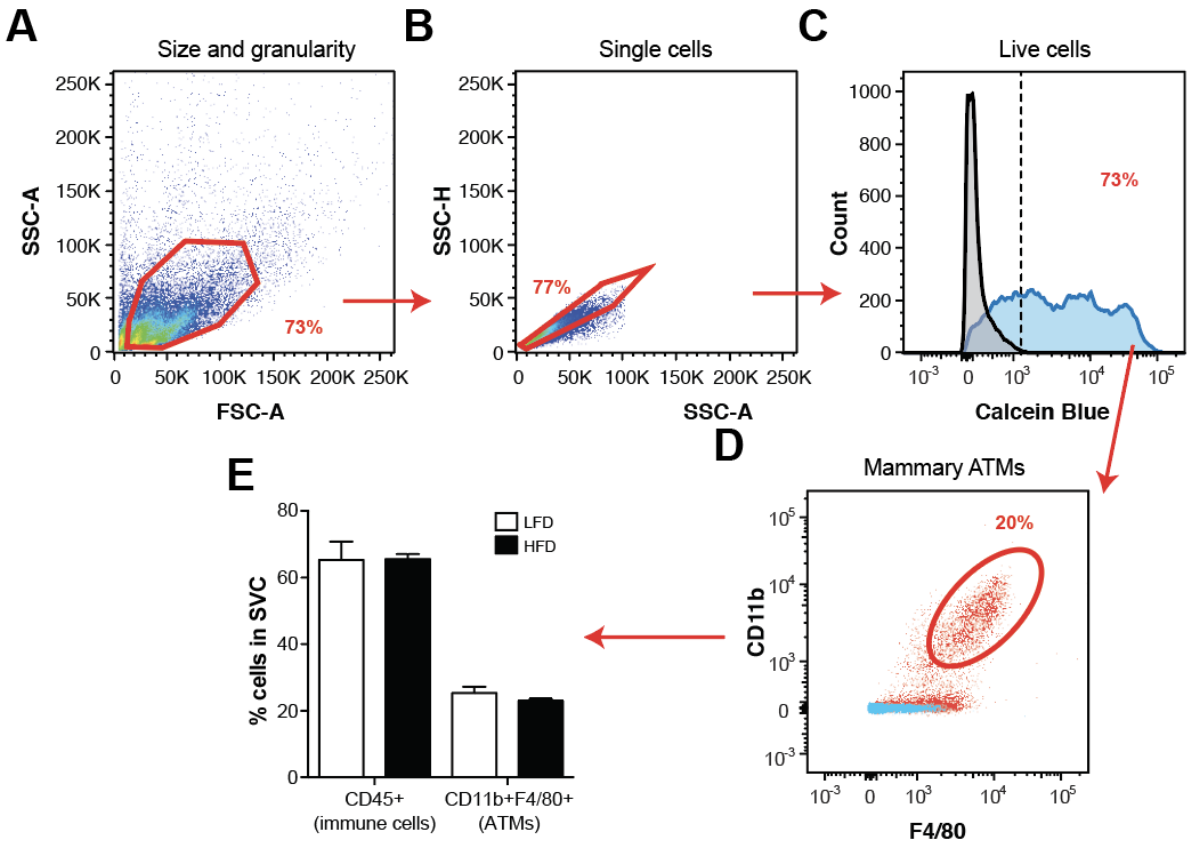


Fig.5. DIO did not increase the percentage of ATM in stromal vascular cells of mice mammary fat pad : Panel A-D: Flow cytometry workflow for characterizing mammary adipose tissue macrophages. Panel E: Female C57BL/6 mice were fed a LFD or HFD for 12 weeks. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5 mice/group.

DIO increased the inflammatory cytokine expression in mammary ATMs.

Since DIO did not increase the mammary ATM number in mammary adipose tissue, we next determined whether DIO could increase the pro-inflammatory cytokine expression in female C57BL/6 mice fed the HFD for 12 weeks. To examine the inflammatory status of mammary ATMs, we purified them from lean and obese mice using CD11b magnetic beads (Fig. 6A) and measured mRNA expression of inflammatory cytokines. We found that mammary ATMs from obese mice had elevated Tnfα, Il1β, and Il6 levels in comparison to those from lean mice (Fig.6B). Thus, although DIO did not induce mammary ATM accumulation in mice, it did increase ATM inflammation in mammary fat; the latter observation is similar to what has been widely reported in visceral fat depots (Gyorki et al., 2009). During early tumor progression, there is another type of macrophage, which is called tumor-associated macrophage (TAM) that also play a role in tumor-initiation and formation (Sainz et al., 2016). Therefore, we also tested if obesity promotes the inflammation of TAMs. Obesity promoted the gain in tumor weight in C3Tag mice (Fig.6C); however obesity did not promote infiltration of TAMs (Fig.6D) or expression of inflammatory cytokines in TAMs as assessed following isolation from tumors (Fig.6E). Even the proteomic analysis of secreted factors from TAMs isolated from lean or obese mice showed no difference (Fig.6F). Thus, obesity does not affect the recruitment of TAMs nor confer a pro-inflammatory phenotype to TAMs, and a change in TAM phenotype cannot explain the increase in CSCs caused by obesity.

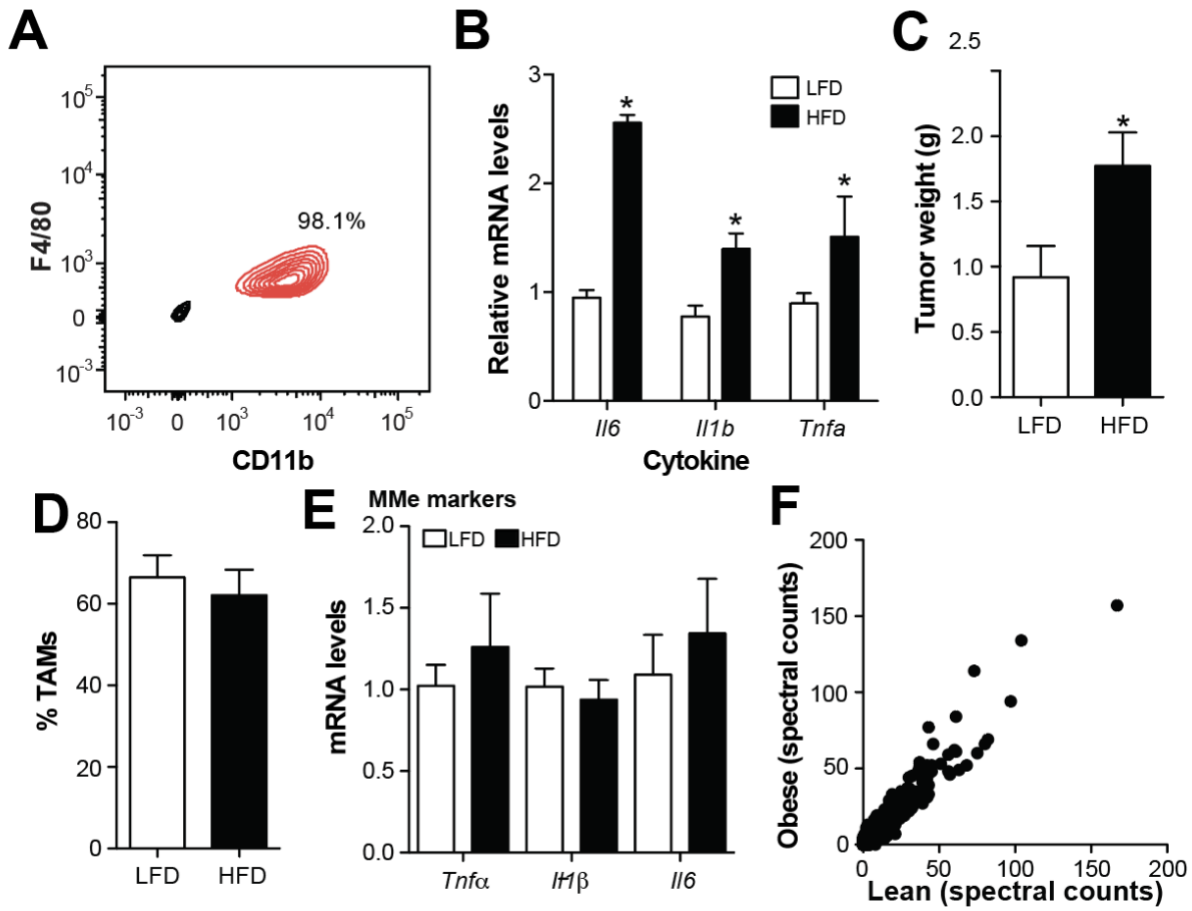
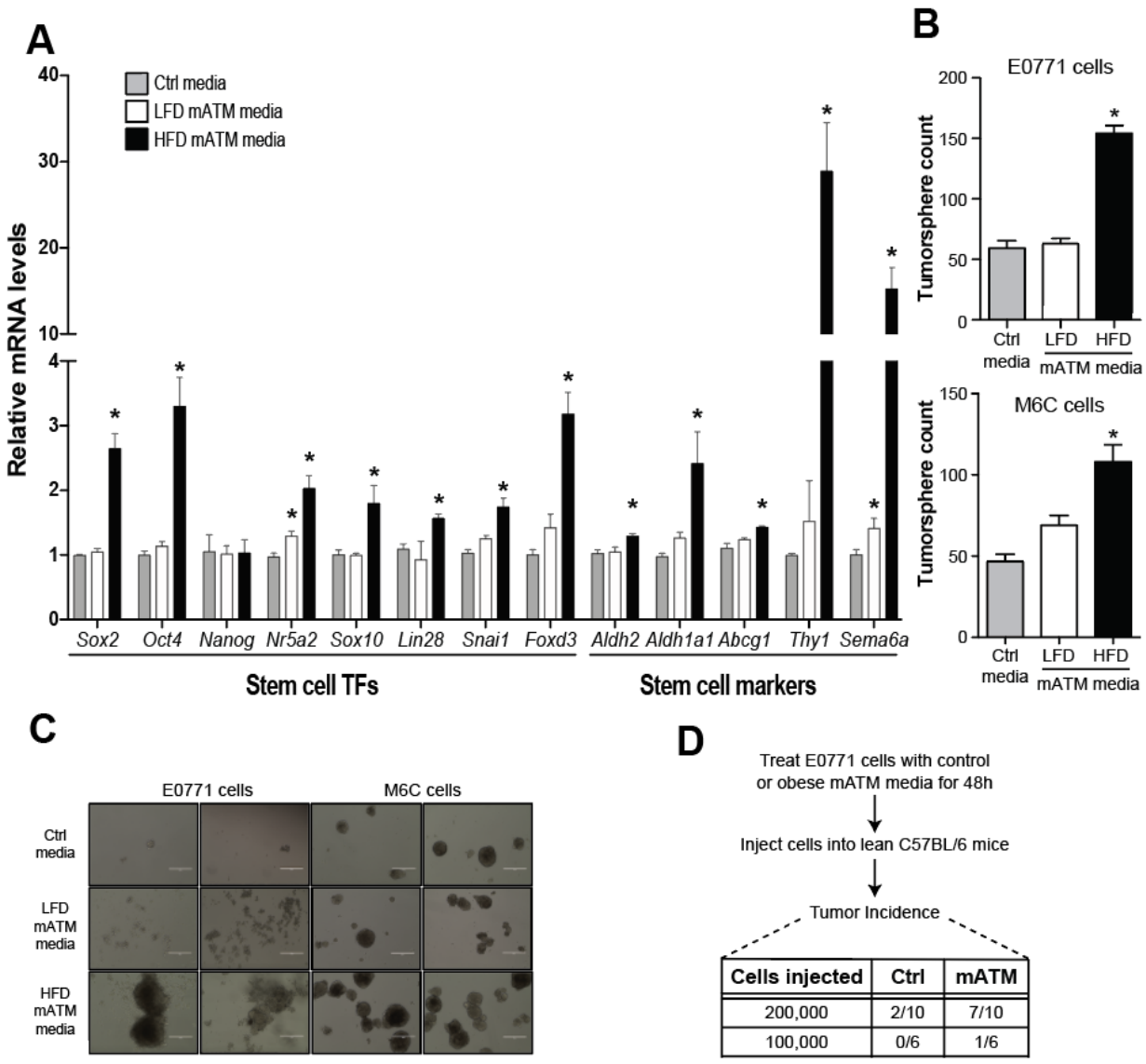


Fig.6. Female C57BL/6 mice were fed a LFD or HFD for 12 weeks. Panel A: Purity of mammary ATMs isolated from mice. Panel B: Pro-inflammatory cytokine expression in mammary ATMs. Panel C: Tumor weight (C3Tag mice). Panel D: Percentage of CD11b+ and F4/80+ positive TAMs quantified by flow cytometry. TAMs were quantified and interrogated by RT-PCR (Panel E) and by proteomics (Panel F). Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5/group.

Obese mATMs promote stem-like properties in TNBC cells.

We next investigated if pro-inflammatory inflamed obese mammary ATMs support stem-like properties in TNBC cells. To explore this, we plated collected conditioned media from an equal number of mammary ATMs from lean and obese mice, since inflammatory cytokines are secreted into the conditioned media and determined whether this media could i) induce the expression of the stem cell transcription factors (Sox2, Oct4, Nanog, Nr5a2, Sox10, Lin28, Snai1, Foxd3) and stem cell markers (Aldh2, Aldh1a1, Abcg1, Thy1, and Sema6a) in TNBC cells, and ii) promote TNBC cell tumorsphere formation *in vitro* and tumor-initiating potential *in vivo*, functional assays associated with the CSCs-phenotype (Lee et al., 2016). To collect conditioned media, we plated 1 million mammary ATMs in 300 ul of serum free DMEM in 24-well plate for 24hrs.

Whereas treatment with conditioned media from lean mammary ATMs had no effect on stem cell marker expression in E0771 cells, conditioned media from obese mammary ATMs increased the expression of stem cell markers in these cells (Fig.7A). Obese mammary ATM media also induced tumorsphere formation of E0771 and M6C cells (derived from C3(1)-TAg mice), but lean mammary ATM media could not (Figs.7B-C). Moreover, pre-treating E0771 cells with obese mammary ATM media increased their tumor-initiating potential *in vivo* (Fig.7D).



Cells injected	Ctrl	mATM
200,000	2/10	7/10
100,000	0/6	1/6

Fig.7. Obese mATMs promote stem-like properties in TNBC cells. Panels A-D: TNBC cells were treated with control media (Ctrl) or media conditioned by mammary ATMs from lean (LFD) or obese (HFD) mice. Panel A: Stem cell marker expression in E0771 cells. Panel B: Tumorsphere formation in E0771 and M6C cells. Panel C: Representative images of tumorspheres. Panel D: Tumor incidence following injection of E0771 cells into mammary fat. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group for *in vitro* assays and n=6-10 mice/group as depicted in the figure. White scale bar shown in images are 200um.

Summary and Discussion

Together, these findings suggest that during obesity mammary ATMs acquire a pro-inflammatory phenotype. These inflamed mammary ATMs secrete factors that can promote stem-like properties in TNBC cells, including stem gene expression and tumor-initiation as assessed by limiting dilution assays, consistent with a pro-tumorigenic phenotype.

In this study, we focused on mammary ATMs during obesity and its effects on breast cancer cells. However, we know from the previous study that visceral and subcutaneous macrophages are metabolically activated during obesity (Kratz et al., 2014). It is likely that macrophages from other sources might play a role in promoting stem cell-like phenotype in cancer cells. For example, understanding the role of omental macrophages in ovarian cancer and colon cancer progression during obesity. To test this, I used conditioned media collected from visceral adipose tissue macrophages (CD11b macrophages) and investigated if it can promote stemness in breast cancer cells. I found that visceral adipose tissue macrophage-conditioned media did not promote stemness. In fact, it was inducing cell death (Data know shown). One possibility is that visceral ATMs are relatively more inflamed than mammary ATMs (Data not shown) and is therefore toxic to breast cancer cells. It is also possible that colon and ovarian cancer can survive in higher-grade inflammation and thus, visceral ATMs can still promote cancer progression due to obesity. Another possibility is that factors secreted from visceral ATMs during obesity are distinct from that of mammary adipose tissue macrophages. However, these hypotheses require further investigation and is critical for understanding the role of obesity in promoting various other cancers such as ovarian and colon cancer.

CHAPTER 5. OBESE MAMMARY ATMS ADOPT A METABOLICALLY ACTIVATED (MME) PHENOTYPE AND CONTRIBUTE TO THE ENHANCED TNBC TUMOR FORMATION

Introduction

Our previous findings demonstrate that during obesity mammary adipose tissue macrophages acquire pro-inflammatory and pro-tumorigenic macrophage phenotype. However, pro-inflammatory macrophage phenotype is often associated with an anti-tumorigenic M1-like macrophage phenotype. Therefore, we next characterized obese mammary adipose tissue macrophages to further identify the mechanism by which these macrophages promote a CSC-like phenotype. We have previously shown that obesity induces metabolic activation of macrophages, termed 'metabolically activated macrophages' or MMes (Kratz et al., 2014), produced by exposure to high levels of insulin, glucose, and palmitate, conditions characteristic of obesity and diabetes and are inflamed. Importantly, this pro-inflammatory macrophage phenotype is mechanistically distinct from classically activated M1 macrophages. Using a combination of proteomics, we have previously identified cell surface proteins unique to MMes (ABCA-1, PLN2, CD36) and M1 (CD38 and CD319) macrophages that can be used as markers to investigate the ATM phenotype together with inflammatory cytokine expression. Although we have shown that MMe macrophages are present in visceral and subcutaneous adipose tissue depots in obese humans and mice (Kratz et al., 2014), their presence in mammary adipose tissue has not been investigated. This is important because obesity can elicit substantially diverse effects on different adipose tissue depots (Sonne et al., 2017). Therefore, we next explored the nature of the pro-inflammatory mammary ATM phenotype in obese mice. To determine whether

metabolically activated ATMs contribute to TNBC tumorigenesis during obesity, we took advantage of our previous work, which showed that NADPH oxidase 2 (NOX2) is required for metabolic activation of macrophages. Depleting NOX2 from macrophages block the induction of pro-inflammatory cytokine expression in macrophages upon activation by free fatty acid (FFA), but not by LPS. Thus, NOX2 depletion blocks metabolic activation but not the classic activation of macrophages (Coats et al., 2017). Here, we use this Nox-2-dependence to ask if the metabolic activation of macrophages is required for obesity-associated TNBC tumor formation.

Results

DIO in female mice promotes the expression of MMe(s) markers and not M1 markers.

To determine whether obesity induced an MMe-like phenotype in mammary ATMs, we first compared mammary ATMs from lean and obese female C57BL/6 mice for mRNA expression of markers diagnostic of the M1 (Cd40, Cd38) and MMe (Cd36, Plin2) phenotypes (Kratz et al., 2014). We found that obesity induced the expression of MMe markers, but not M1 markers, in mammary ATMs (Fig.8A). Likewise, we found that conditioned media from mammary-adipose tissue derived from obese mice promotes the expression of MMe markers in BMDMs relative to lean mice (Fig.8B). These findings suggest that the milder DIO that develops in female mice (Dorfman et al., 2017) is sufficient to support an MMe phenotype in ATMs and that MMe, rather than M1, is the dominant pro-inflammatory macrophage phenotype in mammary adipose tissue of obese mice.

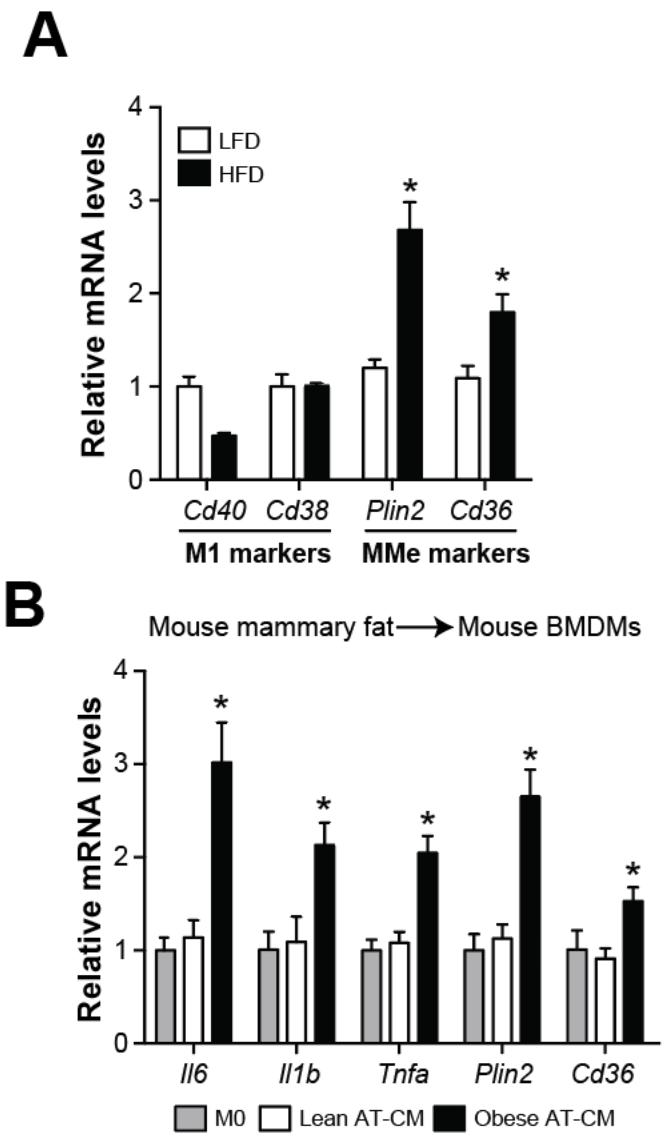
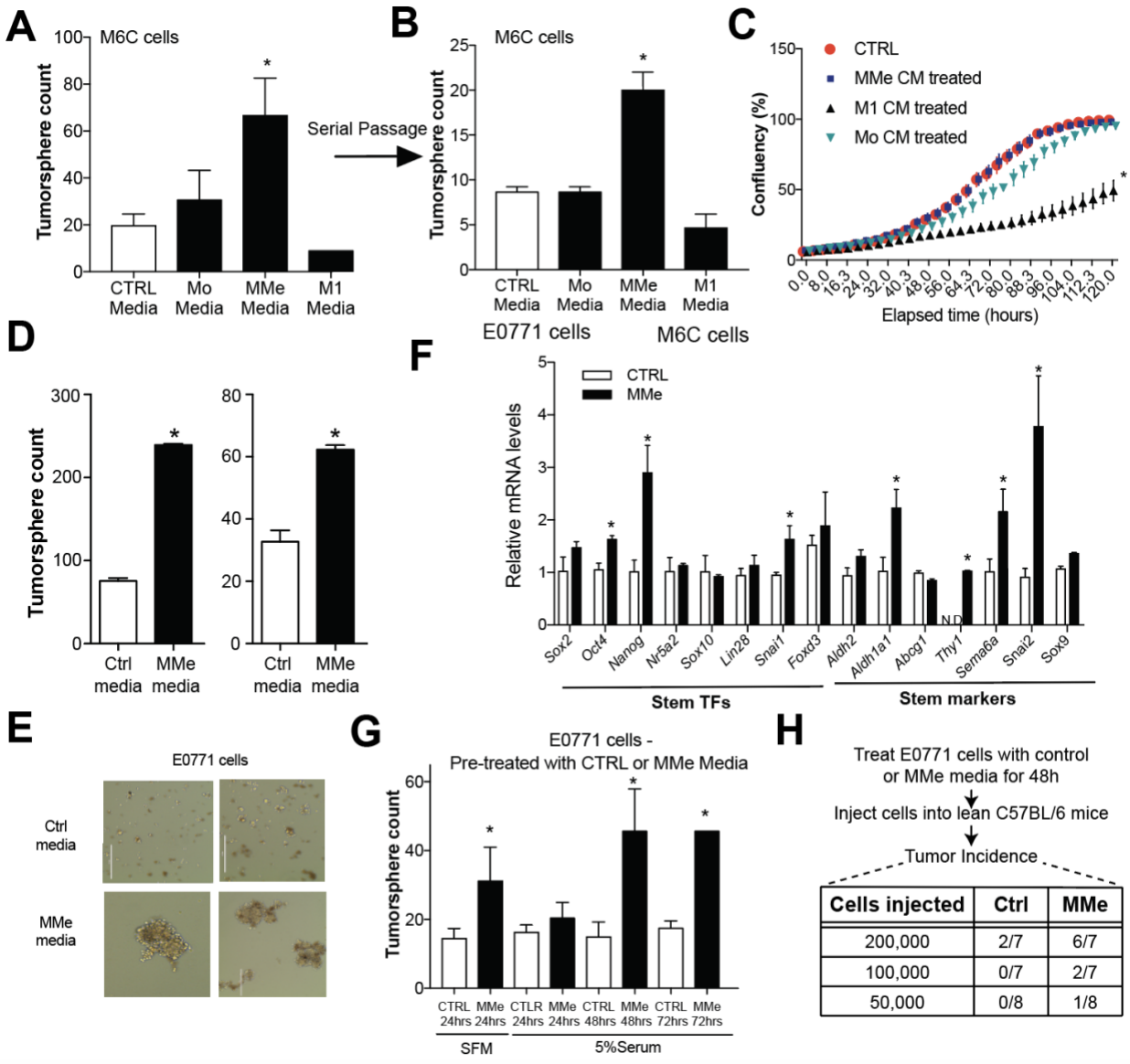


Fig.8. DIO in female mice promotes the expression of MMe(s) markers and not M1 markers. Panel A: M1 and MMe marker expression in mammary ATMs from lean and obese C57BL/6 mice. Panel B: Inflammatory cytokine expression in mammary ATMs from lean and obese C57BL/6 mice. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=4/group.

***In vitro* MMe(s) mimic the effects of obese mATMs on TNBC stemness.**

To further determine if obesity promotes metabolic activation of macrophages, we next determined whether *in vitro*-derived MMe macrophages could mimic the stem cell promoting properties of obese mammary ATMs. We isolated bone marrow-derived macrophages (M0), metabolically-activated them with a mixture of palmitate, insulin, and glucose (MMe) or classically-activated them with LPS and INFγ, and collected their conditioned media. Conditioned media from MMe but not M1 or Mo macrophages induced the tumorsphere formation in M6C cells (Fig.9A), and when tumorspheres were serially passaged in the absence of conditioned media the effect was maintained (Fig.9B). The increase in proliferation cannot explain the increase in tumorsphere growth because MMe did not promote the proliferation of cancer cells (Fig.9C). However, M1 macrophage-conditioned media significantly inhibited proliferation of M6C cells (Fig.9C), which is consistent with the observation that M1 macrophages inhibited tumorsphere growth (Figs.9A-B) and are considered anti-tumorigenic. We further showed that conditioned media from MMe macrophages induced the tumorsphere formation, expression of stem-like markers (Oct4, Nanog) in E0771 cells (Figs.9D-F). Pretreating E0771 cells with MMe conditioned media is sufficient to induce tumorsphere growth *in vitro* and the tumor-initiating potential *in vivo* (Figs.9G-H).



Cells injected	Ctrl	MME
200,000	2/7	6/7
100,000	0/7	2/7
50,000	0/8	1/8

Fig.9. *In vitro* MMe(s) mimic the effects of obese mATMs on TNBC stemness. Panels A-H: BMDMs (M0) were metabolically activated (MMe) or classically activated (M1) *in vitro*. Panel A-B: M6C cells tumorsphere count when macrophage media was added to the mammosphere media. Panel C: M6C cell proliferation assay. Panel D: TNBC cells tumorsphere count. Panel E. Representative tumorspheres images. Panel F: Stem cell TF-

Fig.9. continued.

expression. Panel G: Tumorsphere count of E0771 cells when cancer cells were pretreated with macrophage conditioned media in condition and period as shown in the figure. Panel H: Tumor incidence following injection of E0771 cells into mammary fat. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group for *in vitro* assays and n=7-8mice/group.

Pro-tumorigenic phenotype of metabolically active macrophages *in vitro* is Nox2 dependent.

Previously Becker lab showed that NADPH oxidase 2 (NOX2) is required for metabolic activation of macrophages (Coats et al., 2017; Kratz et al., 2014). Depleting NOX2 from macrophages block the induction of pro-inflammatory cytokine expression in macrophages upon activation by free fatty acid (FFA). Since, inflammation is linked with CSC maintenance and proliferation we next determined whether deleting Nox2 (Cybb-/-) in *in vitro*-derived MMe macrophages could diminish their ability to promote TNBC stem-like properties. As previously described, deleting Nox2 lowered inflammatory cytokine expression in MMe macrophages (Fig.10A). Moreover, deleting Nox2 in MMe macrophages attenuated their ability to induce stem-like marker expression and tumorsphere formation in E0771 and M6C cells *in vitro* (Figs.10B-C). This decrease in CSC-like properties is not complete, which can be explained by incomplete inhibition of inflammatory cytokine expression in macrophages by NOX2 depletion. Thus, the pro-inflammatory and stem cell promoting properties of *in vitro*-derived MMe macrophages are in part dependent on Nox2.

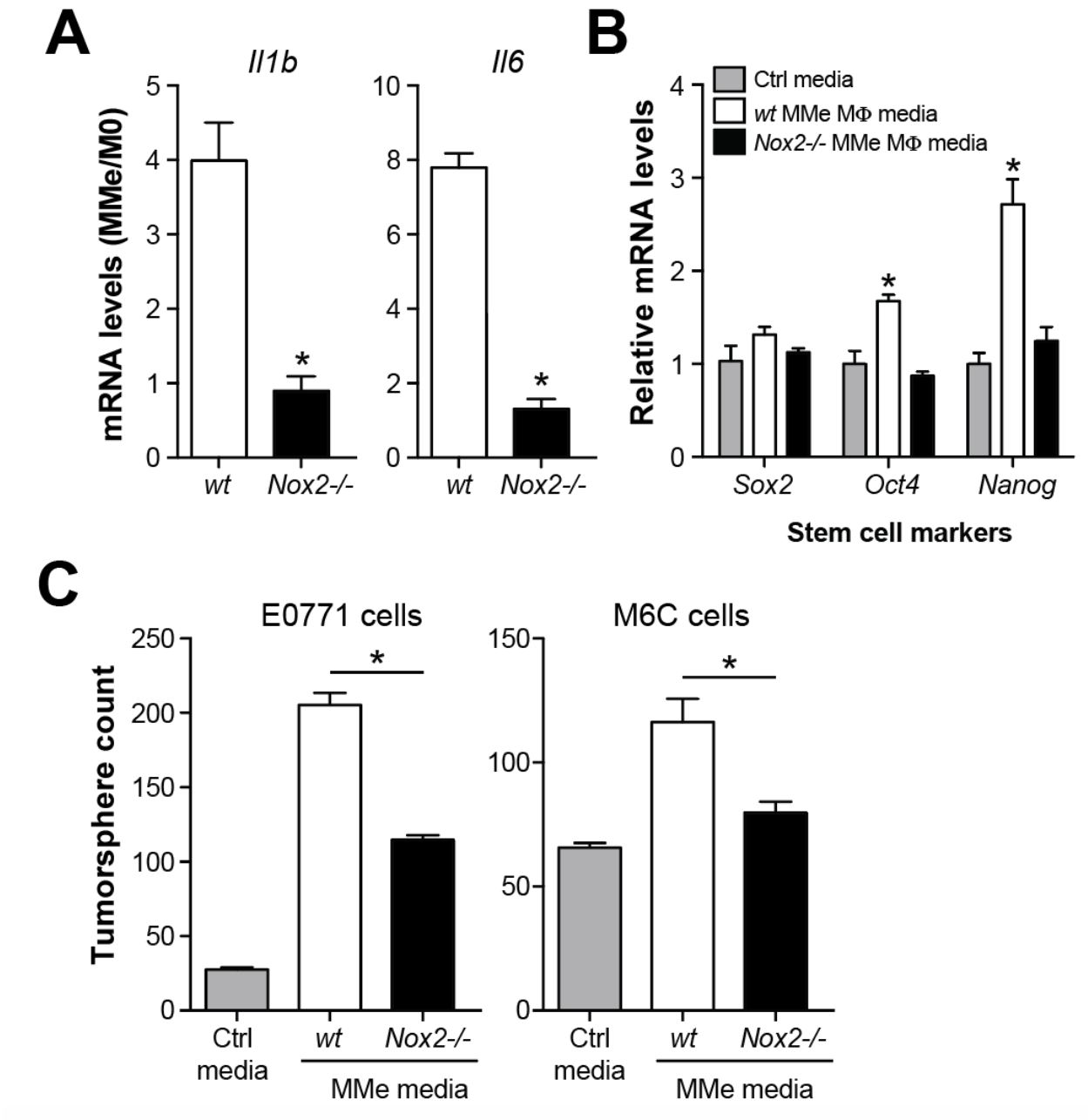


Fig.10. Pro-tumorigenic phenotype of metabolically active macrophages *in vitro* is dependent on Nox2. Panel A-C: Wt. and Nox2-/- BMDMs were metabolically activated. Panel A: Cytokine expression in MMe macrophages. Panels B-C: Effect of MMe macrophage media on TNBC stem cell marker expression (Panel B), and tumorsphere formation (Panel C). Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group.

Myeloid cell-specific Nox2 depletion does not affect obesity-induced metabolic phenotype.

To study the effect of Nox2 on TNBC cells tumor-formation potential, we first characterize the metabolic phenotype of myeloid cell-specific Nox2-deficient obese mice to make sure inhibiting macrophage inflammation does not affect mammary adipose tissue homeostasis. To explore this, we fed myeloid cell-specific Nox2-deficient mice (mNox2-/-) and littermate controls (wt) a HFD for 12 weeks. Deleting myeloid cell Nox2 in female mice did not appreciably impact the metabolic phenotype following 12 weeks of HFD. As previously described in male mNox2-/- mice (Coats et al., 2017), female mNox2-/- mice fed a HFD gained more body weight and adipose tissue mass than wt. mice, but fasting glucose and insulin levels were unaffected (Figs. 11A-D). Mammary and visceral adipose tissue health and liver fat accumulation were also unaffected at this time point (Figs.11E-F), which is important because, at least in males, mNox2-/- mice develop late-onset visceral lipoatrophy and hepato-steatosis after 16 weeks of HFD (Coats et al., 2017) which can affect cancer progression. Therefore, we decided to inject cancer cells after mice were HFD for 10 weeks to give six weeks of time for tumor development and progression before we expect mice to develop visceral lipoatrophy and hepato-steatosis that could affect cancer progression.

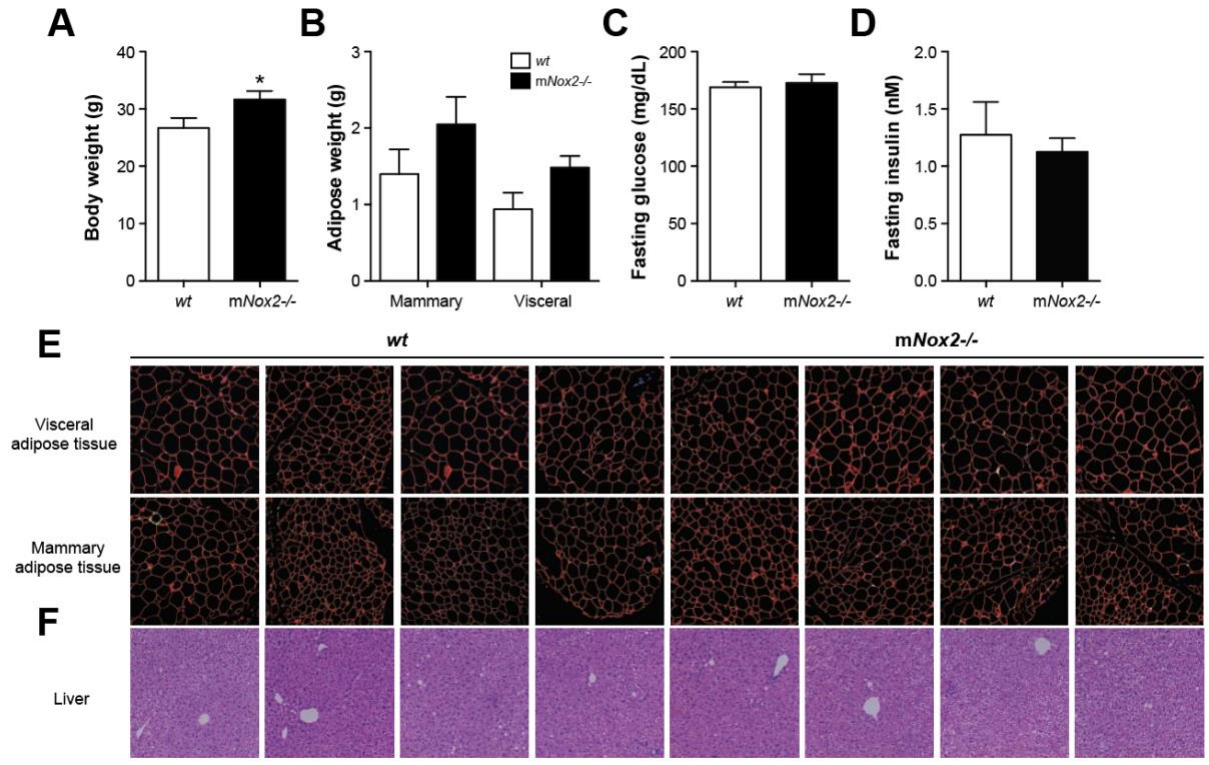


Fig.11. Characterizing metabolic phenotype of mNox2-/- obese mice. Control Nox2fl/fl mice (wt.) and myeloid cell-specific LysM-cre+/- Nox2fl/fl mice (mNox2-/-) were fed a HFD for 10 weeks. Panel A: Body weight. Panel B: Mammary and visceral adipose tissue weight. Panels C-D: Fasting glucose and insulin levels. Panel E: IHC on mammary and visceral adipose tissue. Panel F: Liver H&E staining. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=4-8/group.

Depleting myeloid cell-specific Nox2 in obese mice attenuated the metabolic activation of mammary adipose tissue macrophages.

After finding that Nox2 deletion does not affect the mammary adipose tissue health and other obesity-related phenotypes, we next determined whether NOX2 is required for the metabolic activation of obese mammary adipose tissue macrophages. To test this, we fed myeloid cell-specific Nox2-deficient mice (mNox2-/-) and littermate controls (wt) a HFD for 12 weeks. Although the percentage of mammary ATMs was elevated in mNox2-/- mice (Fig.12A), analysis of these ATMs revealed attenuated expression of all MMe markers tested, including Tnfα, Il6, Il1β, Abca1, Cd36, and Plin2 (Fig.12B). Thus, these female mNox2-/- mice afford an opportunity to study the role of MMe macrophages in TNBC tumor formation during obesity in the absence of substantive changes to the overall metabolic phenotype (see Fig.11).

Depleting myeloid cell-specific Nox2 in obese mice attenuated the pro-tumorigenic effect of obese mammary ATMs on TNBC cells.

To determine the impact of deleting Nox2 in myeloid cells in obese mice on induction of TNBC CSC-like properties, we isolated mammary ATMs from obese wt or mNox2-/- mice and measured their capability to promote TNBC stem-like properties. Mammary ATMs from obese mNox2-/- mice had a decreased ability to promote stem cell transcription factors (Sox2, Oct4, Nanog, Nr5a2, Sox10, Lin28, Snai1, Foxd3) and stem cell markers (Aldh2, Aldh1a1, Abcg1, Thy1, and Sema6a) (Fig. 13A) and tumorsphere formation of E0771 cells (Fig.13B). Similar to *in vitro*- derived MMe(s), the decrease in CSC-like properties by Nox2 depletion in obese mammary adipose tissue macrophages is partial. These results suggest Nox2 is required for full induction of CSC-like properties in TNBC cells by obese mammary adipose tissue macrophages.

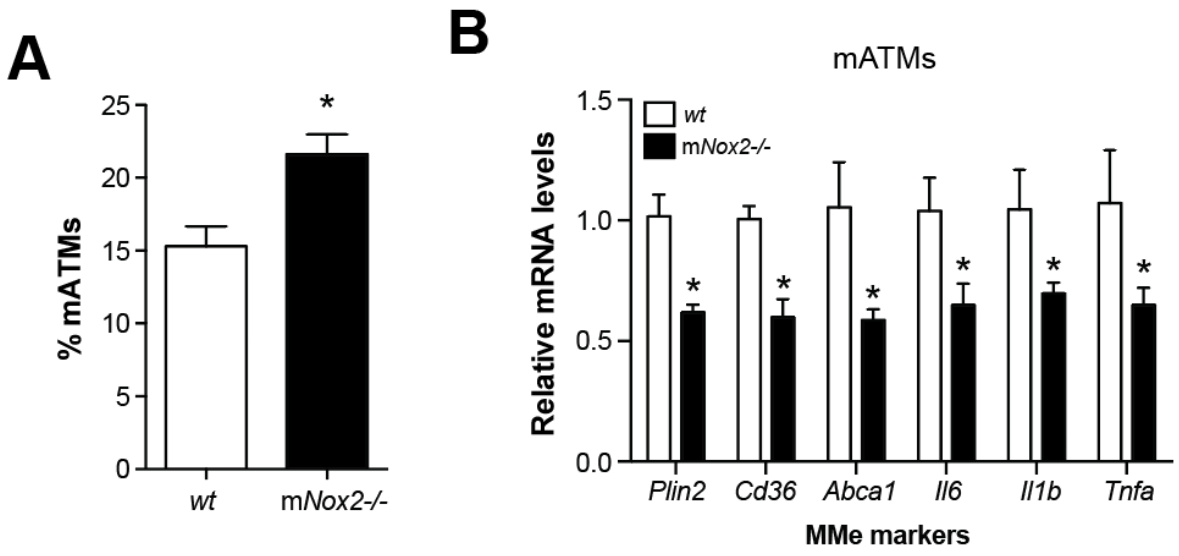


Fig.12. Depleting myeloid cell specific Nox2 in obese mice attenuated the metabolic activation of mammary adipose tissue macrophages. Panel A-B: Control $Nox2^{fl/fl}$ mice (wt.) and myeloid cell-specific LysM-$cre^{+/-}$ $Nox2^{fl/fl}$ mice (mNox2-/-) were fed a HFD for 12 weeks. Panel A: Percent ATMs in mammary adipose tissue. Panel B: MMe marker expression in mammary ATMs. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=4-5/group.

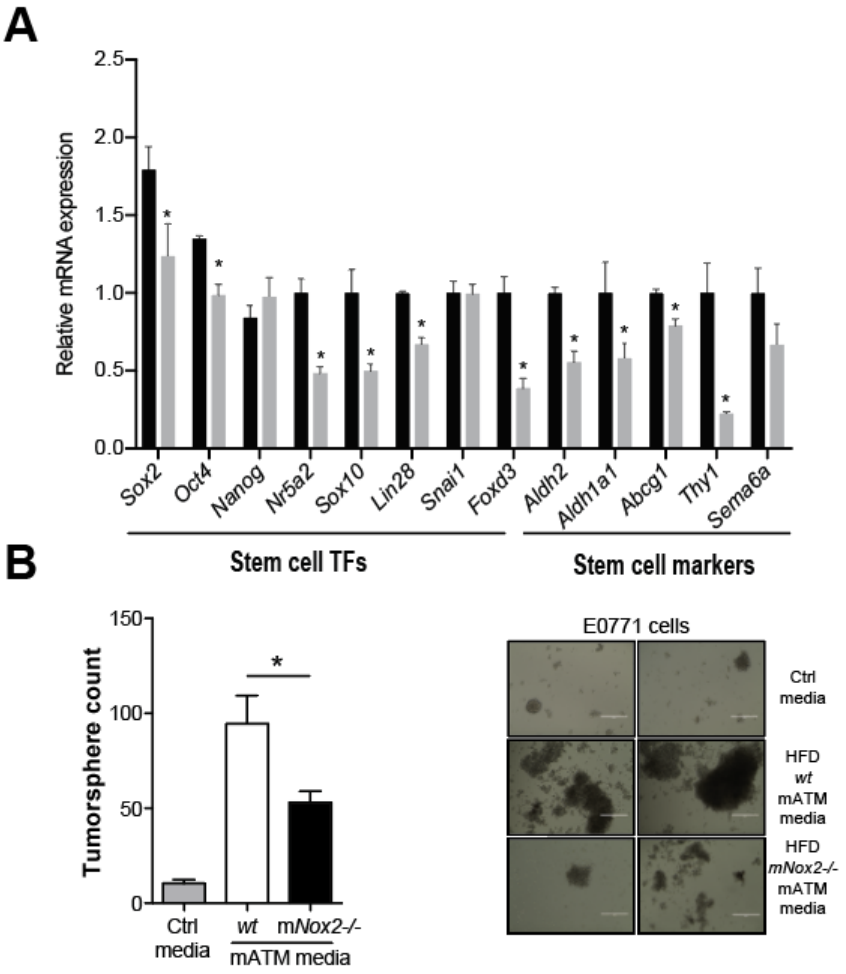
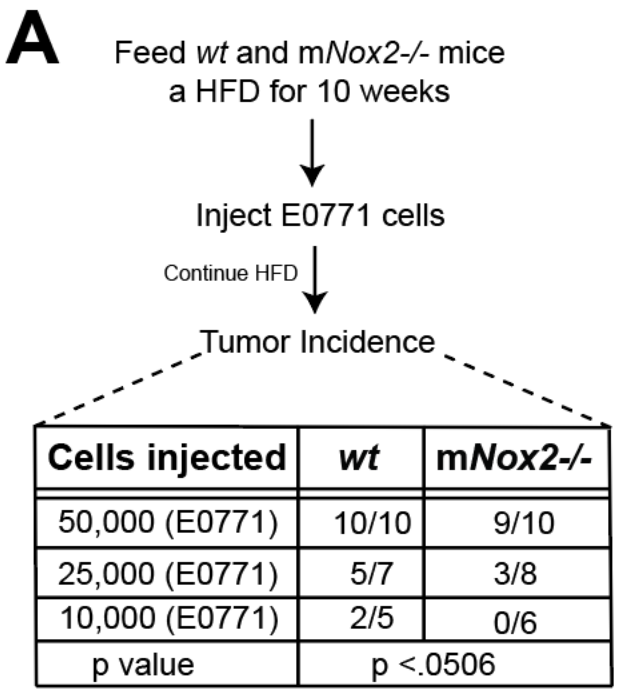


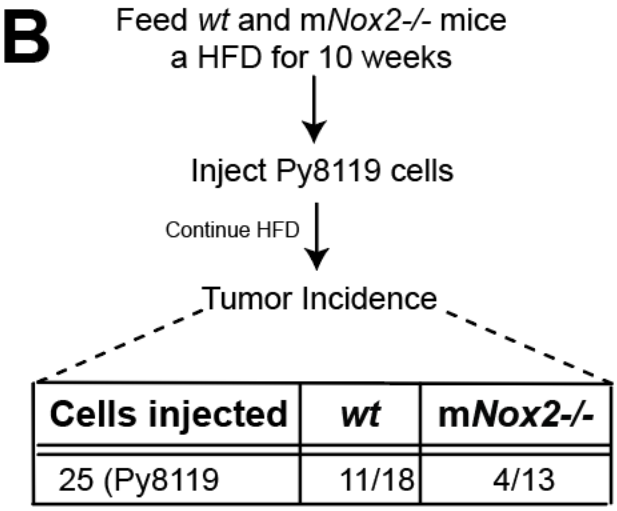
Fig.13. Depleting myeloid cell specific Nox2 in obese mice attenuated the pro-tumorigenic effect of obese mammary ATMs on TNBC cells. Panels A-B: Control $Nox2^{fl/fl}$ mice (wt) and myeloid cell-specific LysM-$cre^{+/-}$ $Nox2^{fl/fl}$ mice (mNox2-/-) were fed a HFD for 12 weeks. After which mammary ATMs were isolated and seeded in serum free media to collect conditioned media to test the effect on cancer cell stemness. Panel A: Expression of stem cell markers. Panel B: Tumorsphere counts with representative images of tumorspheres. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group.

Depleting myeloid cell-specific Nox2 in obese mice attenuated the effect of obesity on TNBC tumor formation.

To determine the impact of deleting Nox2 in myeloid cells on TNBC tumor formation during obesity, we used a limiting dilution assay to investigate whether deleting Nox2 from myeloid cells could attenuate the ability of DIO to promote the tumor-initiating potential of TNBC cells *in vivo*. Deleting Nox2 from myeloid cells decreased tumor incidence in obese mice injected with E0771 or Py8119 cells (Fig.14 A-B). This decreased tumor incidence was not absolute, which may be explained by i) the inability of Nox2 deletion to completely block MMe phenotype and the induction of stem-like properties in TNBC cells (see. Figs.10B-C, and 13B), and ii) the slight increases in body weight, mammary fat mass, and mammary ATM number in mNox2-/- mice (see Figs.11A-B, and 12A). This result highlights the contribution of MMe macrophages in mammary fat to the increased TNBC tumor formation during obesity.



Cells injected	*wt*	m*Nox2-/-*
50,000 (E0771)	10/10	9/10
25,000 (E0771)	5/7	3/8
10,000 (E0771)	2/5	0/6
p value	p <.0506	



Cells injected	*wt*	m*Nox2-/-*
25 (Py8119	11/18	4/13

Fig.14. Depleting myeloid cell specific Nox2 in obese mice attenuated the effect of obesity on TNBC tumor formation. Panel A-B: Control $Nox2^{fl/fl}$ mice (wt) and myeloid cell-specific LysM-$cre^{+/-}$ $Nox2^{fl/fl}$ mice (mNox2-/-) were fed a HFD for 10 weeks. After which tumors were injected and mice stayed on HFD. Tumor incidence following injection of E0771 or Py8199 cells into mammary fat. Tumor incidence p value is based on extreme limiting dilution analysis (ELDA).

Summary and Discussion

Together, we showed that obese mammary adipose tissue macrophages are metabolically activated in three ways. First, obesity induces expression of metabolically active macrophages markers and not that of classically activated M1-macrophage markers or mammary adipose tissue macrophage markers. Second, conditioned media from mammary adipose-tissue in obese mice can metabolically activate the bone-marrow-derived macrophages. This observation is similar to what we observed previously in visceral and subcutaneous adipose tissue. Third, *in vitro*-derived MMe (but not M1) macrophages promote the CSC-like properties in TNBC cells. Thus *in vitro*-derived MMe macrophages mimic the pro-tumorigenic effect of obese mammary adipose tissue macrophages. Furthermore, we showed that Nox2 is required for the expression of pro-inflammatory cytokines in macrophages upon activation by FFA or obesity and thus required for the complete metabolic activation of mammary ATMs *in vivo*. It also suggests that the obesity-associated increase in TNBC cells tumor-initiating potential is in part dependent on Nox2-mediated metabolic activation of mammary adipose tissue macrophages.

How does Nox2 regulate inflammatory cytokine expression during metabolic activation of macrophages is still unclear. Preliminary studies from our lab suggest the role of reactive oxygen species (ROS) generated by NOX2 during metabolic activation since inhibiting ROS via antioxidants attenuates the expression of inflammatory cytokines. ROS act as a signaling molecule and an understood mechanism of redox signaling involves H2O2-mediated oxidation of cysteine residues within proteins. Further studies (as discusses in future directions) are required to understand the regulation of expression of inflammatory cytokines in MMe via Nox2 and ROS and use of Nox2 inhibitors as possible preventive or treatment option for obese women

with increased risk of TNBC. A more detailed analysis is required to understand the broader impact of Nox2 on MMe secreted factors using cytokine array, proteomics, and RNA-sequencing. In addition, the inability of Nox2 deletion to completely block MMe phenotype and the induction of stem-like properties in TNBC cells suggests that Nox2 independent factors may contribute to MMe-induced stemness in cancer cells. An unbiased approach such as cytokine array, proteomics, and RNA-sequencing can help us in identifying novel factors secreted by metabolically activated macrophages that may promote cancer stemness and progression.

CHAPTER 6. METABOLICALLY ACTIVATED MAMMARY ATMS RELEASE CYTOKINES THAT SIGNAL THROUGH GP130 ON TNBC CELLS TO PROMOTE THEIR STEM-LIKE PROPERTIES.

Introduction

In the previous chapter we showed that Nox2 mediates metabolic activation of macrophages, including expression of pro-inflammatory cytokines, and is partly required for MMe-induced CSC-like properties in TNBC cells. Next, we wanted to determine the contribution of inflammatory cytokines to MMe-induced TNBC stemness. Previous studies showed that pro-inflammatory cytokines are critical for transformation, mammosphere formation, and tumorigenesis (Arnold et al., 2015). One well-studied pro-inflammatory cytokine is IL-6, which binds to glycoprotein 130 (GP130) to induce STAT3 phosphorylation and promote changes in differentiated cancer cells by increasing expression of stem cell markers and self-renewal properties (Korkaya et al., 2011). IL-6 is elevated in metabolically activated mammary ATMs from obese mice and attenuated by deleting Nox2 (see Figs.6A and 12B). Because IL11, OSM, LIF, CNTF, and CTF1 can also signal through GP130 (Silver and Hunter, 2010), and have the potential to promote stemness in cancer cells, we examined their regulation in mammary ATMs, and the role of their receptor, GP130, in the induction of stem-like properties in TNBC cells by obese mammary ATMs.

Results

Metabolically activated macrophages activate the GP130/STAT3 axis in TNBC cells.

First, we examined the regulation of GP130 ligands in obese mammary ATMs and found that obesity increased GP130 ligands transcript expression in mammary ATMs (Figs.15C). Moreover, treating E0771 or M6C cells with mammary ATM conditioned media from obese relative to lean mice induced STAT3 phosphorylation (Fig.15D), a key effector of GP130 signaling. *In vitro*-derived MMe macrophages similarly up-regulated the expression of all GP130 ligands tested and their media also induced STAT3 phosphorylation in E0771, M6C, and Py8119 cells (Figs. 15A-B). Thus, metabolically activated macrophages derived from obese mammary adipose tissue or *in vitro*, have increased expression of many GP130 ligands.

Deletion of Nox2 in myeloid cells attenuates the GP130 ligand expression and activation of GP130/STAT3 axis in TNBC cells.

We next determined whether GP130 ligand expression was lowered in mammary ATMs from obese mNox2-/- mice, a perturbation that attenuated both the MMe phenotype of mammary ATMs and the ability of obesity to promote tumor formation (see Figs.13-14). We found that mammary ATMs from obese mNox2-/- mice induced less GP130 ligand expression (Fig.16A) and had a diminished capability to induce STAT3 phosphorylation in E0771 cells (Fig.16B), relative to mammary ATMs from obese wt. mice. These findings suggest that Nox2 mediated metabolic activation of macrophages promotes the expression of GP130 ligands.

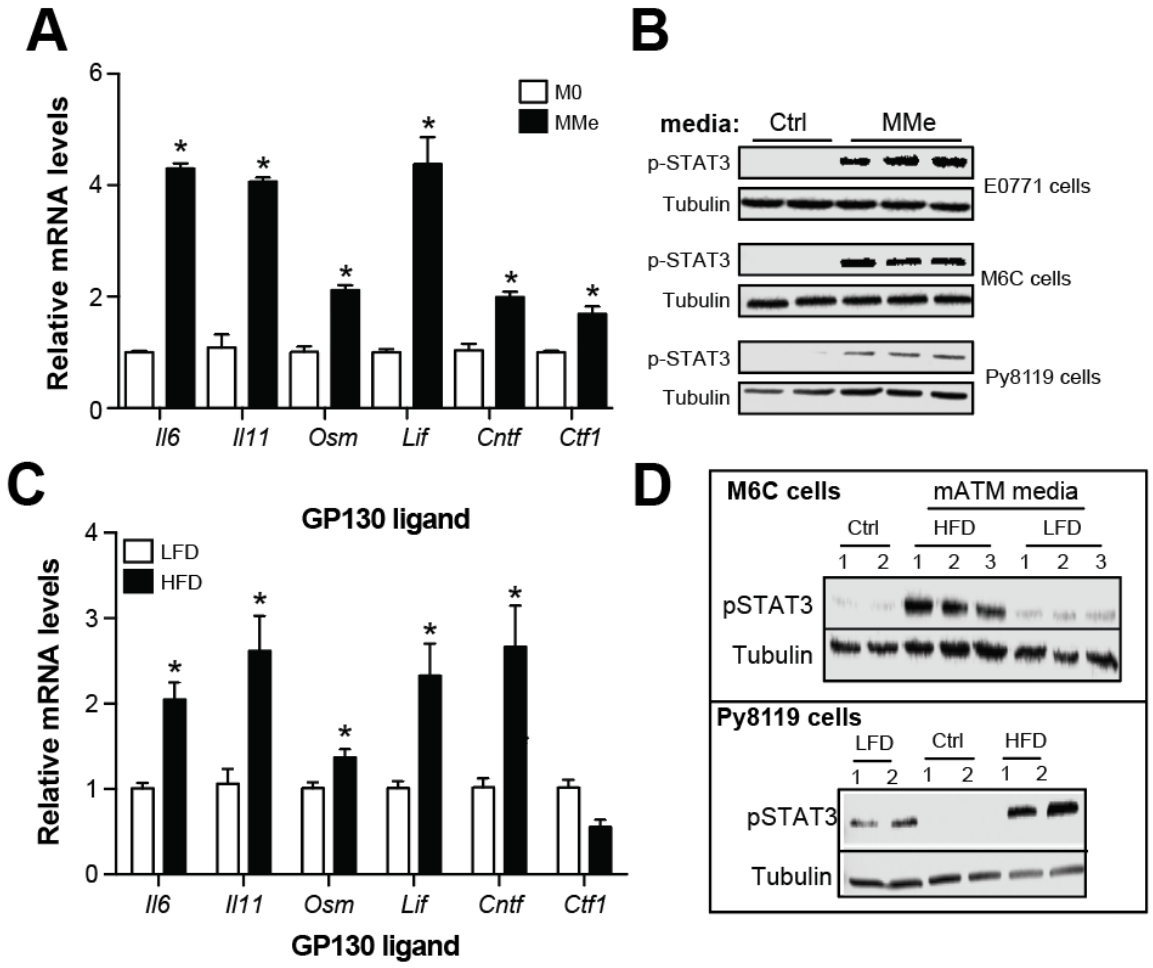


Fig.15. Metabolically activated macrophages activate GP130/STAT3 axis in TNBC cells. Panel A: GP130 ligand expression by *in vitro* metabolically activated macrophages. Panel B: Effects of *in vitro* metabolically activated macrophages media on STAT3 phosphorylation in TNBC cells. Panel C: GP130 ligand expression by mammary ATMs isolated from lean and obese mice. Panel D: Effects of mammary ATM media on STAT3 phosphorylation in TNBC cells. Numbers 1,2 and 3 represents conditioned media collected from mammary ATMs isolated from 3 different sets of lean and obese mice. In each set we have 2 obese mice and 5 lean mice. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=4/group. N

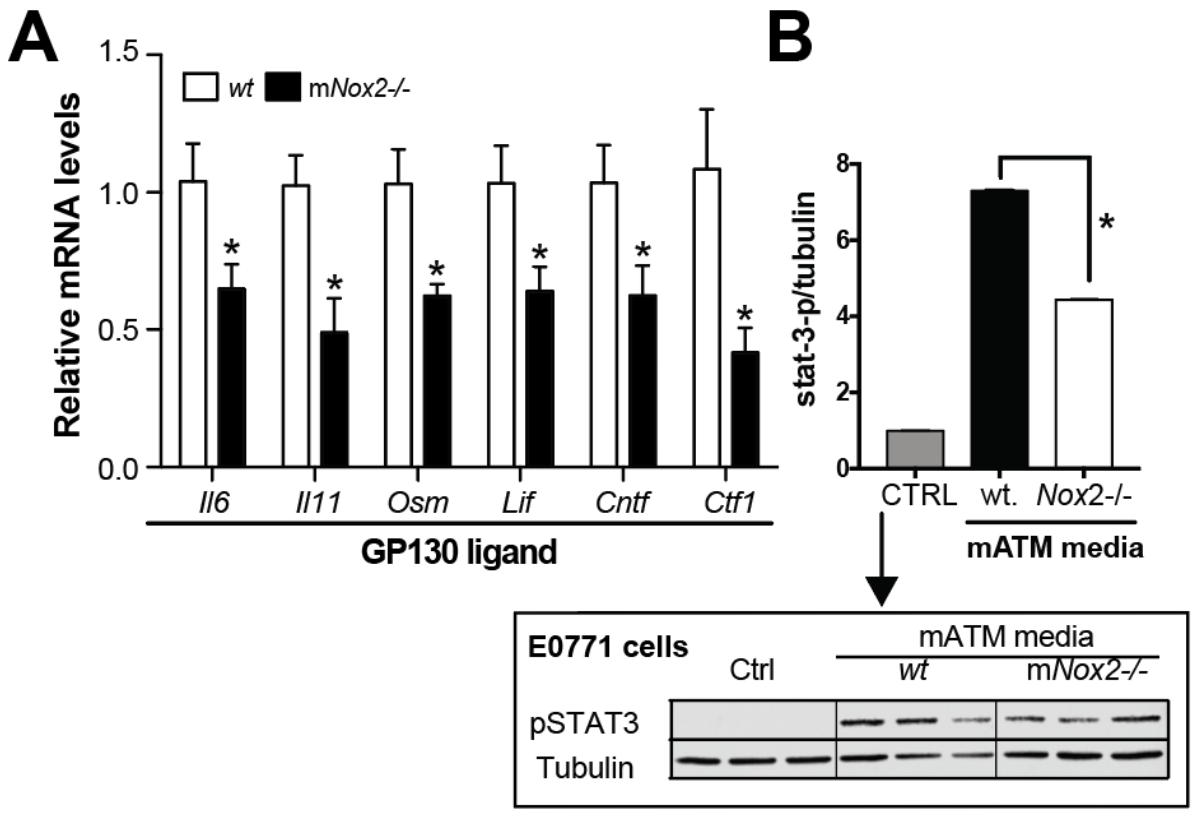


Fig.16. Deletion of Nox2 in myeloid cells attenuates the GP130 ligand expression and activation GP130/STAT3 axis in TNBC cells. Panel A: GP130 ligand expression by mammary ATMs isolated from wt and Nox2-/- obese mice. Panel B: Western blot analysis of STAT3 phosphorylation in TNBC cells with quantification. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3-4/group.

GP130 is required for obese mammary ATMs to promote stem-like properties in TNBC cells.

Next, we determined if these GP130 ligands are required for MMe to induce stemness in TNBC cells. Because each of the GP130 ligands elevated in obese mammary ATMs binds distinct receptors that heterodimerize with GP130 (Silver and Hunter, 2010), we depleted GP130 to attenuate signaling from multiple ligands. We used shRNA to knockdown GP130 levels in E0771 cells, confirmed knockdown using several clones (Fig.17A), and studied effects of obese mammary ATMs on TNBC cells. We found that knocking down GP130 attenuated the ability of obese mammary ATM media to induce STAT3 phosphorylation and to increase stem cell marker expression in E0771 cells (Figs.17C and E). Knocking down GP130 also lowered the ability of *in vitro*-derived MMe macrophages to induce STAT3 phosphorylation and tumorsphere growth in E0711 cells (Figs.17B and D), suggesting that this pathway may be utilized by MMe macrophage to promote stem-like properties in TNBC cells.

Knocking down GP130 in cancer cells attenuated the effect of obesity on TNBC tumor-forming potential.

GP130 signaling pathway in cancer cells is required for MMe macrophages to promote stem-like properties in TNBC cells. Next, we explored whether GP130 signaling was required for DIO to promote the tumor-initiation potential of TNBC cells *in vivo*. To test the relevance of GP130 signaling in cancer cells during obesity, we injected sh-control or sh-Gp130 E0771 cells into lean and obese female mice and monitored tumor incidence. Gp130 knockdown in E0771 cells attenuated the ability of obesity to promote tumor formation *in vivo* (Fig.18), and this decrease was comparable to the effect observed in obese mNox2-/- mice (see Fig.14).

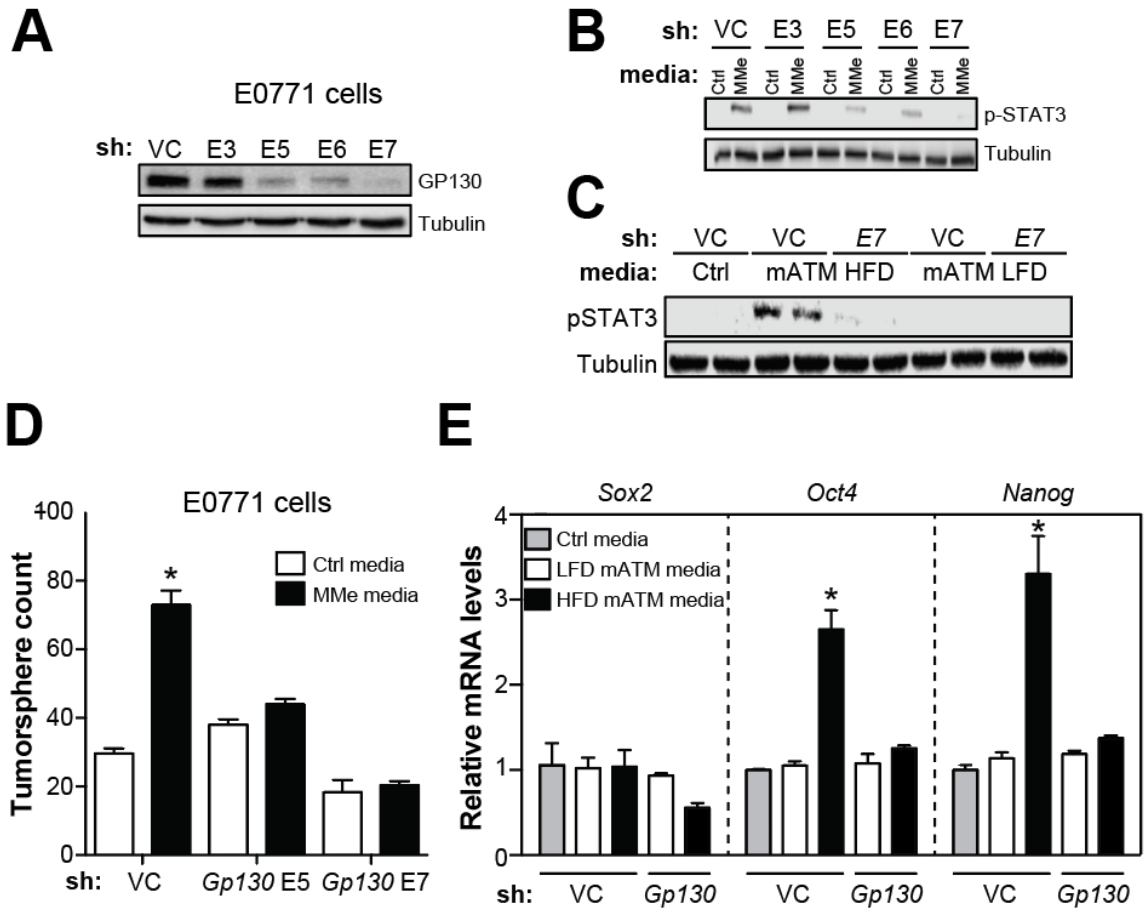
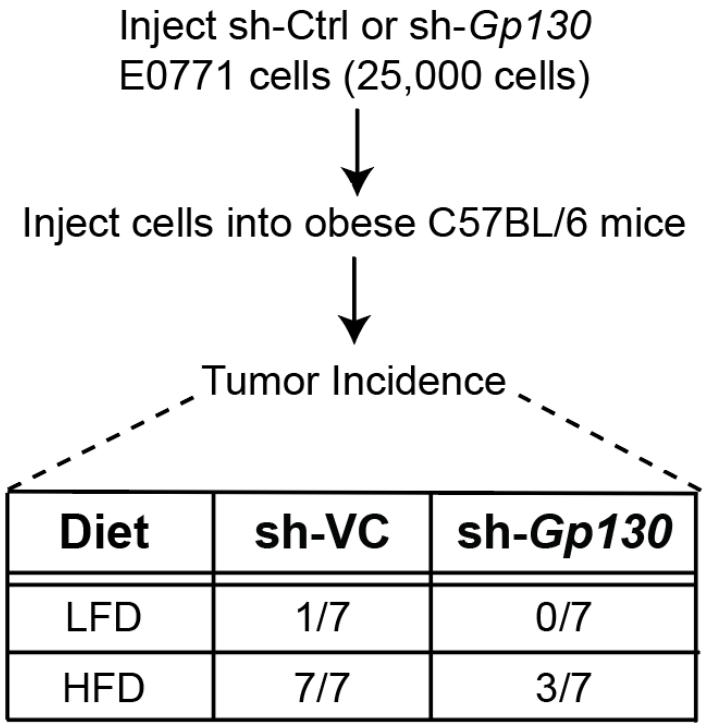


Fig.17. GP130 is required for obese mammary ATMs to promote stem-like properties in TNBC cells. Panel A: E0771 cells were treated with vector control shRNA (VC) or Gp130 shRNA (E3, E5, E6, E7) and GP130 knockdown was confirmed by western blotting. Panels B-E: Effect of GP130 knockdown on the ability of in MMe and mammary ATM media to induce stemness in cancer cells. Panels B-C: STAT3 phosphorylation. Panel D: Tumorsphere count. Panel E: Stem cell marker expression. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group.



Diet	sh-VC	sh-*Gp130*
LFD	1/7	0/7
HFD	7/7	3/7

Fig.18. Knocking-down GP130 in cancer cells attenuated the promotion of tumor-initiation potential of cancer cells during obesity. Control $Nox2^{fl/fl}$ mice (wt) and myeloid cell-specific LysM-$cre^{+/-}$ $Nox2^{fl/fl}$ mice (mNox2-/-) were fed a HFD for 12 weeks. Tumor incidence following injection of sh-VC or sh-Gp130 (E7) E0771 cells into mammary fat of lean or obese C57BL/6 mice.

Summary and Discussion

Our findings suggest that obesity-induced or *in vitro* palmitate-induced metabolic activation promotes the expression of GP130 ligands in MMe macrophages in a Nox2-dependent manner. Inhibiting the action of these GP130 ligands on cancer cells by knocking-down GP130, a common receptor for many pro-inflammatory cytokines in cancer cells attenuates the effect of obesity and obese mammary ATMs on TNBC stemness.

Breast cancer cells are known to secrete IL-6, LIF and other cytokines (Conze et al., 2001; Li et al., 2014b), which then act in an autocrine fashion to induce stemness in cancer cells. Thus, inhibition of stemness due to GP130 knockdown could be in part due to inhibition of autocrine signaling. To confirm the role of MMe macrophage secreted Gp130 ligands in inducing cancer stemness, we can use the following approaches. First, detection of GP130 ligands in the MMe macrophage-conditioned media. We used ELISA to detect GP130 ligands in the conditioned media, but we did not detect any GP130 ligands. One possibility is that these cytokines are present at a lower concentration (<1ng/ml) relative to the lower limit of detection, which is approximately 1ng/ml. This limitation can be overcome by concentrating the conditioned media. Second, deplete specific Gp130 ligands using neutralizing antibodies in MMe-conditioned media and test their requirement in inducing stemness in cancer cells. To fully explore the effect of MMe macrophages on cancer cells, such as changes in gene expression linked to EMT, metabolism, etc., an unbiased approach should be used. For example, RNA-Seq analysis of cancer cells treated with or without MMe conditioned media would provide us with in-depth knowledge of the effect of MMe macrophages on cancer cells.

CHAPTER 7. WEIGHT LOSS REDUCES OBESITY-ASSOCIATED MACROPHAGE INFLAMMATION AND TUMOR FORMATION IN MAMMARY FAT.

Introduction

Obesity (after smoking) is the largest preventable risk factor for cancer and several other diseases. Obese patients are at higher risk of several metabolic disorders including diabetes and cancer. Weight loss is associated with improvements in obesity-related metabolic disorders such as improvement in function of pancreatic β-cell and insulin sensitivity of liver and skeletal muscle (Dixon, 2009; Franz, 2017; Heymsfield and Wadden, 2017; Mackenzie et al.; Mitchell et al., 2015). Similarly, TNBC patients identified as obese at the time of diagnosis have increased the risk for tumor relapse. Weight loss through a combination of nutrient intervention and exercise has been shown to reduce the risk of tumor relapse and improve five-year disease-free survival (Davis and Kaklamani, 2012; Ligibel et al., 2017). In preclinical models, weight loss can reduce experimental metastasis in mice. Furthermore, weight loss has been shown to reduce mammary adipose tissue inflammation. Based on these findings we next determined whether weight loss could reverse the metabolic activation of macrophages, induction of GP130 ligands expression by obese mammary adipose tissue macrophages and an increase in TNBC cells tumor-forming potential.

Results

Weight loss reverses the mammary ATM inflammation.

To test the effect of weight loss, we fed female C57BL/6 mice a HFD for three months and then switched them to a LFD to induce weight loss (Fig.19A). As a control, we fed mice the LFD for

the entire duration. As expected, diet-switching in five weeks reduced body weight, fasting glucose, and mammary and visceral fat mass (Figs.19B-D).

Our analyses of mammary ATMs from the mice showed that weight loss due to diet switch completely reversed their expression of GP130 ligands and inflammatory cytokines (Figs.20A-B). However, levels of the MMe markers Cd36 and Plin2 levels remained elevated in mammary ATMs (Fig.20B), perhaps due to the excess lipid released by adipose tissue during weight loss.

Weight loss reverse the effect of obesity on tumor incidence of TNBC cells.

To determine the effect of weight loss on the tumor-initiating potential of TNBC cells, we fed female C57BL/6 mice a HFD for three months and then switched them to a LFD to induce weight loss. Five weeks after switching diet we injected E0771 cells into the mammary fat pad of lean mice and mice that have lost weight (Fig.19A). We found that weight loss eliminated the increased tumor-forming capability of E0771 cells during obesity (Fig.21).

Summary and Discussion

Together, these data suggest that weight loss can reverse expression of pro-inflammatory cytokines, including GP130 ligands in MMe macrophages and obese ATM in mammary fat. Likewise, weight loss also reversed the effect of obesity on TNBC tumor formation, even though body weight and mammary and visceral fat mass remained significantly elevated relative to lean mice. These data further indicate the relevance of GP130 ligands and MMe(s) in obesity-associated TNBC and that diet changes in association with weight loss may be sufficient to reverse the pro-tumorigenic effects of obesity on cancer stemness.

In clinic weight loss is achieved using change in diet, caloric restriction and exercise, all of which has different effects on weight and metabolic profile of patients. It would be important to explore all these techniques alone or in combinations to mimic the weight loss methods in human and understand the effect on metabolic activation of macrophages and tumor formation.

Another interesting observation we made was that upon weight loss inflammation is completely attenuated in mammary adipose tissue macrophages. However, MMe phenotype such as PLN2 and CD36 is not completely reversed. One explanation to this is that during fasting adipocytes secrete FFAs to maintain homeostasis and upon an increase in FFA availability, MMe uptake the FFA, activates P62, which then suppresses the TLR2 and NOX2 induced inflammation (Kratz et al., 2014). This can be tested by analyzing increased lipid uptake in mammary adipose tissue macrophages upon weight loss using immunohistochemistry. Similarly, exercise also cause adipocytes to release FFA, which may attenuate macrophage inflammation in adipose tissue. Thus, it is an interesting idea that needs to be investigated and holds clinical relevance for various diseases including cancer.

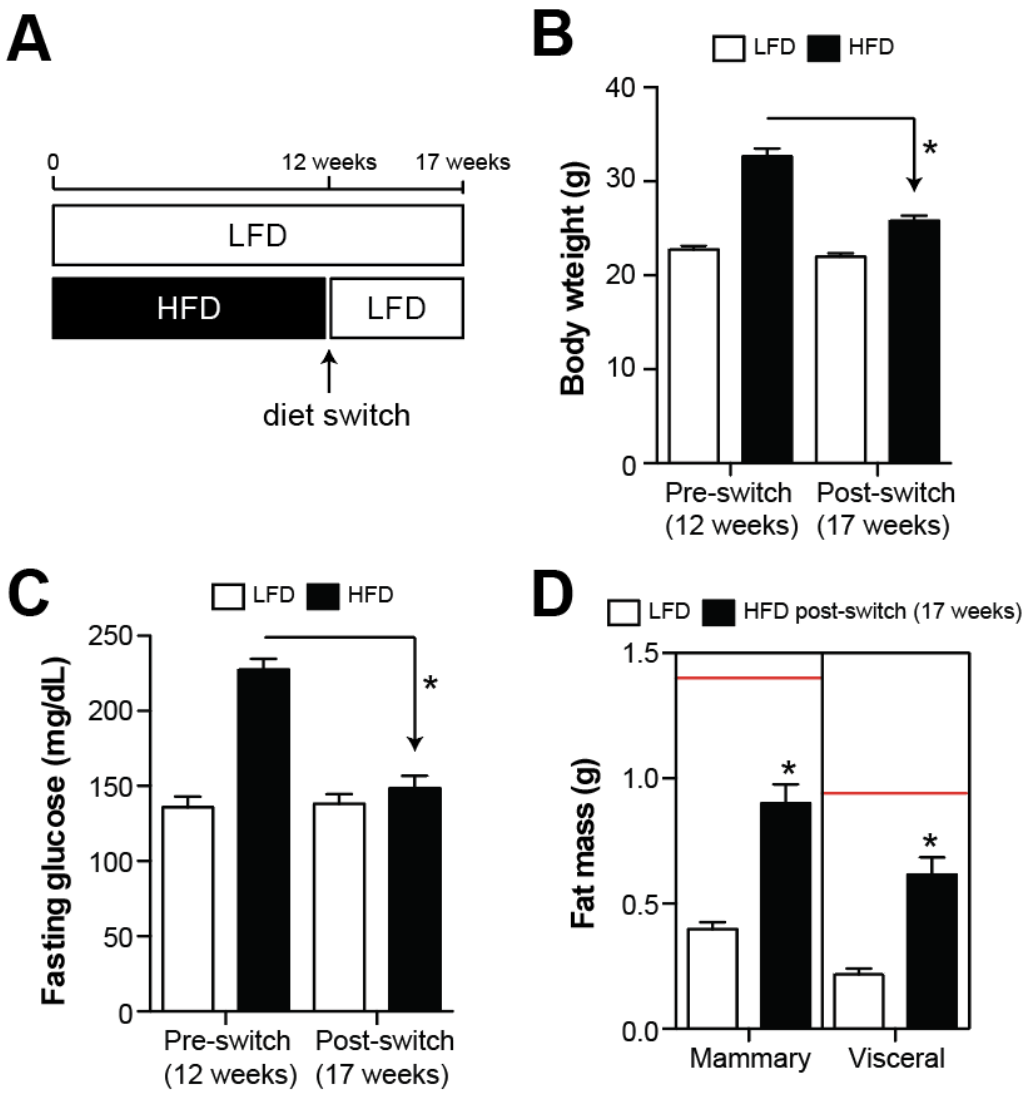


Fig.19. Weight loss reverses obesity-associated metabolic phenotypes. Panel A: Female C57BL/6 mice were fed a LFD (control), or HFD for 12 weeks. Starting week 13 obese mice were switched back to a LFD for five weeks to induce weight loss (HFD switch). At this time metabolic phenotype was characterized. Panel B: Body weight. Panel C: Mammary and visceral adipose tissue weight. Panel D Fasting glucose levels. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5-7 mice/group. Red lines indicate values obtained from C57BL/6 mice fed a HFD for 12 weeks (re-presented from Fig.4).

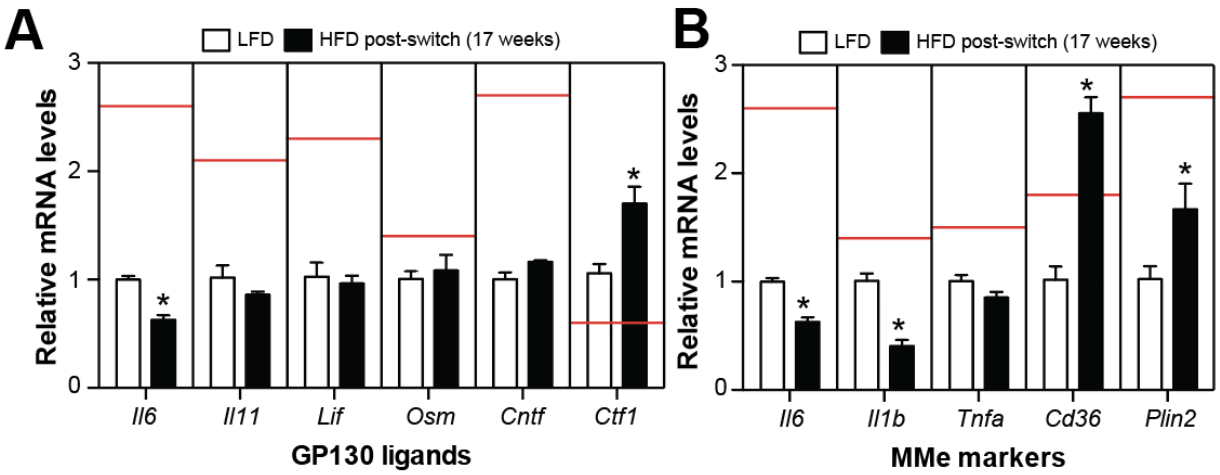
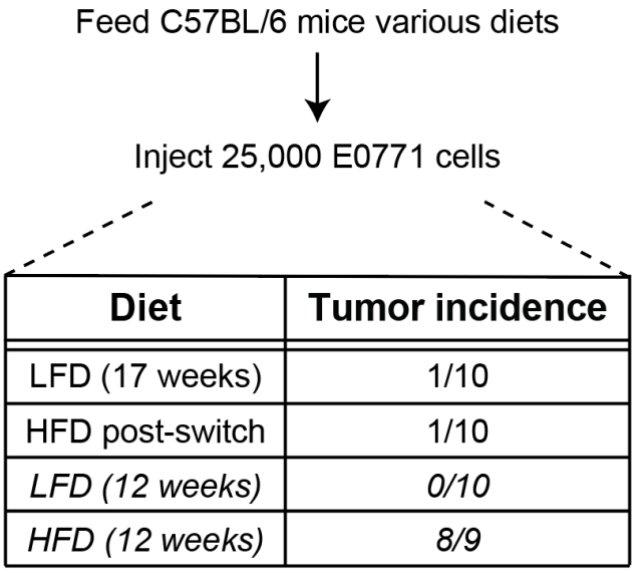


Fig.20. Weight loss reduces MMe macrophage inflammation. Female C57BL/6 mice were fed a LFD (control), or HFD for 12 weeks. Starting week 13 obese mice were switched back to a LFD for five weeks to induce weight loss (HFD switch). At this point mice sacrificed to isolate mammary ATMs to analyze the metabolic activation. Panel A: GP130 ligand expression in mammary ATMs. Panel B: MMe marker expression in mammary ATMs. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5 mice/group. Red lines indicate values obtained from C57BL/6 mice fed a HFD for 12 weeks (re-presented from Fig.4).



Diet	Tumor incidence
LFD (17 weeks)	1/10
HFD post-switch	1/10
LFD (12 weeks)	*0/10*
HFD (12 weeks)	*8/9*

Fig.21. Weight loss reverse the effect of obesity on tumor incidence of TNBC cells. Female C57BL/6 mice were fed a LFD (control), or HFD for 12 weeks. Starting week 13 obese mice were switched back to a LFD for five weeks to induce weight loss (HFD switch). At this point, E0771 cancer cells were injected into the mammary fat pad and mice were continued on LFD until the end of experiment (additional 7 weeks). Tumor incidence following injection of E0771 cells into mammary fat. Tumor incidence at 12 weeks of LFD and HFD are re-presented from Fig.1 for comparative purposes.

CHAPTER 8. HUMAN MME MACROPHAGES ARE PRESENT IN MAMMARY ADIPOSE TISSUE OF OBESE WOMEN.

Introduction

Having shown that DIO promotes an MMe phenotype in mouse mammary ATMs, which overexpress cytokines that signal through GP130 to promote the stem-like properties of TNBC cells in mice, we investigated whether this mechanism may also be operative in humans.

Results

Obesity increases the accumulation of metabolically activated macrophages in breast of obese women.

We determined whether MMe macrophages were increased in mammary adipose tissue of obese women (BMI > 30kg/m^2) in comparison to non-obese women (BMI < 30kg/m^2) undergoing mammary reductions. To this end, we isolated the SVC from mammary adipose tissue and studied ATMs (defined as $CD45^+CD11b^+CD14^+CD206^+$) by flow cytometry (Fig.22).

The number of ATMs (defined as $CD45^+CD11b^+CD14^+$) in the SVC from mammary fat was significantly elevated in obese women relative to non-obese women (Fig.23C). To explore the activation status of mammary ATMs, we stained them with antibodies against M1 markers (CD319 and CD38) and MMe markers (ABCA1 and CD36). These analyses showed that MMe ATMs, and not M1 ATMs, accumulate in mammary adipose tissue during obesity (Figs.23A-C). Indeed, the percent of mammary ATMs expressing MMe markers was significantly and

positively correlated with BMI ($p<0.001$, $R^2 = 0.67$), while the percent M1 ATMs was not correlated ($p=0.14$, $R^2 = 0.14$) (Fig.23D).

Interestingly, the majority of mammary ATMs from non-obese and obese subjects stained positive for MMe markers (Fig.23A), suggesting that obesity did not induce a phenotypic switch in ATMs towards the MMe phenotype. Instead, we observed an increase in the number of MMe macrophages in the SVC of obese mammary fat (Fig.23C). These data are in contrast to what we observed in mammary adipose tissue in mice, where obesity-induced MMe marker expression in ATMs without altering their number indicating that ATMs were reprogrammed to MMes rather than increasing their number (see Figs.5E and 8A). Thus, although the mechanisms are different between mice and humans, the common endpoint is an increased presence of MMe macrophages in mammary adipose tissue during obesity.

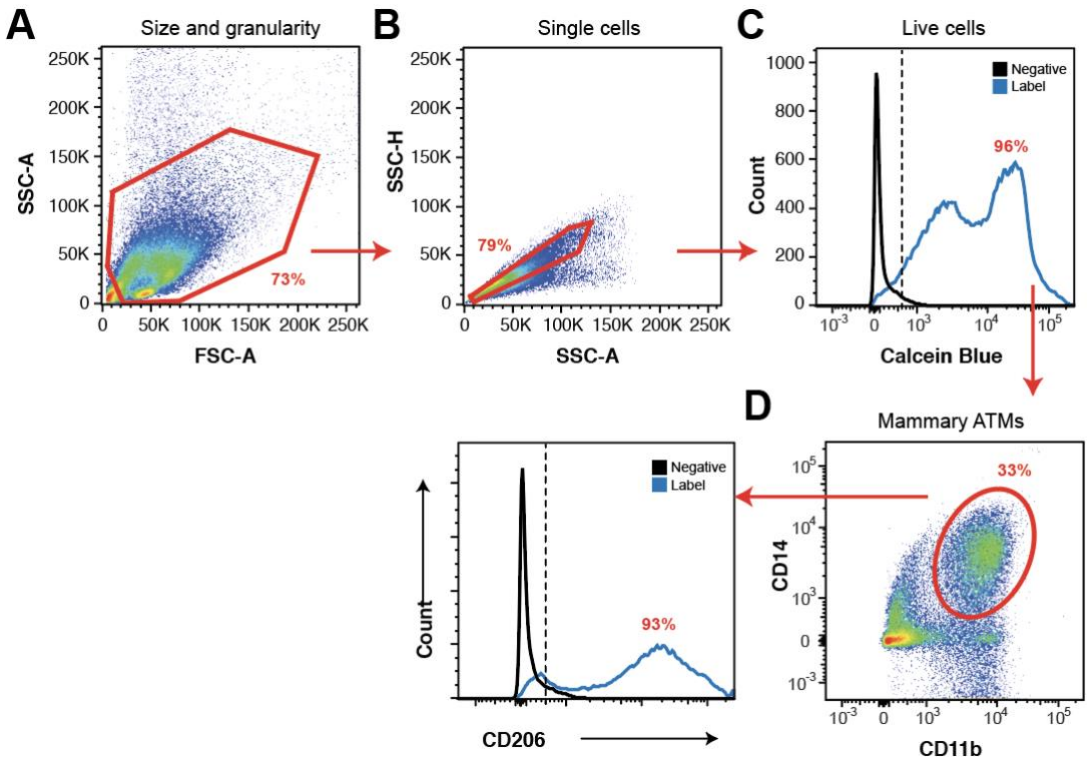


Fig.22. Flow cytometry workflow for characterizing human breast adipose tissue macrophages.

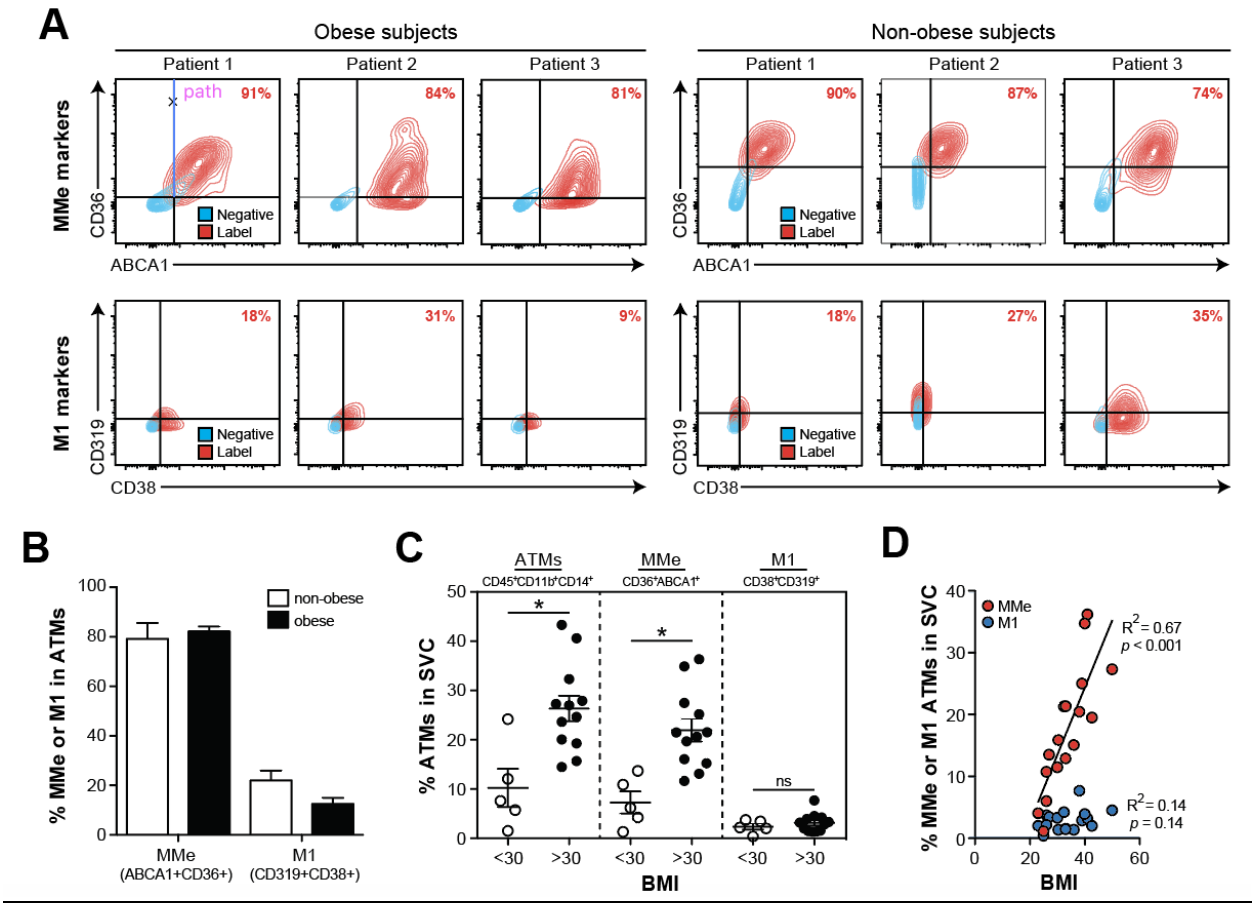


Fig.23. MMe macrophages are present in mammary fat of obese women, and promote stem-like properties in TNBC cells. Panels A-C: Mammary adipose tissue was obtained from non-obese (BMI<30, n=5) and obese (BMI>30, n=12) women. Panel A: Flow cytometric analysis of mammary ATMs. One subject is shown as an example. Panel B: Percent MMe markers and M1 markers in ATMs. Panel C: Percent ATMs, MMe ATMs, and M1 ATMs in the stromal vascular cells (SVC) obtained from mammary fat. Panel C: Linear regression analysis of percent MMe and M1 ATMs in mammary fat with BMI. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=5 non-obese human sample and n=12 obese human samples.

Human metabolically activated macrophages signal through GP130 to promote stem-like properties in human TNBC cells.

Next, we determined whether human mammary ATMs could signal through GP130 to promote human TNBC cell stem-like properties. Because we were unable to isolate enough ATMs from mammary adipose tissue of lean subjects, and the number of purified mammary ATMs from obese subjects was limiting, we could only test this hypothesis using a limited number of assays. We found that conditioned media from mammary ATMs from three obese women induced STAT3 phosphorylation and stem cell marker expression in SUM159 cells (Figs.24A-D), a human TNBC cell line (Neve et al., 2006). Importantly both of these effects were diminished when GP130 was knocked down in SUM159 cells by shRNA (Figs.24B-D). To provide additional evidence that human MMe macrophages can support GP130-mediated stemness in TNBC cells, we isolated human monocyte-derived macrophages (M0) and exposed them to MMe polarizing stimuli *in vitro*. *In vitro*-derived human MMe macrophages over-expressed all GP130 ligands tested, including IL-6, OSM, CNTF, CTF1, and LIF (Fig.25A). Moreover, human MMe macrophage media induced STAT-3 phosphorylation, stem cell marker expression, and tumorsphere growth of SUM159 cells (Figs.25B-D), and all of these effects were attenuated by GP130 knockdown (Figs.25B-D).

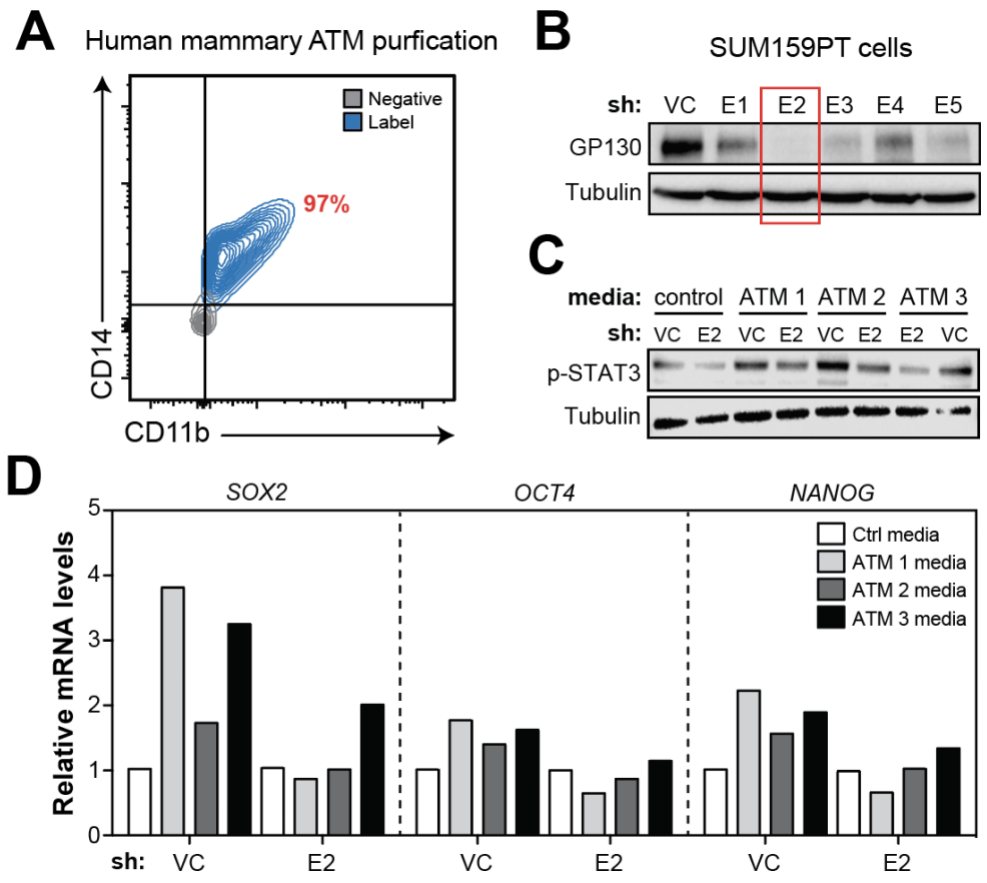


Fig.24. Obese women breast adipose tissue-derived macrophages promote stem-like properties in TNBC cells. Panel A: Purification of mammary ATMs from obese women. Panel B: Human SUM159PT cells were treated with control shRNA (VC) or GP130 shRNA (E1-E5) and GP130 knockdown was confirmed by western blotting. Panels C-D: Effect of GP130 knockdown (E2) on the ability of obese mammary ATM media, from three independent donors, to induce STAT3 phosphorylation (Panel C) and stem cell marker expression (Panel D) in SUM159PT cells.

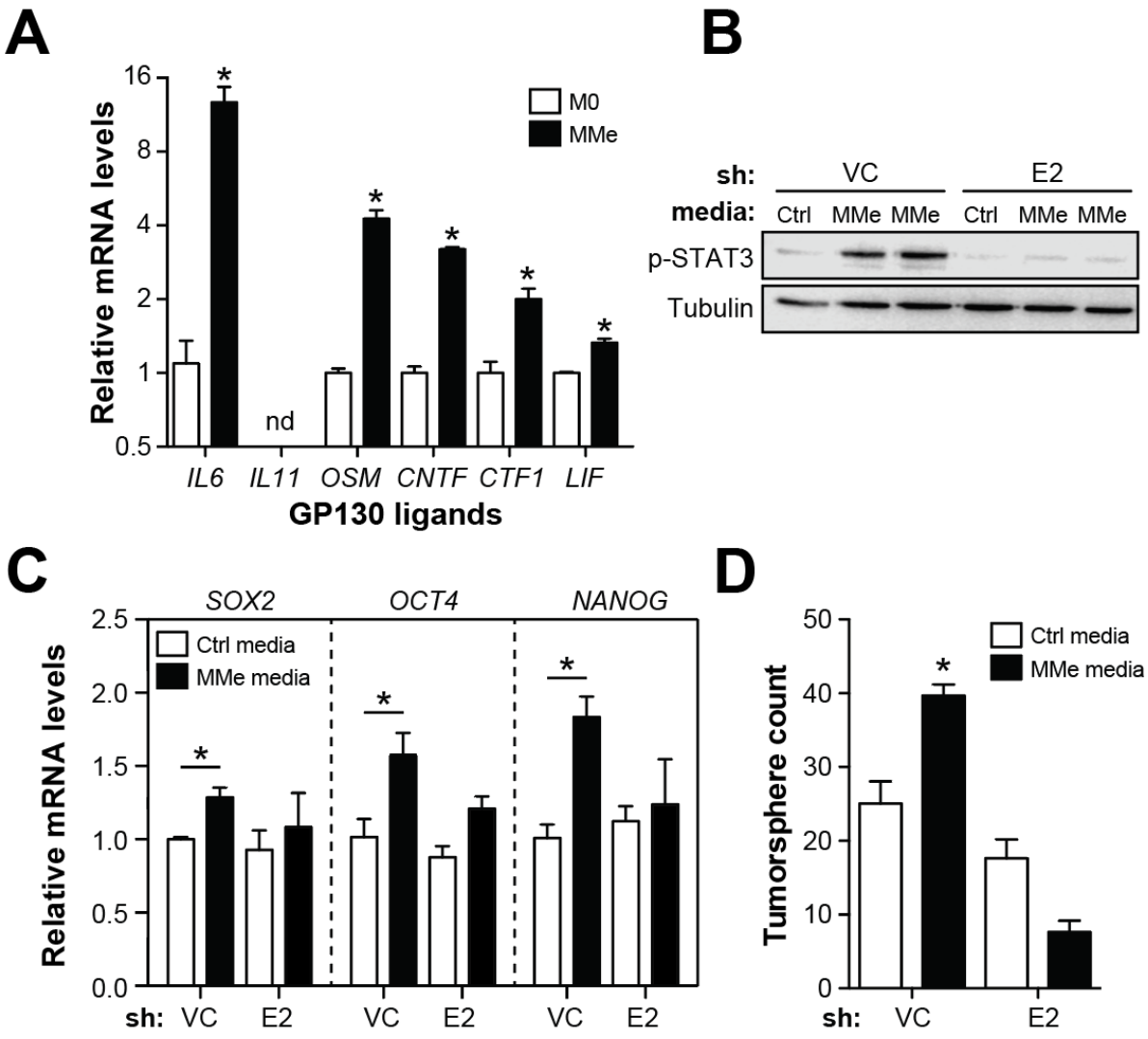


Fig.25. Human-blood derived metabolically activated macrophages signals through GP130 to promote stem-like properties in human TNBC cells. Panel A: GP130 ligand expression in unstimulated (M0) and metabolically activated (MMe) HMDMs. Panels B-D: Effect of GP130 knockdown (E2) on the ability of human MMe media to induce STAT3 phosphorylation (Panel B), stem cell marker expression (Panel C), and tumorsphere formation (Panel D) in SUM159PT cells. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group.

Summary and Discussion

Collectively, these findings suggest that during obesity, MMe macrophages accumulate in mammary adipose tissue and that human MMe macrophages, like their mouse counterparts, can promote stem-like properties in TNBC cells through GP130 signaling.

Contrary to our finding in mouse, breast adipose tissue macrophages isolated from both obese and non-obese human express MMe markers. One explanation could be that non-obese humans are overweight and not lean and that overweight condition is sufficient to metabolically activate macrophages. However, obesity is required to increase the accumulation of MMe macrophages in human breast adipose tissue. Another explanation could be that these non-obese patients are not completely healthy people and are coming for breast reduction due to some underlying pathological condition which can cause metabolic activation of macrophages. Metabolic activation of non-obese patients can be an artifact because breast adipose tissue samples are sitting out for long before it reaches lab (minimum of 4-6 hours), which may lead to the of FFA from the adipocytes and metabolic activation of macrophages. Last but not least, this difference could be due to the different physiology of mouse and human breast adipose tissue. To understand this in more detail, please refer to future directions where I have discussed the alternative and better technical ways to investigate the presence of MMe macrophages in human breast (lean or obese) in detail.

CHAPTER 9. MME MACROPHAGES PROMOTE TNBC CELL INVASIVENESS.

Introduction

In previous chapters, we showed that MMe macrophages promote a CSC-like phenotype in TNBC cells. CSC-like properties are often associated with an increased metastatic property of cancer cells (Radisky and LaBarge, 2008). Here we extended our studies to characterize the effect of MMe macrophages on TNBC cell invasiveness using a combination of *in vitro* and *in vivo* approaches.

Results

MMe macrophages promote tumor cell invasiveness.

To investigate the effect of MMe macrophages on TNBC cells invasiveness, we pretreated TNBC cells with CM from BMDM derived MMe macrophages. We found that invasion of human MDA-MB-231 or BM1 breast tumor cells was potentiated by pre-treatment with conditioned media from MMe macrophages but not un-stimulated MM0 macrophages (Fig.26B). Similar data were obtained using MMe macrophages produced by treatment with mammary adipose tissue conditioned media obtained from lean and obese mice (Fig.26A). Next, we determined if MMe macrophages can promote the TNBC intravasation into the blood *in vivo*. We found that co-injecting MMe macrophages with MDA-MB-231 tumor cells into mammary fat pads of nude mice promote tumor cell intravasation into the bloodstream (Figs.26C-D). Importantly the inability of M0 macrophages to promote intravasation suggests that the reprogramming in mice of injected macrophages to a TAM-like phenotype cannot explain our findings. In addition, the tumor growth was not changed by co-injecting macrophages with

TNBC cells. This is important because tumor size can affect the number of cells intravasated into the bloodstream. Together, our data suggest that MME macrophages promote the cancer cells intravasation. To test if metabolic activation is required for MMe(s) to potentiate cancer cells invasiveness, we used wt or Nox2 ko BMDMs and treated them with palmitate. We found that invasion of human MDA-MB-231 cells was potentiated by pre-treatment with conditioned media from MMe macrophages but not from un-stimulated MM0 macrophages or Nox2 ko MMe macrophages (Fig.27). Thus, Nox2 dependent metabolic activation of macrophages is required for MMe to potentiate invasiveness of TNBC cells.

Summary and Discussion

Collectively, these studies suggest that MMe macrophages release specific factors that exacerbate tumor cells invasiveness both *in vitro* in a Boyden chamber assay and *in vivo* in mice. Increased intravasation into the bloodstream from primary tumor is an important step in metastasis, and hence MMe macrophages contribute to increasing metastasis. Furthermore, Nox2 is required for MMe macrophages to promote the cancer cell invasiveness. Further studies are required using in mammary adipose tissue macrophages from mice and human, more TNBC cancer cell lines transfected with or without GP130 shRNA and syngeneic mouse experiments with Nox2 ko to confirm the role of MMe macrophages and GP130 ligands in potentiating cancer cell invasiveness.

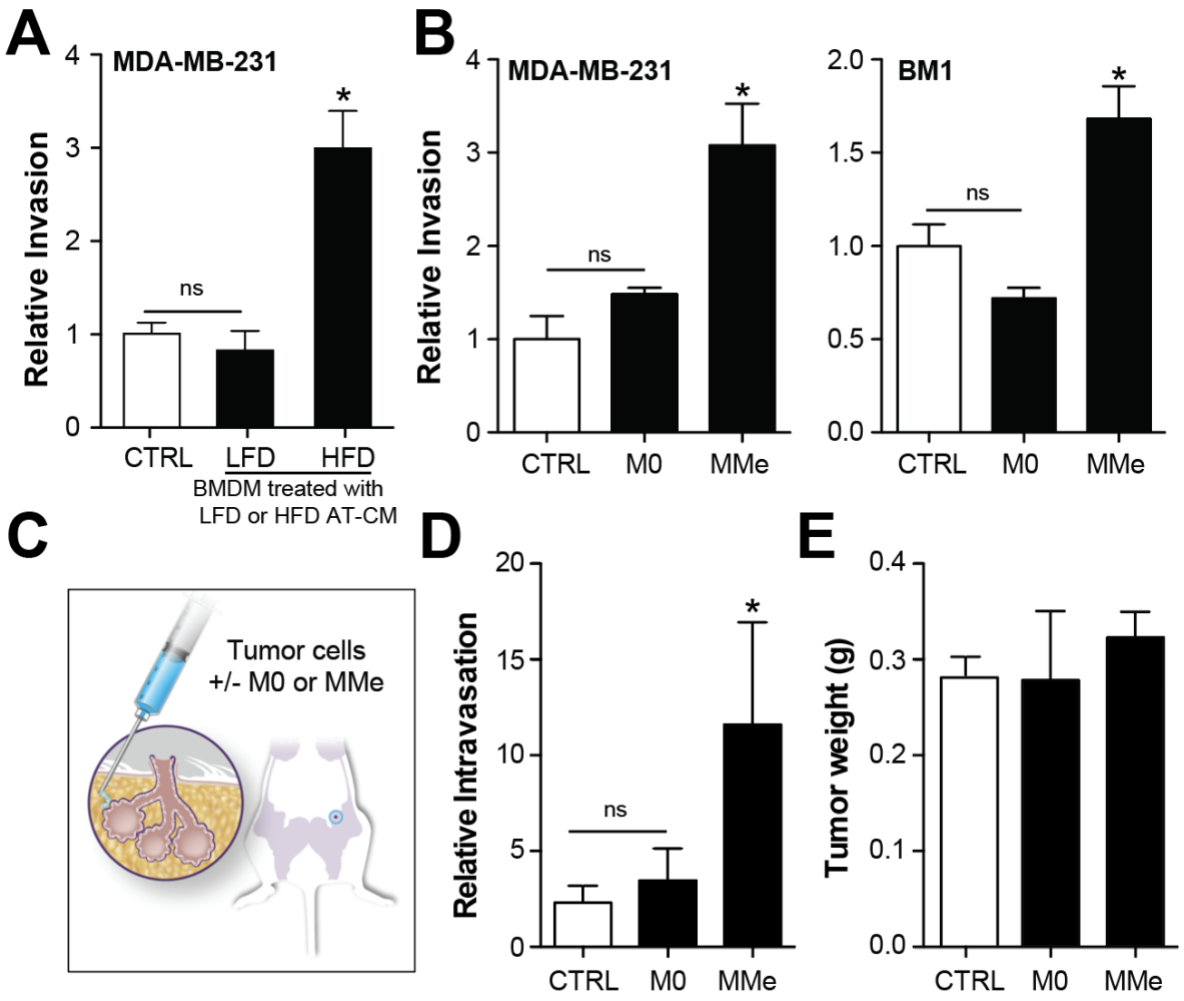


Fig.26. MMe macrophages promote tumor cell invasiveness. Panel A-B: TNBC cells pretreated with BMDM conditioned media treated with mammary adipose tissue conditioned media from lean or obese mice (Panel A) or BMDMs treated with palmitate (Panel B). Panel C: MDA-MB-231 cells +/- M0 or MMe macrophages were injected into mammary fat of nude mice and tumor were allowed to develop for 4 weeks. Panel D: Intravasation of tumor cells into blood (determine by the ratio of human GAPDH to mouse Gapdh). Panel E: Tumor weight. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3-5/group.

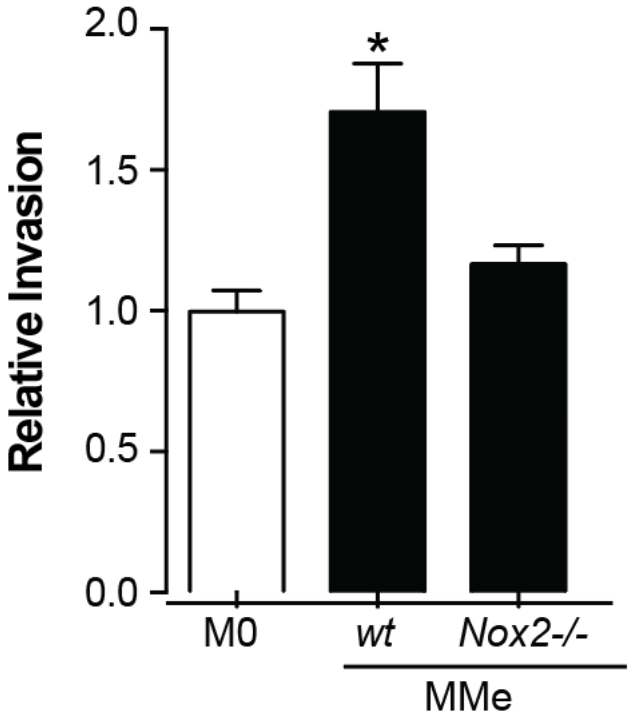


Fig.27. Nox2 are required for MMe macrophages to promote cancer cell invasion. Boyden chamber invasion assay with BM-1 cells treated with media from MMe (wt, Nox2-/-) or Mo macrophages. Results are mean ± SEM. *, $p<0.05$ Student's t-test. n=3/group.

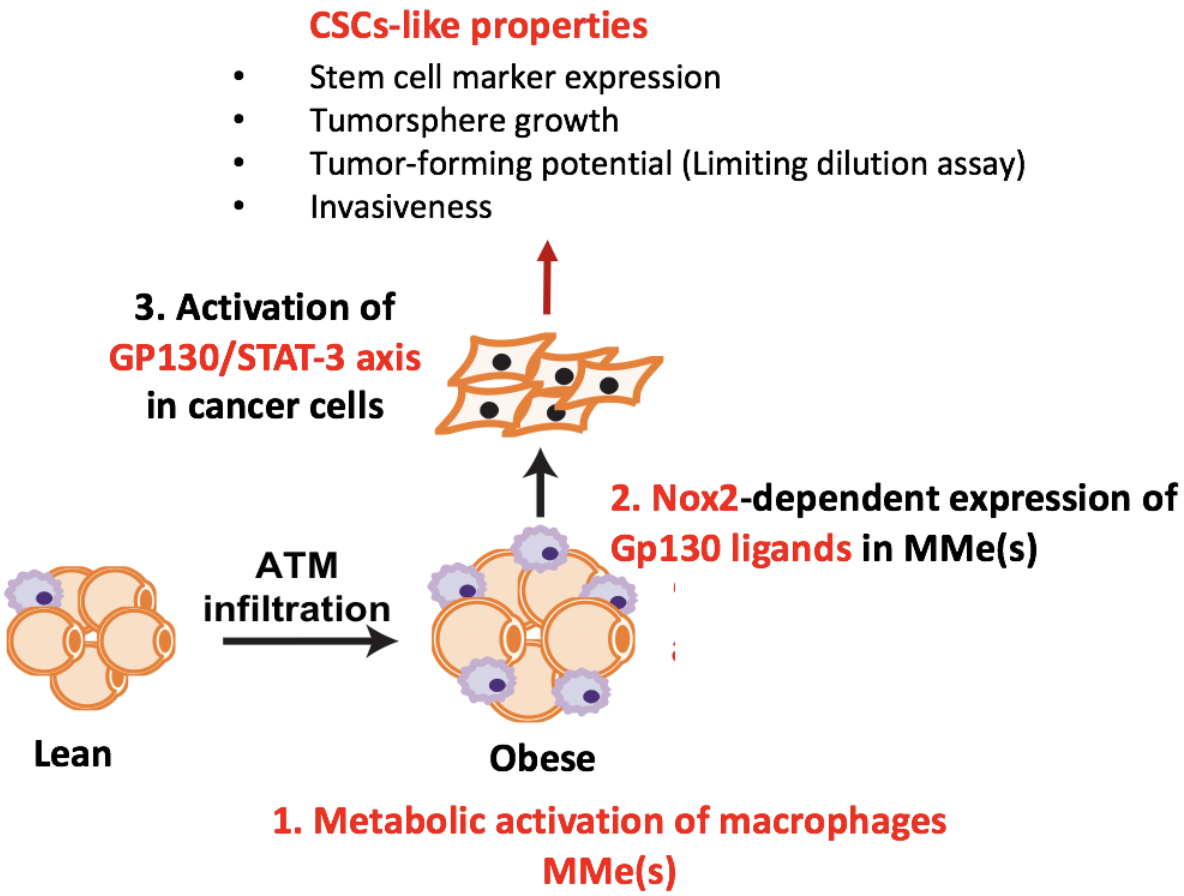


Fig.28. Graphical summary: 1. MMe macrophage are the predominant phenotype of macrophages in mammary adipose tissue of mice and human. 2. These MMe macrophages express pro-inflammatory cytokines in a Nox-2 dependent manner. 3. Cytokines activate the GP130/STAT3 signaling axis in cancer cells and potentiate CSCs-like phenotype.

CHAPTER 10. DISCUSSION

Our findings support a model wherein DIO induces an MMe phenotype in mammary ATMs, which overexpress cytokines that signal through GP130 to induce stem-like properties in TNBC cells (Fig.28). Three lines of evidence support the idea that metabolically activated mammary ATMs promote TNBC tumor formation during obesity. First, we show that MMe is the predominant ATM phenotype in mammary adipose tissue of obese humans and mice mammary. Second, we demonstrate that *in vitro*-derived murine and human MMe macrophages mimic the effects of obese mammary ATMs on stem-like properties of TNBC cells, both in terms of mechanism and function. Third, we show that ablating Nox2, a gene required for MMe macrophage polarization, attenuated the ability of obese mammary ATMs to promote TNBC stemness *in vitro*, and the ability of obesity to promote TNBC tumor formation *in vivo*. The importance of this mechanism is underscored by our demonstration that it is utilized by *in vitro* and *in vivo*-derived MMe macrophages to induce stem-like properties in multiple TNBC cell lines of murine and human origin. Our demonstration underscores the importance of this mechanism that it is utilized by *in vitro* and *in vivo*-derived MMe macrophages to induce stem-like properties in multiple TNBC cell lines of murine and human origin.

These findings, together with our previous work (Kratz et al., 2014), suggest that the MMe phenotype may be broadly applicable to ATMs in multiple adipose tissue depots during obesity in humans and mice and that these cells perform a wide array of functions that impact the metabolic phenotype and its associated co-morbidities. We previously showed that MMe-like ATMs in visceral fat over-express inflammatory cytokines to exacerbate insulin resistance. during early obesity, but also protect against insulin resistance in prolonged obesity by clearing

dead adipocytes through a lysosomal exocytosis pathway (Coats et al., 2017). Our new findings suggest that MMe-like ATMs in mammary fat overexpress GP130 ligands that promote TNBC stem-like properties, and it is possible that a similar MMe macrophage mechanism may promote ER+ breast cancer and other obesity-associated cancers arising in adipose tissue rich environments. For example, ovarian, colon, and brain cancer. However, deciphering the contribution of MMe macrophages on tumor formation in ER+ breast cancer may be challenging because obesity is also known to promote ER+ breast cancer tumorigenesis by increasing local production of estrogen in adipose tissue (Subbaramaiah et al., 2011). Further studies are required to test the role of MMe macrophages in other obesity-associated cancer in adipose tissue rich environment. Furthermore, obesity is associated with systemic inflammation, i.e., pro-inflammatory cytokines such as IL-6 have been found to be elevated in blood serum of obese patients and is associated with poor prognosis (Nakashima et al., 2000; Salgado et al., 2003). It would be of interest to evaluate the presence of other GP130 ligands in the serum of obese patients and their prognostic value as well as their role in promoting obesity-associated cancer that is not in adipose tissue rich environment.

Inflammatory cytokines (such as IL-6) arising from tumor cells or an altered tumor microenvironment can signal through GP130 to induce JAK/STAT3 signaling and drive cancer cell growth and stemness (Korkaya et al., 2011). Constitutively activated STAT3 has been implicated in the initiation of many types of cancer, including breast cancer (Ling and Arlinghaus, 2005), as well as the promotion of invasion, migration, epithelial-to-mesenchymal transition, and cancer stem cell self-renewal and differentiation(Korkaya et al., 2011; Yuan et al., 2015). Our findings suggest that the chronic metabolic activation of ATMs in mammary fat promotes TNBC tumor stemness through this mechanism. Thus our findings corroborate with the

idea that stem-like properties may be activated by mutation or epigenetic regulation of key genes in cancer cells, or by signals from the tumor microenvironment (Karnoub et al., 2007; Liu et al., 2015). Cancer cells with stem-like properties are known to promote tumor initiation, and metastasis in mice (Grivennikov et al., 2010). In fact, the CSC-like phenotype is associated with tumor relapse and distance metastasis in TNBC patients. Our preliminary findings support this hypothesis as Nox2 mediated metabolically active macrophages potentiate the cancer invasiveness. Further studies are required using mammary ATMs and cancer cell lines transfected with or without GP130 shRNA and syngeneic mouse experiments with Nox2 knock-out to confirm the role of MMe macrophages and GP130 ligands in potentiating cancer cell invasiveness. Nonetheless, our study provides potential targets to treat obesity-associated TNBC.

Importantly, disabling this MMe-GP130-stemness pathway (by deleting Nox2 in macrophages or attenuating GP130 in TNBC cells) did not completely block increased tumor formation during obesity. It is therefore likely that additional DIO-induced factors can contribute to this phenotype. For example, leptin, an adipocyte-derived hormone that is increased during obesity, has been shown to promote cancer cell stemness in breast cancer by phosphorylating STAT-3 via leptin-receptor (Chang et al., 2015). Hyperinsulinemia, which is an increase in insulin level during obesity due to insulin resistance can also promote CSCs-like phenotype in cancer cells by activating PI3K/AKT and Ras/MAPK pathway via insulin growth factor receptor. Hyperinsulinemia has been linked with increased breast cancer development (Poloz and Stambolic, 2015). During obesity, altered extracellular matrix mechanical properties caused by fibroblast accumulation promotes tumorigenesis. Free fatty acids (FFA) can also promote stem-like properties in cancer cells by activating the Wnt/β-catenin and TGF-β signaling pathways (Nath et al., 2015), and during obesity, the increased adipose tissue mass and adipocyte insulin

resistance would increase FFA levels in mammary adipose-tissue. These increased FFA levels would also stimulate MMe polarization in mammary ATMs, which our findings suggest substantially contributes to TNBC cell stemness during obesity.

More generally, our findings reinforce the idea that tumorigenesis is regulated both by intrinsic properties of cancer cells (i.e., genetic alterations) as well as the tissue-specific niche in which the tumor develops (Polyak et al., 2009). From this perspective, obesity may be conceptualized as a pathological state that facilitates tumorigenesis by creating tumor permissive conditions in multiple tissues. For example, our studies demonstrated that obesity-induced ATM inflammation in mammary fat promotes TNBC tumor formation, while previous studies showed that obesity-induced neutropenia in the airway facilitates TNBC tumor metastasis (Quail et al., 2017), and obesity-associated TAM inflammation promotes tumor growth by inducing angiogenesis (Kolb et al., 2016). Although the specific mechanisms underlying these observations are distinct, they can be integrated through obesity's ability to induce chronic inflammation. Indeed, chronic inflammation, from a multitude of underlying sources (e.g., ulcerative colitis, Hepatitis C, pancreatitis), is known to promote cancer cell stemness and increase the risk of many types of cancers (Multhoff et al., 2012).

The strong correlation and mechanistic link between tumor-associated macrophages (TAMs) and tumor growth and progression emphasize the contribution of macrophages in disease progression and value of TAMs as an effective therapeutic target. It remains to be deciphered to what extent adipose tissue macrophages contribute to these processes, especially in tumors growing within or adjacent to adipose tissue. Our mechanistic insight into the role of metabolically activated

adipose tissue macrophages in TNBC progression reveals a number of potential therapeutic approaches to counteract the effects of obesity.

We have identified NOX2 as a potential target for treating obesity-associated TNBC cancer. We have previously shown that NOX2 mediated ATM inflammation during obesity induce insulin resistance (Coats et al., 2017). Here we showed that NOX2 induced the expression of pro-inflammatory cytokines in MMe macrophages induces TNBC stemness. Together, our work suggests that by targeting NOX2 we can manage obesity-induced insulin resistance and reduce obesity-associated TNBC risk and progression. In line with this, recent studies have identified that depleting or inhibiting NOX2 in tumor reduced tumorigenesis, and metastasis in several cancers including lung cancer (Han et al., 2016), hepatocellular cancer (Eun et al., 2017), and melanoma (Aydin et al., 2017). Although the source of NOX2 in these studies has not been identified NOX2 is mostly found in macrophages and neutrophils (Singel and Segal, 2016)(Patriarca P et al., 1971; Babior BM et al., 1976). Our finding together with previous studies suggest that suppressing NOX2 or interfering NOX2 function in the tumor microenvironment may be an important approach to prevent oxidative-stress-related carcinogenesis. NOX2 inhibitors such as gp91sd-tat (Smith et al., 2015) may be an attractive approach for attenuating MMe macrophage inflammation in obesity-driven TNBC.

GP130 is another target we identified to block the effect of MMe on TNBC progression. GP130 inhibitors such as Bazedoxifene (BZA) (Xu and Neamati, 2013), may also be useful for blocking the ability of MMe macrophage-derived cytokines to induce TNBC cell stemness. Like NOX2 inhibitor, BZA will target obesity-associated metabolic conditions including cancer. BZA and conjugated estrogen prevent diet-induced obesity, hepatic steatosis, and type 2 diabetes in mice

(Barrera et al., 2014). However, this approach may require the development of new GP130 inhibitors because MMe macrophages overexpress many GP130 ligands and BZA ability to inhibit the GP130/STAT3 pathway is limited to IL-6 and IL-11 (Wu et al., 2016; Xiao et al., 2017).

Finally, JAK1/2 inhibitors may have therapeutic value in blocking signaling downstream of MMe-derived cytokine interactions with GP130. JAK2 is overexpressed in TNBC and has been shown to be important for CSC survival and growth, and TNBC metastasis. The JAK1/2 inhibitor Ruxolitinib is currently in clinical trials for the treatment of TNBC cases positive for phosphorylated STAT3 staining (Stover et al., 2018). It is possible that tumors evolving in obese microenvironment might be dependent on JAK/STAT3 signaling even in the absence of JAK overexpression. Future studies will be needed to test the dependency of obesity-associated TNBC on JAK/STAT3 signaling and efficacy of these therapeutics in treating obesity-driven TNBC. However, this strategy is limited because GP130 activates several downstream signaling pathways which are responsible for cancer growth and metastasis. Such as PI3K/AKT/mTOR, MAPK/ERK, and NFkB pathway. Combination therapies such as PI3K, AKT or mTOR inhibitor might be a better therapeutic strategy. Further studies are required to test the role of MMe macrophages inactivation of these downstream signaling pathways in TNBC cells.

Obesity is one of the largest preventable risk factors for cancer and several other diseases such as type-2 diabetes and stroke. Lifestyle changes or invasive intervention such as bariatric surgeries causing weight loss has shown to reverse some of the pathologies cause due to obesity. For example, moderate weight loss (5–10% reduction) from baseline weight is associated with clinically meaningful improvements in obesity-related metabolic risk factors such as

improvement in pancreatic β-cell function and the sensitivity of liver and skeletal muscle to insulin. Likewise, TNBC patients who are obese at the time of diagnosis have increased risk factor for tumor relapse. Weight loss through nutrient intervention and exercise has shown to reduce the risk of tumor relapse and improve five-year disease-free survival. Furthermore, weight loss can reduce experimental metastasis in mice by reversing the lung inflammation caused by neutropenia (Quail et al., 2017). Moreover, weight loss has been shown to reduce mammary adipose tissue inflammation. In line with this, we found that weight loss can reverse mammary adipose tissue macrophage inflammation and the effect of obese mammary adipose tissue macrophages on cancer cells stemness. Notably, our studies combined with previous work suggest that obesity-associated chronic inflammation and its effects on tumorigenesis as well as other diseases may be reversed by caloric restriction, highlighting the potential therapeutic value of weight loss intervention.

CHAPTER 11. FUTURE DIRECTIONS

Extending the clinical significance of MMe markers in the context of cancer

Obesity is a risk and prognostic factor for TNBC. We showed that during obesity both human and mice mammary adipose tissue is enriched with metabolically active macrophages and that these MMe macrophages can promote the tumor-forming potential of cancer cells. However, our analysis is limited to the presence of MMe macrophages in the mammary adipose tissue of obese human in the absence of cancer. Also as discussed in the introduction, ~20% of lean adults display WAT inflammation, and insulin resistance, classified as metabolic obesity and ~50% of obese individuals remain metabolically healthy (Stefan et al., 2018). Thus, BMI alone cannot accurately capture immune and metabolic dysfunction in an individual. Therefore, in order to extend the clinical relevance of MMe macrophages we need to collect more patient data and build database on the presence of MMe macrophages in mammary adipose tissue (and other fat depots) in lean, overweight and obese patients, and also in mammary adipose tissue adjacent to DCIS and use this information to correlate percentage of MMe macrophages with mammary adipose tissue inflammation, pathological complete response to neoadjuvant chemotherapy, tumor-relapse at metastatic site, median disease/progression-free survival.

In our work we use flow cytometry to identify MMe markers; however, there are several limitations to this technique. First, it only works with fresh tissue samples. Second, it is not high throughput. Third, it gives information about only MMe cell surface markers but cannot be used for inflammatory cytokine expression. Fourth, most importantly it is challenging to get fresh cancer patient samples. The alternative approach is the use of immunohistochemistry (IHC) with paraformaldehyde fixed tissue sections. Unlike flow-cytometry, it is high throughput, requires

small tissue section. But most importantly, paraformaldehyde fixed sections are easily accessible through tissue banks. However, immunostaining MMe markers and secreted inflammatory cytokines using IHC have been proven complicated in our hands. One approach to this problem is the use of fluorescent in-situ hybridization (FISH), which detects expression of mRNA. The advantage of this technique is it can be used to quantify the presence of both MMe markers and pro-inflammatory GP130 ligands and will show the physical distribution of MMe macrophages in mammary adipose tissue surrounding DCIS and possibly in the DCIS. Although, we and others have demonstrated that TAMs isolated from obese tumors are not different in the expression pro-inflammatory cytokine expression; but these studies are done using TAMs isolated from late-stage tumors, where the tumor microenvironment will primarily regulate macrophage phenotype. However, it is possible that during early DCIS, we can identify MMe macrophages in or right next to the DCIS assuming that effect of obesity is more prominent on the macrophage phenotype than few cancer cells during early tumorigenesis.

In addition, studying the correlation between the presence of MMe in mammary fat depot and the presence of inflammatory cytokines in the blood serum will provide information if serum inflammation is a useful marker for mammary fat inflammation across patients with a range of BMI.

A recent study (Hao et al., 2018) showed that circulating adipose fatty acid binding protein (A-FABP) is elevated in obese breast cancer patients and promotes obesity-associated breast tumor development by enhancing CSC-like properties of cancer cells. A whole-body knockdown of A-FABP prevented tumor development in a spontaneous mouse model. They propose that A-FABP directly promotes the stemness of cancer cells via IL-6/STAT-3 signaling

pathway. However, *in vivo* evidence of A-FABP present in the mammary adipose tissue or binding to cancer cells is lacking. A-FABP is a protein which is expressed by adipocytes and macrophages. It is responsible for the transfer of FFA between different cell compartments and is required for foam-cell formation (macrophage loaded with fat) during obesity and atherosclerosis(Makowski et al., 2001). It is likely that A-FABP is promoting CSC-like properties in-part by promoting the MMe phenotype in macrophages as we showed that mammary adipose tissue conditioned media and FFA promotes metabolic activation of macrophages. However, further studies are required to test this hypothesis. One should characterize the adipose tissue macrophages isolated from wt and A-FABP-/- obese mice and analyze for MMe marker and inflammatory cytokine expression. If A-FABP promotes metabolic-activation of macrophages, it can be used as a diagnostic marker for the presence of MMe macrophages in obese and cancer patients.

Effect of MMe macrophages on metastasis

Several clinical evidence exit that links CSC-phenotype to metastasis. For example, the combination of CSC-markers (CD133, CD44, and CD166) can successfully identify patients at low-, intermediate-, and high-risk of recurrence and metastasis in colorectal carcinoma (Horst et al., 2009). Also, circulating tumor cells (CTCs) have been shown to express stem cell markers (Aktas et al., 2009; Kasimir-Bauer et al., 2012). A study demonstrated that in 66.7% of breast cancer patients, CTCs demonstrate a stem cell phenotype (35.2% CD44 (+)/CD24 (-/low) or 17.7% ALDH1 (high)/CD24 (-/low)) (Theodoropoulos et al., 2010). CTCs obtained from colon cancers patients that express CD133, carcinoembryonic antigen and cytokeratin (stem-cell

markers) have a poor prognosis due to metastasis than those individuals whose CTCs did not express these markers in (Alix-Panabières et al., 2008; Yang et al., 2015).

Several experimental pieces of evidence also exist that mechanistically link CSCs to metastasis-inducing cells. One of the key steps in the metastatic cascade is the migration of tumor cells away from the primary tumor and requires cancer cells to undergo epithelial to mesenchymal transition (EMT). EMT process is a critical aspect of CSCs, and thus CSCs are intrinsically more migratory relative to non-CSCs. In fact, the mesenchymal phenotype marker Zeb1 facilitate the acquisition of stem cell-like properties (Peter, 2010). Likewise, over-expressing Ras or Her2/neu give rise to a stem-like subpopulation of CD44high/CD24low cells with an enhanced EMT phenotype (Radisky and LaBarge, 2008). Many common molecules and signaling pathways have been identified that play a crucial role in maintaining CSC phenotype as well as in EMT process. For example, both BMI1 and TWIST1 regulates stemness and EMT in breast cancer cells (Hong Ren et al., 2016). Wnt/β catenin signaling is another example which regulates breast cancer metastasis by inducing stem-like phenotype in cancer cells (Jang et al., 2015). Together these studies demonstrate the importance of CSC-like phenotype in metastasis. We have shown that obesity promotes CSC-like phenotype in TNBC cells and increase the expression of stem markers. It is likely that MMe also promotes expression of epithelial-mesenchymal transition marker such as vimentin, twist1, snail, slug, etc. Moreover, our preliminary data also showed that MMe promotes the invasive property of MDA-MB-231 cells both *in vitro* and *in vivo* and therefore, it is likely that MMe(s) play a role in promoting obesity-associate TNBC metastasis. Further studies are warranted to test this hypothesis as metastasis is the primary reason for cancer-associated death and obesity is linked to poor prognosis in TNBC patients. Some key experiments include a). Identification of the pro-tumorigenic factor(s) released by MMe

macrophages using cytokine microarrays, proteomics, knockout mice (Nox2-/- mice), perturbations in cancer cells (Gp130 shRNA) will provide mechanistic insight and b). Complementary invasion and metastasis assays using cancer cells pretreated with mammary ATMs isolated from lean and obese mice and humans will provide *in vivo* validation for the functional and mechanistic insights obtained.

Effect of MMe macrophages on chemo-resistance and tumor relapse

Chemotherapy is currently mainstream anticancer therapy for TNBC treatment. Chemotherapeutic agents kill rapidly dividing cancer cells by damaging DNA and/or inhibiting mitotic division (Malhotra and Perry, 2003). However, chemotherapy misses slowly dividing and non-dividing cancer cells. CSCs, which are relatively quiescent cells in the tumor, are resistant to chemotherapy, and because of their ability to self-renew and give rise to other tumor cells, they are shown to be responsible for tumor relapse. In fact, minimal residual diseases are highly enriched for cancer stem cells(Ghiaur et al., 2012). For example, residual breast tumor cells persisting after chemotherapy are enriched for phenotypic breast CSCs (Creighton et al., 2009). In myeloma, a strong inverse correlation was found between CSC numbers and progression-free survival in patients after chemotherapy (Huff and Matsui, 2008). These data provide evidence of clinical relevance for CSCs. We have shown that obesity promotes CSC-like phenotype in TNBC cells via inflammatory cytokines secreted by metabolically activate macrophages. It would be of utmost clinical relevance to investigate the effect of MMe(s) on inducing chemo-resistance in TNBC cells. This would provide mechanistic understanding of the poor progression-free survival of obese TNBC patients relative to lean patients. Developing therapies

that target inflammation and CSC holds promise for more effective treatment of obesity-associated TNBC.

Role of obesity/MMe-induced CSC-phenotype in immune escape to promote the tumor formation

We showed that obesity promotes the tumor-forming potential of TNBC cells. A fewer number of cancer cells are required to form the tumor in immune-competent obese mice than in lean mice. Conceptually, this requires a subset of malignant cells which is capable of initiating tumors but is also not eliminated by immune cells. These criteria are fulfilled by CSCs that are pluripotent and immune-privileged. Thus, it's an interesting hypothesis that obesity-induced CSC-phenotype promotes immune-privilege and therefore, promote the tumor-forming potential of TNBC cells in immune competent mice. Several mechanisms have been reported by which CSCs can exempt immune surveillance. For example, enriched PD-L1 expression in cancer stem-like cells (CSCs) (Hsu et al., 2018), and epigenetic silencing of antigen processing and presentation genes (e.g., TAP1 and TAP2) and co-stimulatory molecule genes (e.g., CD80) in CSCs (Sultan et al., 2018) contributes to CSC immune evasion. We can test this hypothesis by injecting E0771 and Py8119 cancer cells in lean and obese mice and after a week look for the T-cell infiltration and activation using flow-cytometry. We can test the expression of PD-L1, TAP1, and TAP2, and CD80 on cancer cells isolated from lean and obese mice tumor and on cancer cells pretreated with MMe conditioned media *in vitro*. NOX2 perturbation in macrophages and GP130 perturbation in cancer cells will further provide mechanistic insight into the role of inflammation and CSC phenotype in promoting immune privilege. Based on the mechanistic findings on how obesity/MMe-induced CSC-phenotype escape immune

surveillance, appropriate immunotherapy might be beneficial in treating obesity-associated TNBC.

Macrophage based therapy to treat obesity-associated TNBC

Our findings have identified several potential therapeutic targets such as Gp91, GP130, and JAK1/2. A large body of work is present focusing on inhibiting the JAK/STAT pathway in cancer and ruxolitinib is currently in clinical trials for TNBC patients. However, targeting cancer cells and downstream signaling in cancer cells always leads to resistance and tumor relapse due to the nature of cancer cells to evolve and mutate as well as due to the redundancy in signaling pathways in cancer cells. Targeting cells of tumor microenvironment have few advantages such as they don't evolve as fast as cancer cells due to lack of mutations, and have relatively less redundancy in signaling pathways compared to cancer cells. Therefore, instead of targeting the signaling pathways in cancer cells, targeting the adipose tissue macrophages that are the source of inflammatory cytokines might be more beneficial. We showed that during obesity human breast adipose tissue is enriched with macrophages, almost 3-fold more than lean patients (See figure xx). This suggest that macrophage-based cancer therapies might be more effective in obesity-associated breast cancer. We further showed that obesity-associated macrophages reprogramming can be reversed by weight-loss intervention. This suggests that mammary adipose tissue macrophages are plastic and macrophage plasticity can be therapeutically exploited by reprograming pro-tumorigenic macrophages to a less pro-tumorigenic or antitumorigenic macrophage phenotype.

1. Reprograming MMe macrophage to M1-like macrophages:

Following are the current methods to reprogram pro-tumorigenic macrophage to anti-tumorigenic macrophage phenotype. 1. Antibody-mediated activation of costimulatory CD40, 2. blocking of IL-10, 3. delivery of immune-stimulatory cytokines such as IL-12, or 4. the administration of Toll-like receptor agonists or 5. targeting intracellular signaling molecules such as PI3Kinase γ with small-molecule inhibitors, and 6. Changing the transcriptional profile of macrophages using TMP195, a new Class IIa Histone Acetyl Deacetylase (HDAC) inhibitors (Cassetta and Pollard, 2017; Guerriero et al., 2017). Anyone or combination of these strategies potentially will work better in obese patients due to an increase in macrophage infiltration to the surrounding adipose tissue. In particular, the administration of the Toll-like receptor agonists that can block the FFA-binding to the TLR2 and thus be blocking GP130 ligand expression, and simultaneously activate tumoricidal macrophage phenotype. One disadvantage of this approach is that it can cause a sudden hike in inflammation (cytokine-shock syndrome) in obese patients if used systemically as several adipose tissue depots are enriched with macrophages and can be detrimental. To avoid cytokine release syndrome often associated with immunotherapy (Shimabukuro-Vornhagen et al., 2018) local administration of therapeutic agents might be a better strategy.

2. Reprograming macrophage to inhibit the expression of GP130 ligands and thus blocking the pro-tumorigenic effect of MMe macrophages:

In this study, we identified ATMs as the source of GP130 ligands (and probably other inflammatory cytokines) and the expression of these pro-inflammatory cytokines is dependent on NOX2. NOX2 are considered "professional" ROS-producing enzymes (Singel and Segal, 2016), which is present in neutrophils and macrophages (Patriarca P et al., 1971; Babior BM et al., 1976). Recent studies have identified the role of NOX2 in tumorigenesis, and metastasis in several cancers including lung cancer (Han et al., 2016), hepatocellular cancer (Eun et al., 2017), and melanoma (Aydin et al., 2017). Besides, NOX2 was found expressed in breast cancer cells and primary breast tumors (Mukawera et al., 2015). Knocking down NOX2 resulted in significant reduction of IKKε expression and tumor growth (Mukawera et al., 2015). Our study has identified the role of NOX2 in regulating macrophage phenotype and obesity-associated TNBC. Thus, making NOX2 an interesting target for cancer therapy. Since NOX2 is involved in regulating macrophages inflammation and insulin resistance (Kratz et al., 2014), NOX2 inhibitors can be used as a prevention strategy to control insulin resistance and the risk of developing cancer. However, there are seven isoforms of NOX, structurally very similar with varied functions in cancer. Some isoforms are shown linked with reduced tumor development (Aydin et al., 2017). Therefore, it is important to inhibit NOX2 selectively. With the advancement in the development of a more specific NOX2 inhibitor, Gp91-ds-TAT (Smith et al., 2015), it is now possible to explore the role of inhibiting NOX2 inhibitors in cancer progression. Pre-clinical studies are required to test the action of Gp91-ds-TAT in regulating a). Macrophage inflammation in various adipose tissue depot by isolating ATMs and analyzing mRNA expression of inflammatory cytokines, b). Insulin resistance which can be measured by testing

fasting blood glucose and insulin, c). Cancer prevention, which can be tested by studying tumor-incidence in syngeneic TNBC mouse models on HFD treated with or without Gp91-ds-tat, and by injecting E0771 or Py8119 in obese mice treated with or without Gp-91-ds-tat to test their tumor-forming potential, and d). Cancer progressing, which can be studied by treating lean and obese mice bearing tumor with Gp-91-ds-tat to see the effect on tumor growth and metastasis. Although NOX2 is an attractive therapeutic target, we need to be cautious in treating morbidly obese patients as our previous paper (Coats et al., 2017) showed the important role played by NOX2 during late-stage obesity.

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