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The Characterization of Angiopoietin-like Protein 4 Overexpression in Triple Negative Breast Cancer

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The Characterization of Angiopoietin-like Protein 4 Overexpression in Triple Negative Breast Cancer

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ABSTRACT

Triple Negative Breast Cancer (TNBC) is highly invasive and metastatic with approximately 15% of patients developing liver metastases. The primary treatment of metastatic TNBC is chemotherapy, however, there is an increased chance of resistance to this therapeutic technique. If Breast Cancer Liver Metastasis (BCLM) is left untreated most patients survive only 4 to 8 months with a very rare 5-year survival. Therefore, it is imperative to analyze markers and molecular pathways that TNBC cells use to progress, invade, and metastasize to the liver. The aim of this study was to examine the overexpression of angiopoietin-like 4 (ANGPTL4) in TNBC cells *in vitro* and *in vivo*.

Experimentally, ANGPTL4 mGFP was overexpressed in the MDA-MB-231(TNBC) cell line using lentiviral-mediated transduction. This expression was then confirmed using western blot analysis, ELISA, and fluorescence microscopy.

The intracellular localization of ANGPTL4, Tat-interactive protein p60 (Tip60) and Aurora Kinase A (AURKA) were evaluated by subcellular fractionation, western blot analysis, and immunofluorescence microscopy. This study showed that ANGPTL4 is localized in the nucleus and is a chromatin-bound protein. This implies that ANGPTL4 could potentially bind to DNA and affect transcriptional regulation. In addition, the findings of this study showed that TIP60 and AURKA, predictive interactors of ANGPTL4 are in the nucleus and compartmentalizes with ANGPTL4. This suggests that their association with ANGPTL4 could drive tumor progression and metastases. A mammosphere assay was performed to evaluate the "stemness" capability of TNBC cells expressing ANGPTL4. The results of the mammosphere assay promote the expansion of the cell colonies in the ANGPTL4 mGFP overexpression in the TNBC cell line. This indicates that the ANGPTL4 mGFP expressing cell line have stem-cell like

renewal potential *in vitro*. In this study, the soft agar colony formation assay was performed to measure the prospective tumorigenic ability of the ANGPTL4 mGFP cell line. The ANGPTL4 mGFP cell line exhibited enhanced anchorage-independence which speaks to the possible tumorigenic capacity of the cells to be used in *in vivo* studies.

Subsequently, a mouse model was used to evaluate the metastatic ability of ANGPTL4 primed TNBC cells *in vivo*. The findings show that MDA-MB-231 ANGPTL4 mGFP cell line had bigger primary tumors compared to the mGFP cell line expressing endogenous ANGPTL4. Furthermore, ANGPTL4 mGFP cell line resulted in more metastatic lesions on the liver compared to the MDA-MB-231 mGFP cell line. Collectively, these findings imply that ANGPTL4 is a driver of TNBC proliferation and metastases and therefore could be used as a therapeutic target for TNBC treatment.

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I. INTRODUCTION

I.1 The Molecular Subtypes of Breast Cancer

Breast cancer is the world's most diagnosed disease ((Breast cancer now most common form of cancer: WHO taking action, 2021) and, because of its heterogeneous nature, evidence suggests that with different histopathological and biological features there are distinct behaviors that lead to different treatment responses. Due to these responses, specific therapeutic approaches should be given for breast cancer treatment ((Blows, et al., 2010).Thus, the accurate grouping of breast cancers into clinically relevant subtypes is the first important step for therapeutic decision-making ((Dai, et al., 2015).

Consequently, there are five major subtypes of breast cancer ((Molecular Subtypes of Breast Cancer, 2021) determined by the presence or absence of the hormone receptor (HR) and the human epidermal growth factor receptor (HER2/neu). The hormone receptor can be an estrogen receptor or a progesterone receptor. HR+ (positive) means that tumor cells have receptors for the hormone estrogen or progesterone that promote the growth of HR+ tumors, whereas HER2+ means that tumor cells produce high levels of a protein called HER2/neu, which has been associated with aggressive types of breast cancer. In addition, high or low levels of the nuclear protein Ki-67 is used to detect how quick cells grow (Li, Jiang, Chen, & Zheng, 2015), (Sun & Kaufman, 2018).

The first subtype of breast cancer is luminal A, and it has the tendency to progress slowly and, as a result, has the best prognosis. This type of breast cancer is HR+, HER2 – (negative), and has low levels of the protein Ki-67. Second, luminal B breast cancer is considered HR+, and either HER2+ or HER2- with high levels of Ki-67 (National Institute of Health. Surveillance, Epidemiology, and End Results Program , 2017). Aggressive basal-like, triple-negative breast

cancer (TNBC) is HR- and HER2- (Keam, et al., 2011), (National Institute of Health. Surveillance, Epidemiology, and End Results Program , 2017), (Soliman, Khalil, & Antonia, 2014). In addition, HER2-enriched breast cancer is hormone-receptor negative and HER2+, and they tend to grow faster than luminal cancers and have a potentially worse prognosis, (National Institute of Health. Surveillance, Epidemiology, and End Results Program , 2017), (Soliman, Khalil, & Antonia, 2014). However, they are usually successfully treated with targeted therapies aimed at the HER2 protein, such as Herceptin. Furthermore, normal-like breast cancer is similar to luminal A disease. However, its prognosis is slightly worse than luminal A cancer's prognosis (Herr, et al., 2019)

Table 1.1 - Summary showing the characterizations of the breast cancer subtypes and the percent survival of each one.

Breast Cancer Subtype	Estrogen (ER)	Progesterone (PR)	Human Epidermal Growth Factor Receptor (HER2)	Ki-67 Levels	5-year survival Percentage (%)
Luminal A	+	+	-	**Low**	**94.3**
Luminal B	+	+	+/-	**High**	**90.5**
TNBC	-	-	-	**High**	**76.9**
HER2-enriched	-	-	+	**High**	**84.0**
Normal-like	+	+	-	**Low**	**91.0**

I.2 Triple Negative Breast Cancer Overview

TNBC is defined by the absence of an estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. It is estimated that out of the worldwide breast cancer

burden, approximately 170,000 cases are TNBC and account for 10-20% of invasive breast cancers ((Boyle, 2012), (Foulkes, Smith, & Reis-Filho, 2010), (Kumar & Aggarwal, 2016). TNBC incidence has been associated with women of color, mutations in breast cancer gene susceptibility protein 1/2 (BRCA 1/2), women of Jewish ancestry, onset at a younger age, higher-grade tumors, more advanced tumor grade at diagnosis, and increased recurrence ((Byrski, et al., 2009) (Comen, et al., 2011), (Dent, et al., 2007), (Foulkes, et al., 2003), (Kirk, 2010) (Lundqvist, Andersson, Ahlberg, Nilbert, & Gerdtham, 2016), (Mavaddat, et al., 2012), (Musolino, et al., 2007), (Prat & Perou, 2011), (Philipovskiy, Corral, Dwivedi, Heydarian, & Gaur, 2019), (Turner, Tutt, & Ashworth, 2004).

Epidemiological studies have reported that TNBC is more common in women of African, Hispanic, and Jewish ancestry ((Bauer, Brown, Cress, Parise, & Caggiano, 2007), (Carey, et al., 2006), (Comen, et al., 2011), (Jiagge, et al., 2016), (Kohler, et al., 2015), (Philipovskiy, Corral, Dwivedi, Heydarian, & Gaur, 2019), (Sørlie, et al., 2001). Additionally, multiple findings have examined higher prevalence and incident rates, worse clinical outcomes, and mortality rates of TNBC in African American, Hispanic and Jewish women compared to TNBC in European American women (Bonotto, et al., 2014), (Clark, Rodriguez, Snyder, Hankins, & Boehning, 2012), (DeSantis, et al., 2019), (Hunter, 2000), (Siegel, Miller, & Jemal, 2016).

The frequency of TNBC in women of color with low survival could be accounted for by genetic risk and socioeconomic factors. Although (Stewart, et al., 2019) reported that African-American women with TNBC showed enhanced BRCA1 gene, BRCA1 is a lower pathogenic variant compared to the increased BRCA1 mutation incidences in European American women (Nanda, et al., 2005), (Qian, et al., 2017), (Stewart, et al., 2019). This is due to the abundance of sequence variations associated with the BRCA1 gene in African-American women which

inhibits BRCA1 function at a lower rate than in European women (Szabo & King, 1997). In addition, (Santonja, et al., 2018) reported that Hispanic women with breast cancer have the highest incidence of pathogenic BRCA1 mutations and this genetic factor may heavily contribute to the high mortality rates of women with TNBC. (Kwon, et al., 2010) found that more than half of the Jewish women in their study with TNBC carried a BRCA founder mutation. As a result, there was a prevalence of BRCA mutations among young and older Jewish women with TNBC. Furthermore, there was a significant proportion of Jewish women with TNBC and BRCA2 mutations, meaning that TNBC was not limited to only BRCA1(Comen, et al., 2011).

In addition, numerous socioeconomic factors that influence the access to standard care, novel treatments, limited healthcare, and inclusion in clinical trials contribute to the overall poor prognosis of African American and Hispanic women diagnosed with TNBC (Hossain, et al., 2019), (Lara-Medina, 2011), (Patel, et al., 2010), (Siddharth & Sharma, 2018), (Sturtz, Melley, Mamula, Shriver, & Ellsworth, 2014), (Elrafei, et al., 2014). Many women of color with breast cancer have subpar access to appropriate treatment; as a result, there is delayed diagnosis and a greater tumor burden. The delay in diagnosis could be because of little or no hospitals near the locations where these women reside, the inability to seek a leave of absence from work due to a lack of affordability, and no or little access to health insurance (Hossain, et al., 2019), (Parise & Caggiano, 2018). Latina women are more likely to be uninsured, have lower educational accomplishments, and have higher poverty rates than European American women (Chlebowski, et al., 2005). Language, cultural, and financial barriers also play a significant contribution to a higher mortality rate of TNBC in Hispanic Women (Mack, Pavao, Tabnak, Knutson, & Kimerling, 2009). Thus, the inaccessibility to health care leads to limited access to standard care and timely treatment and as result poor overall survival. Moreover, women of color with breast

cancer generally have a lower participation in clinical trials than their white counterparts, due to the poor communication and language barriers regarding these trials and their lack of confidence and trust in medical research. This could be because of the poor care in previous clinical experiments; for example, the treatment of African-American men in the infamous Tuskegee Syphilis Study and the study of genetic etiology of aggressive behavior in African-American boys (Alsan & Graziani, 2019), (Scharff, et al., 2010), (Shavers & Brown, 2002), (US Preventive Services Task Force, et al., 2019).

TNBC has a more aggressive clinical route with an early age of presentation compared to the other breast cancer subtypes. The mean age at diagnosis is significantly younger for the TNBC group at 53.0 years compared with other groups at 57.7 years (Dent, et al., 2007). Similarly, patients in the TNBC group had a 2-fold increase in grade III tumors compared to the other breast cancer subtypes, and the mean tumor size was larger in the triple-negative group than in any group (Dent, et al., 2007). (Collett, et al., 2005) evaluated cancers diagnosed in a screening program between 1996 and 2001 and found that TNBC was more likely than other breast cancers to present in the interval between regular mammograms. Also, (Brown, et al., 2008) reported that TNBC tumors were significantly larger, and they were more likely to present as stage II or III of the disease.

Most TNBCs are high grade invasive ductal carcinomas (IDCs) with characteristics such as apocrine differentiation, breast cancer with medullary features, and metaplastic breast carcinomas (MBCs) (Foulkes, et al., 2010). TNBCs with apocrine differentiation demonstrate the same growth pattern as IDC, differing only in their cytological appearance. The cells are characterized by abundant eosinophilic granular cytoplasm (Type A) and multiple nucleoli (Vranic, et al., 2010), (Yerushalmi, Hayes, & Gelmon, 2009). Thus, these apocrine cells can

proliferate into atypical lesions. TNBC with medullary features display clearly defined oval borders, a syncytial growth pattern, and lymphocytic infiltration with high mitotic activity (Kleer, 2009) (Lakhani, Ellis, Schnitt, Tan, & van de Vijver, 2012) (Pedersen, Schiødt, Holck, & & Zedeler, 1990). Lastly, MBCs encompass a range of tumors with squamous or mesenchymal differentiation (Weigelt, et al., 2008). These tumors are most often high grade, with easily visible abnormal nuclear size and shape with increased mitotic activity. TNBCs with MBCs are resistant to chemotherapy and have a worse outcome compared to the other subtypes (Jung, et al., 2015)

TNBC is known to have frequent recurrence after diagnosis and more aggressive visceral metastases which are more likely to occur in the lungs, brain, liver and less likely to spread to the bone (Criscitiello, Azim, Jr, Linn, & Sotiriou, 2012), (Smid, et al., 2008). A Canadian series reported by (Dent, et al., 2007) evaluated over 1,500 women diagnosed with TNBC which showed a higher proportion of local and distal recurrence and death compared to non-TNBC patients.

Chapter I.3 Triple Negative Breast Cancer Therapeutic Treatment Options

Patients with TNBC derive no advantages from molecularly targeted treatments, such as endocrine therapy and Herceptin, because they lack the proper targets for these drugs. The standard approach to treat TNBC has been to use cytotoxic therapeutics, but the chemotherapies lack the desired selectivity and chemoresistance is a significant problem, demonstrating 90% of drug failures in metastatic cancers (Longley & Johnston, 2005). Initially, TNBC patients respond well to neoadjuvant treatments. Unfortunately, there is a possibility of relapse in patients in the first 5 years in comparison with other breast cancer subtypes (Cinkaya, Akin, & Sengul, 2016). Nevertheless, neoadjuvant chemotherapy remains the standard care for TNBC patients (Rouzier, et al., 2005). Currently, neoadjuvant anthracycline–cyclophosphamide (AC-scheme)

chemotherapy treatment appears to be more efficient, although there have been reports on resistance developed for these drugs (Geisler, et al., 2001).

In addition, use of anthracyclines (A), taxanes (T), or platinum compounds are also utilized as adjuvant therapy which works to disrupt cancer cell survival (Liedtke, Mazouni, Hess, André, & Pusztai, 2008), (Petrelli, De Stefani, Raspagliesi, Lorusso, & Barni, 2014), (Silver, et al., 2010). (Jones, et al., 2009) published that four-cycle regimens of docetaxel and cyclophosphamide are more effective than four-cycle regimens of anthracycline and cyclophosphamide which resulted in longer overall survival of TNBC patients. As a result, the use of non-anthracycline taxane-containing regimens has become more common. Adjuvant chemotherapy reduces the risk of recurrence, particularly in patients with tumors greater than one centimeter. However, A+T treatment increases the risk of cardiac toxicity in older patients (Schreiber, et al., 2020).

Breast-conserving surgery has been studied as a therapeutic approach to treat TNBC. (Frasci, et al., 2009) evaluated tests to determine the prognostic effects of mastectomy over lumpectomy. In TNBC the sole choice of the surgical approach does not improve the prognosis or inhibit the local tumor recurrence (Freedman, Anderson, Li, & Nicolaou, 2009). A lumpectomy followed by radiation therapy (RT) could be an option. However, in TNBC the benchmark treatment is neoadjuvant therapy, and it is preferred before surgery.

Similar to breast surgery RT is part of the treatment regimen for TNBC, though there are drawbacks (Dawood, Broglio, Buzdar, Hortobagyi, & Giordano, 2010). TNBC is considered an aggressive subtype which is susceptible to radiotherapy and evidence points out that TNBC BRCA1 abnormal expression is highly radiosensitive (Abdulkarim et al., 2011). Although

radiotherapy after mastectomy can improve the results of TNBC, radioresistance is a major contributor to radiotherapy failure (He, et al., 2018), (Yin, et al., 2016).

Additionally, the use of small molecules to enhance specific delivery and action toward the direct molecular target has been analyzed as a potential treatment option. Aptamers are molecules made up of nucleotides, generally in a range of 50 deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) bases, that are evolved to bind to specific molecular targets. Their small size makes them suitable to reach molecular targets, therapeutic targets, protein complexes, and cancer cells (Kulbachinskiy, 2007). Aptamers are easy and cheap to produce but their degradation in the bloodstream is a clear disadvantage.

TNBC has the worst outcome for immune response treatment because of the higher expression of the metastasis promoting genes as well as the decreased expression of metastasis inhibiting genes. Consequently, within the TNBC tumor microenvironment, there are latent lymphocytes and antigen-presenting cells that do not respond correctly to tumor cells. Recently, strategies have been implemented to "push" these cells from their quiescent state and initiate a response that slows the progression of the tumor (Li, Qiu, Lu, Jiang, & Wang, 2018), (Ruffell, Affara, & Coussens, 2012). It is suggested that cytotoxic T-lymphocyte antigen-4 (CTLA-4) helps in the downregulation of the immune response (Linsley, et al., 1994). So, the current efforts are directed to activate immune system response by using CTLA-4 inhibitors such as ipilimumab. The challenge, however, is that CTLA-4 inhibitors aggressively activate T-cells, generating different systemic adverse effects (Maker, et al., 2005).

DNA damage of the genome occurs because of base changes, replication errors, free radicals, chemical agents, cancer therapeutics, and UV radiation (Friedberg, et al., 2006), (Ward, 1988). Double-stranded DNA breaks (DSBs) caused by these agents are detrimental to

eukaryotic cells so, two DNA repair mechanisms are used: nonhomologous end-joining and homologous recombination (HR). DSBs produced by collapse of the replication fork are primarily repaired by HR because the broken ends use homologous sequences from sister chromatids or regions on the chromosomes to prime repair synthesis (Chen, Yang, & Pavletich, 2008) (Rothstein, Michel, & Gangloff, 2000). Thus, HR is a DNA metabolic process that plays a vital role in preserving the genome and providing critical support for DNA replication and telomere maintenance (Degrassi, Fiore, & Palitti, 2004), (Shrivastav, De Haro, & Nickoloff, 2008).

Moreover, HR is dependent on functional BRCA1/2 pathways since BRCA maintains genome stability by regulating HR according to the type of DNA damage (Prakash, Zhang, Feng, & Jasin, 2015), (Sadeghi, et al., 2020). Germline mutations in either the BRCA1/2 genes are associated with a high risk of developing TNBC (Ahn, Kim, Kim, & Jeong, 2016), (Chen, et al., 2018), (Evans, et al., 2011), (Greenup, et al., 2013). When HR is dysfunctional, repair shifts toward an alternate DNA repair mechanism dependent on a class of enzymes called poly-adenosine diphosphate-ribose polymerase (PARP). PARPs are a family of enzymes involved in cellular processes such as genomic stability, DNA repair, cell cycle progression, and apoptosis (Schreiber, et al., 1995), (Skidmore, et al., 1979). PARP-1 functions as a DNA-binding protein, with nuclear localization which associates with DNA strand breaks as part of the base excision repair process (Helleday, Petermann, Lundin, Hodgson, & Sharma, 2008), (Plummer, et al., 2008).

As such, there were preclinical studies that justified the use of PARP inhibitors and highlighted PARP inhibitors as a promising targeted therapy for aggressive TNBC (Mahany, et al., 2008). The optimal treatment strategy is the combination of PARP inhibition with either

cytotoxic DNA-damaging chemotherapeutic agents or with molecular targeted agents that also impair mechanisms of DNA repair since monotherapy with PARP inhibitors is unlikely to induce cancer cell death in BRCA-proficient tumors (Alli, Sharma, Sunderesakumar, & Ford, 2009), (Hastak, Alli, & Ford, 2010), (Papadimitriou, Mountzios, & Papadimitriou, 2018).

However, there are several challenges associated with PARP inhibitor TNBC treatment. These challenges are establishing optimal therapy to couple with PARP, having a defined treatment when treating aberrant TNBC metastases, effective administration of cytotoxic therapies, defining the population most likely to respond to PARP inhibition in the TNBC community, and analyzing candidate biomarkers to predict how patients respond to treatment (Dent, et al., 2009), (Edwards, et al., 2008) (Gelmon, et al., 2010), (Rodríguez-Pinilla, et al., 2006). As a result, investigators must further define TNBC patients most likely to respond to PARP inhibitors since these drugs are transitioning to adjuvant settings and long-term toxicities to normal tissues have not yet been characterized for prolonged DNA repair suppression.

Trophoblast cell surface antigen 2 (TROP-2) is a glycoprotein which is overexpressed in human cancers and, has shown to be a promising therapeutic target (Lipinski, Parks, Rouse, & Herzenberg, 1981). TROP-2 overexpression in metastatic TNBC accelerates the cancer cell cycle and drives cancer growth. This antibody was first recognized as a marker of trophoblast cells and, it was developed looking for a marker for non-small-cell lung cancer (De Leij, Helrich, Stein, & Mattes, 1994), (Stein, Chen, Sharkey, & Goldenberg, 1990), (Stein, Basu, Chen, Shih, & Goldenberg, 1993). (Bardia, et al., 2021) reported that 88% of TNBC primary and metastatic tumors show moderate-to-strong expression of TROP-2. Recently, the FDA granted approval of Trodelvy (sacituzumab govitecan-hziy) a topoisomerase inhibitor conjugate directed to the TROP-2 receptor. This approval was based on the results of the ASCENT trial which was

conducted for the treatment of metastatic TNBC patients. This study showed that there was a significant difference in survival of TNBC patients treated with sacitizumab-14.2 months compared to chemotherapy treatment-6.9 months (Bardia, et al., 2021).

I.4 The Metastatic Cascade

The dissemination of TNBC cancer cells and metastatic growth to distal organs represents a significant health problem. The major secondary sites of metastatic TNBC are the lungs, liver, and brain (Chambers, Groom, & MacDonald, 2002), (Yao, et al., 2019). The metastatic disease is incurable and is the primary cause of death for the majority of TNBC patients. Metastatic spread of tumor cells consists of multiple steps, including local tumor cell invasion, entry into the vasculature, the exit of carcinoma cells from the circulation, formation of micrometastases followed by colonization at the distal sites. One of the earliest mentions of metastases postulated by (Paget, 1989) employing his "seed and soil" theory, proposed that secondary growth of cancer cells (the "seed") is dependent on the compatibility of the distal organ (the "soil"). Currently, this theory has been supported as distinct cancer types metastasize at different and tumor-specific sites (Mack, et al., 2004), (Nguyen, Bos, & Massagué, 2009).

In this process, cancer cells, after leaving the primary tumor, invade other tissues either by moving collectively as epithelial sheets, detached clusters, or as single cells (Friedl & Alexander, 2011).Upon genetic or epigenetic alterations, the TNBC cells at the primary tumor acquire anti-apoptotic properties such as self-renewal, invasion, and migration abilities to distal organs. Cancer stem cells are known for their self-renewal and chemoresistance capability, and it has been shown that TNBC is enriched in cancer stem cells (Honeth, et al., 2008), (Li, et al., 2013). (Ricardo, et al., 2011) analyzed numerous human breast cancers and evaluated that

TNBCs harbor the highest percentage of $CD44^{+}/CD24^{-}$ cancer stem cells, a feature that correlates with metastasis and overall poor survival. Additionally, specific growth factors and cytokines that activate several stemness pathways have been identified as essential drivers of TNBC proliferation and stemness (Marotta, et al., 2011).

During local invasion, the cancer cells undergo epithelial-to-mesenchymal transition (EMT). The crucial steps of tumor metastasis are thought to be enabled by EMT (Ballinger, Kremer, & Miller, 2016), (Griffiths & Olin, 2012). Cells that have undergone EMT lose their epithelial organization and can become more motile and less invasive to move as single cells in a mesenchymal fashion (Mani, et al., 2008), (Thiery, 2002). Transcription factors activate EMT to promote TNBC cell migration and intravasation in the vasculature (Tran, et al., 2014), (Yang, et al., 2004). EMT is also associated with cancer stem cell properties, and as mentioned, TNBC expresses high $CD44^{+}/CD24^{-}$ and EMT markers which is associated with poor survival outcome (Ma, et al., 2014), (Talukdar, et al., 2019), (Wicha, Liu, & Dontu, 2006).

Upon entering the blood vessels, circulating tumor cells express proteins that have pro-survival functions which allow them to attach and infiltrate specific secondary sites. In addition, the binding of platelets with circulating breast cancer cells are essential for their survival and evasion of pro-apoptotic signals (Neophytou, Boutsikos, & Papageorgis, 2018). (Wenzel, Zeisig, & Fichtner, 2010) evaluated that the disruption of platelet-tumor cell interaction inhibited the development of lung metastasis in TNBC murine model. Disseminated cells that survive pro-apoptotic signals in their new environment undergo a dormancy period (Giancotti, 2013).

After extravasation and the escape of the TNBC cells from the dormancy period, genetic modifications occur, and the cancer cells can form micro and macro-metastatic tumors at the

secondary sites (De Craene & Berx, 2013)In this step, the reversal to mesenchymal-to-epithelial transition (MET) phenotype have been shown to be important for metastatic colonization (Gunasinghe, Wells, Thompson, & Hugo, 2010). MET is characterized by epithelial reorganization and in this process, the mesenchymal cells regain the epithelial cell-to-cell junctions for colonization at distal sites (Kalluri & Weinberg, 2009), (Thiery, Acloque, Huang, & Nieto, 2009).

It is important to understand the insights into the aggressiveness of TNBC by verifying the interactions between TNBC, CSC and EMT phenotypes. Therefore, understanding these phenotypes may be promising for exploring the development of novel effective treatments against metastasis of TNBC.

I.5 Triple Negative Breast Cancer Liver Metastasis

Triple Negative Breast Cancer metastases are common in organs such as the liver, brain, and lungs (Liedtke, Mazouni, Hess, André, & Pusztai, 2008), (Hess, et al., 2006). BCLM are present in 15% of patients newly diagnosed with metastatic breast cancer and ultimately 50% of patients with stage four of the disease will develop liver metastases. When BCLM is left untreated, patient survival time is only 4 to 8 months with a very rare 5-year survival. Therefore, early prognosis and detection of liver metastasis is necessary to improve the life expectancy of TNBC patients (Adam, et al., 2006), (Hoe, Royle, & Taylor, 1991), (O'Reilly, Richards, & Rubens, 1990) (Selzner, Morse, Vredenburgh, Meyers, & Clavien, 2000). Patients with BCLM exhibit a wide array of symptoms, which may present asymptomatically during a metastatic screen or may present with upper abdominal fullness, a mass, ascites, jaundice, or weight loss (O'Reilly, Richards, & Rubens, 1990). Typically, an ultrasound or computed tomography (CT)

scan confirms the diagnosis. However, there have been two case studies in which asymptomatic presentation has been shown. Women of Asian and European American descent both conferred with TNBC via an abdominal CT scan, presented with liver metastases (Chue & La Course, 2019), (Ogata, et al., 2015).

The molecular mechanisms underlying breast cancer metastasis have been especially reported for the lungs and bone (Chiu, et al., 2013), (Gao, et al., 2012), (Jiang, et al., 2012), (Minn, et al., 2005), (Suva, Griffin, & Makhoul, 2009), (Zhang, et al., 2013); however, the molecular mechanisms for BCLM have not been as thoroughly described (Lu, et al., 2007). (Parks & Garden, 2001) reported that the biological structure of the liver also makes it highly advantageous. The beneficial features are the abundant blood supply, the fenestrated blood vessels, and the lack of a sub-endothelial basement membrane. This allows for the movement and progression of the breast cancer cells within the liver microenvironment (Ma, et al., 2015).

BCLM is associated with a few characteristics that result in the successful colonization of the secondary organ. These qualities include stem cell properties and proliferation signaling (Hess, et al., 2006), (Homayounfar, et al., 2013). Also, the breast carcinoma cells that secrete cytokines, chemokine receptors, cadherins promote cell adhesion to the endothelium, and integrin complexes which promote breast cancer cells to metastasize to the liver (Ma, et al., 2015), (Wendel, et al., 2012).

I.6 Triple Negative Breast Cancer Liver Metastasis Therapies

A further understanding of the roles of TNBC cells and the liver microenvironment in early breast cancer metastasis is crucial for the development of effective BCLM therapies (Paget,

1989), (Price, Polyzos, Zhang, & Daniels, 1990). Current treatments for TNBC are systemic chemotherapy and radiation as the first-line treatment for cancer that has metastasized (Davis, Eckhardt, Tentler, & Diamond, 2014), (Cardoso, et al., 2018), (Hortobagyi, et al., 2005), (Schmid, et al., 2018), (Senkus, et al., 2015). However, even with these treatments patients ultimately become resistant to palliative therapy and have a poor response to chemotherapy which results in increased mortality in BCLM patients (Yao, et al., 2017).

Raised serum alkaline phosphatase (ALP) has been established in patients with metastatic colorectal cancer but has not yet been evaluated as a diagnostic tool in BCLM. However, there have been small studies suggesting that elevated ALP, though not specific to liver metastasis is still useful in aiding the detection of the liver metastatic disease (Kamby, et al., 1987), (Klompje, Petrelli, Herrera, & Mittelman, 1987), (Warnes, Hine, Kay, & Smith, 1981). (Saif, Alexander, & Wicox, 2005) reported that patients with increased ALP levels were 5.5 times more likely to develop liver metastasis that patients with normal ALP levels. Thus, monitoring ALP levels at each patient's follow-up can be used as an indicator since having liver metastasis is a good guide of disease progression. ALP is a simple, cost-conscious, and sensitive screening tool for detecting liver metastasis and can be developed in future studies of TNBC liver metastasis as a diagnostic tool.

In several countries, such as Japan and Europe, other approved treatment options such as combined bevacizumab/paclitaxel/carboplatin (BCP) have been used for BCLM. BCP therapy has been used in the treatment of non-small cell lung cancers and significant improvements in survival rates have been reported (Sandler, et al., 2006). Additionally, (Ogata, et al., 2015) showed the successful treatment of BCP therapy in metastatic TNBC. Most importantly, the

patient has been in complete remission without additional treatment and has remained disease-free after 5 years. Thus, this treatment has shown future promise for metastatic TNBC.

Alternatively, (Chue & La Course, 2019) demonstrated the use of metronomic chemotherapy as a viable TNBC liver metastasis treatment option. Metronomic chemotherapy uses lower doses of chemotherapy agents with higher dose intensity (the total dose of chemotherapy administered per unit time) while switching chemotherapy regimens every few weeks. This prevents the development of disease resistance and a better chance of achieving long-term survival (Greaves & Maley, 2012). However, drug resistance is still seen as the primary cause of failure of chemotherapy treatment for metastatic cancer and continuing a chemotherapy regimen until disease progression will inevitably increase the likelihood of a chemotherapy-resistant disease.

More accumulated evidence suggested that the metastatic TNBC subtype can be particularly responsive to immunotherapy (García-Teijido, Cabal, Fernández, & Pérez, 2016). (Schmid, et al., 2018) study showed that treatment with atezolizumab–nab-paclitaxel benefitted patients with the programmed cell death-1 (PD-1) signal pathway which has a ligand called PD-L1 that leads to apoptosis of malignant cells for TNBC (González-Cao, et al., 2015), (Pardoll, 2012), (Wimberly, et al., 2015). The presence of PD-L1 may be a result of genetic events leading to constitutive PD-L1 expression on cancer cells versus non-cancer cells in response to a T cell infiltration. Thus, it is important to determine the PD-L1 expression status on tumor-infiltrating immune cells since it can be used as a treatment choice for patients with metastatic TNBC. Currently, Ipilimumab, pembrolizumab (anti-PD-1), and atezolizumab have been approved by the FDA in patients with overexpression of PD-L1 which helps in the therapeutics of TNBC patients with metastatic cancer (Narayan, et al., 2020). Furthermore, two new PD-L1 inhibitors

mepolizumab and nivolumab have been tested in current clinical trials. Unfortunately, 76% of TNBC patients having PD-L1 expression have not shown therapeutic response under mepolizumab therapy (Gibson, Delaune, Szady, & Markham, 2016).

TNBC metastatic cancers can escape an antitumor immune response through the upregulation of regulatory T cells, secretion of immunosuppressive cytokines into the tumor microenvironment, as well as the expression of immunosuppressive proteins (Bohling & Allison, 2008), (Drake, Jaffee, & Pardoll, 2006), (García-Teijido, Cabal, Fernández, & Pérez, 2016), (Smyth, Godfrey, & Trapani, 2001). Unfortunately, this situation is further exacerbated by the immunosuppressive effect of standard-dose chemotherapy (Kang, et al., 2009). Thus, the recruitment of the regulatory molecules to the tumor site may disarm the T-cell effectors and therefore favor the immunosuppressive activity of the tumor. As a result, this outweighs the body's antitumor immune response and consequently promotes tumor progression (Rabinovich, Gabrilovich, & Sotomayor, 2007).

Moreover, a less arduous and more favorable treatment for TNBM liver metastasis is percutaneous ablation where doctors can utilize heat or cold to burn or freeze the tumor through a puncture thereby killing it. Using this method, high doses of ultrasound therapy can be applied to kill the tumor. Medical doctors also reported the establishment of a regional therapy technique when the tumors are multiple or diffuse. This therapy is chemoembolization, which means inserting a catheter that goes directly into the liver’s arteries to deliver very high levels of specialized toxic drugs precisely to the tumor (Yale Medicine, 2021)

Methionine dependence is linked to epigenetic changes in cancer controlled by methylation events and is demonstrated as a promising target of metastatic TNBC (Hoffman,

2015). Cancer cells require increased levels of methionine (MET- dependence) due to the higher overall rates of transmethylation in comparison to normal cells (Coalson, Mecham, Stern, & Hoffman, 1982). (Kaiser, 2020) described the necessity of the cancer cells to have high methionine, is known as the Hoffman effect, analogous to the Warburg effect which describes the glucose overuse of cancer cells. In current research studies, recombinant methionase (o-rMETase) inhibited the growth of the metastasis of cancer cells *in vitro* and *in vivo* (Hoffman, 2015), (Lim, et al., 2020). These results suggest that o-rMETase has the potential as a new effective modality for metastatic TNBC, especially since it can be administered orally without toxicity (Han, Tan, & Hoffman, 2020), (Kawaguchi, et al., 2019).

I.7 Angiopoietin-like protein 4 background and structure

The angiopoietin-like protein 4 (ANGPTL4) was discovered approximately two decades ago and belongs to the angiopoietins family of secreted proteins which plays a role in the formation blood vessels. The eight members of angiopoietins are identified in humans and mice, except ANGPTL5, which is only found in humans (Ito, et al., 2003), (Kim, et al., 2000), (Santulli, 2014). All angiopoietin-like proteins have a C-terminal fibrinogen-like domain and an N-terminal coiled-coil domain.

ANGPTL1 to 4 and ANGPTL6 have been shown to regulate angiogenesis. ANGPTL3 to 6 and 8 are involved in the regulation of lipid metabolism, glucose and energy homeostasis (Koliwad, Gray, & Wang, 2012), (Oike, et al., 2005), (Xu, et al., 2005). (Lee, et al., 2009) evaluated that ANGPTLs 3 and 4 control lipid metabolism by inhibiting the activity of lipoprotein lipase, an enzyme that is responsible for the hydrolysis of triglycerides. ANGPTL4 is primarily present in the liver, adipose tissue, and skeletal muscle (Dijk, et al., 2015), (Kersten, et al., 2009), (Oike, et al., 2005), (Tan, Teo, Sng, Zhu, & Tan, 2012).

ANGPTL4 is evolutionarily conserved among species and the sequence is closely homologous with mice. It is located on chromosome 19p13.3 and comprised of seven exons with a 406-amino acid glycoprotein. ANGPTL4 contains a hydrophobic region that acts as a signal peptide for protein secretion and exhibits several N- and O-glycosylation sites (Grootaert, Van de Wiele, Verstraete, Bracke, & Vanhoecke, 2012). Different studies showed that the nANGPTL4 domain is used to modulate lipid metabolism, whereas the cANGPTL4 domain may be a modulator of the tumorigenesis process (Tan, Teo, Sng, Zhu, & Tan, 2012). ANGPTL4 functions as a LPL inhibitor due to an protein synthesis process mediated by the N-terminal region responsible for its assembly into 3D protein structures. Experimental analyses showed that proprotein convertases like furin, proprotein convertase 5/6, and proprotein convertase subtilisin/Kexin type 3 catalyze the proteolytic processing of the human flANGPTL4 protein via a particular amino acid sequence, which causes the release of the N-terminal region and a C-terminal portion (Lei, et al., 2011), (Yau, et al., 2009). As a result, the shortened form of ANGPTl4 is secreted from the liver, whereas the full-length form is released from adipose tissue. Since ANGPTL4 expression was found mainly in the liver and adipose tissue, it was classified as an adipokine and as such its involvement in lipid metabolism is well characterized. Subsequently, many studies showed ANGPTL4 plays a highly versatile role in angiogenesis, vascular permeability, tumorigenesis, lipid metabolism and inflammation.

I.8 The Role of ANGPTL4 in TNBC

I.8.1 ANGPTL4 and regulatory factors in human cancers

Previous studies have demonstrated that ANGPTL4 is involved in cancer growth, progression, angiogenesis, metabolism, and metastasis (Galaup, et al., 2006), (Kim, et al., 2011),

(Padua, et al., 2008), (Tan, Teo, Sng, Zhu, & Tan, 2012), (Zhu, et al., 2011). In several cancer types, the diverse roles of ANGPTL4 remain controversial and have reported involvement with many different signaling molecules and pathways. Several studies show that high levels of ANGPTL4 are associated with poor prognosis in patients with various solid tumors. This suggests that ANGPTL4 gives the cells the ability to acquire sustained proliferation, evading growth suppressors, resist apoptosis, replicative immortality, angiogenesis, invasion, and metastasis (Hanahan & Weinberg, 2011).

Several studies have reported that the transcription factor hypoxia-inducible factor-1α (HIF-1α) stimulated by hypoxia can induce the upregulation of ANGPTL4 which stimulates angiogenesis (La Paglia, et al., 2017), (Kim, et al., 2011), (Li, et al., 2007), (Zhu, et al., 2011). Additionally, a previous study demonstrated that ANGPTL4 upregulation in endothelial cells plays a key role in the tumorigenesis of Kaposi's sarcoma (Hu, et al., 2016). Furthermore, evidence suggests that ANGPTL4 acts via the transforming growth factor β (TGF-β) signaling pathway to promote tumor invasion and metastasis (Mathieu, et al., 2014). Moreover, ANGPTL4 is the potential target gene for the three isoforms of peroxisome proliferator-activated receptor (PPAR) and PPARs have been implicated in the development of tumors in breast cancer cell lines (Girroir, et al., 2008), (Kim, et al., 2011). For other human cancers, studies reported that ANGPTL4 promotes invasion and metastasis as well (Dong, et al., 2017), (Hu, et al., 2016), (Nakayama, et al., 2011).

These findings implicate ANGPTL4 in cancer progression, invasion, angiogenesis, and metastasis. More importantly, the research suggests that the expression and the role of ANGPTL4 in tumors are dependent on context and tumor type. This suggests the clinical

importance of ANGPTL4 in tumor biology and the research necessary to evaluate an effective targeted therapy against it.

1.8.2 ANGPTL4 Primes Tumor Progression

Increased ANGPTL4 expression has been shown to contribute enhanced tumor progression, metastasis, and poor overall survival. Conversely, studies have reported that the inhibition of ANGPTL4 reduces metastasis and tumorigenesis (Dao, et al., 2020), (Gong, et al., 2019), (Kim, et al., 2011), (Padua, et al., 2008), (Zhao, et al., 2020), (Zhu, et al., 2011), (Wang, et al., 2013). As such it important to understand the role ANGPTL4 plays as a driver in tumor progression.

In the (Gong, et al., 2019) study, the researchers evaluated the role of ANGPTL4 as promoter for TNBC brain metastasis in vivo and to further understand the interaction of the cancer cells with the astrocytes comprising of the human blood brain barrier in vitro. (Wang, et al., 2013) published that TNBC cells passaged in astrocyte cell medium enhanced brain trophism in a murine model. These observations suggested that the medium derived paracrine signaling of growth factors to promote seeding and metastasis of the brain. Thus, (Gong, et al., 2019) evaluated the role of ANGPTL4 expression in TNBC cells in astrocyte medium and found that it was induced by TGF-B and ANGPTL4 was significantly elevated suggesting their roles in tumor progression and metastasis. In the murine model, ANGPTL4 shRNA was expressed in the MDA-MB-231 cell line and reduced the ability of the cancer cells to progress, seed and colonize the brain. As such (Gong, et al., 2019) reported ANGPTL4's contribution to the metastatic lesions of MDA-MB-231 cells in the brain.

In another study, (Padua, et al., 2008) evaluated ANGPTL4's function in TNBC colonization of the lung. The lung is one of the more common sites that TNBC cells can seed and metastasize. To investigate whether ANGPTL4 is involved in the metastatic colonization of the lungs, this research group inhibited the expression of ANGPTL4 in a TNBC cell line and orthotopically injected the TNBC cells in a murine model. They reported that the seeding to the lungs decreased more than 10-fold, suggesting that ANGPTL4 is involved in cancer cell's ability to survive in circulation and seed to the lungs. In addition, they showed that the overexpression of ANGPTL4 in TNBC cells increased lung colonization by these cells. These results provide evidence that ANGPTL4 expression is necessary for the ability of TGFβ to prime the breast cancer cells for invasion of the lungs.

Furthermore, (Padua, et al., 2008) investigated whether ANGPTL4 can affect the extravasation of the breast cancer cells through the endothelial tight junctions of the lung parenchyma. A lung permeability assay was performed in vivo with rhodamine signal. The TNBC cells expressing ANGPTL4 showed a 3-fold increase in the rhodamine signal, showing that ANGPTL4 can disrupt the integrity of the endothelial cell tight junctions and aid in colonization of the lungs. To further understand the role of ANGPTL4 in the TNBC migration through the cell-to-cell endothelial junctions an assay using tissue culture inserts. It was shown that TNBC cells overexpressing ANGPTL4 passed twice as much through the endothelial layers compared to control cells. This demonstrates that Angptl4 disrupts the integrity of vascular endothelial cell layers both in vitro and in the lungs, facilitating the passage of breast cancer cells (Bos, et al., 2009). Jointly, (Padua, et al., 2008) showed that of ANGPTL4 enhances TNBC cells migration, tumor progression, and extravasation to the lungs.

(Dao, et al., 2020) investigated the role of ANGPTL4 fragments in brain metastasis using a murine model. This group found that ANGPTL4 concentration in the serum is directly proportional to the increased risk of brain metastases and shorter survival in patients with breast cancer. Multivariate analyses performed by this research group showed that metastases were significantly associated with elevated ANGPTL4 concentration in the serum. In addition, they evaluated the proliferative, invasive, and migratory abilities of the breast cancer cells expressing nANGPTL4 and cANGPTL4 fragments. It was reported that tumor cells expressing the different fragments of ANGPTL4 had increased proliferation, migration and invasion compared to the wild type cells. For in vivo experiments, mice injected with cells expressing the fragment of nANGPTL4 had a shorter median survival of 19 days compared median time of 23 days for mice injected with wild-type cells. In addition, the mice injected with ANGPTL4 expressing cancer cells had significantly larger lung and brain metastasis compared to the mice injected with wildtype cells.

(Zhao, et al., 2020) reported the association between ANGPTL4 expression and the different breast cancer tissues. They evaluated the expression levels of ANGPTL4 in different breast cancer tissue and shown that 63% of ANGPTL4 is expressed in IDCs, the highest percentage of ANGPTL4 expressed among all the breast cancer tissues. This means that as the malignancy of the tissues increased the rate of ANGPTL4 expression increased. Consequently, ANGPTL4 expression was associated with the worst prognosis in patients with IDC.

Collectively, the *in vivo* and *in vitro* models in these studies of breast cancer have demonstrated the ability of ANGPTL4 to enhance tumor proliferation, migration, disruption of the endothelium cell-to-cell junction, invasion, and metastasis.

1.8.3 ANGPTL4's Role in Anoikis Resistance

Tumor cells abnormal growth suggests that anoikis should follow due to the stresses the cells are experiencing and, as a result cell growth progression should halt. However, proliferation, cell migration and metastasis continue to occur. This suggests that the cells are no longer reliant on the extracellular matrix for attachment and that the tumor cells have undergone anoikis resistance. Anoikis resistance is defined as the cells ability to evade cell death when there is loss of attachment to the extracellular matrix.

(Zhu, et al., 2011) reported that enhanced ANGPTL4 expression promotes anoikis resistance to tumors via autocrine adhesion mimicry. It is noted that cANGPTL4 was detected and elevated in many human tumor cells (Hanahan & Weinberg, 2011).

In their study, suppression of ANGPTL4 in the cancer cells formed 85% less colonies on a soft agar colony formation assay compared to the control cell line. In addition, in the anoikis assay the ANGPTL4 shRNA cell line was more susceptible to anoikis and resulted in 30% more apoptotic cells than the control cell line.

The anoikis resistance effect of ANGPTL4 is mediated by reactive oxygen species (ROS). ANGPTL4 activates ROS which leads to an anti-apoptotic and anchorage-independent signals resulting in the tumor cells not being reliant on the extracellular matrix for normal growth. cANGPTL4 specifically binds to integrins βeta-1 and βeta-5 on tumor cells and activates pro-survival pathways, which further stimulates NADPH oxidase 1 activation via autocrine signaling (Zhu, et al., 2011). However, paracrine signaling can occur if the tissue expressing elevated cANGPTL4 is near the tumor (Desgrosellier & Cheresh, 2010). Downstream, this further triggers the inhibition of apoptotic proteins resulting in resistance to anoikis and favoring tumor survival and growth.

In a separate study, (Liao, et al., 2017) evaluate EGF-induced ANGPTL4 in tumour cells and anoikis resistance capability. The study showed that the secretion of EGF-induced ANGPTL4 was inhibited in ANGPTL4 shRNA cells which resulted in the reduction of EGF-induced anoikis resistance. Conversely, the reduction of anoikis resistance could be reversed by treating the ANGPTL4 shRNA cells with recombinant cANGPTL4. Consequently, they evaluated that EGF-induced ANGPTL4 by autocrine signaling significantly enhanced anoikis resistance. Moreover, (Terada & Nwariaku, 2011) demonstrated that tumour derived ANGPTL4 increases the production of ROS, resulting in activation of survival pathways to enhance anoikis resistance.

1.8.4 ANGPTL4's Role in Hypoxia and Angiogenesis

Hypoxia induced by the overconsumption of oxygen by the rapidly growing tumor cells results in an oxygen deficit. This hypoxic environment triggers the onset of angiogenesis, which forms new blood vessels to supply oxygen, nutrients and remove metabolic waste products supporting tumor survival (Bertout, Patel, & Simon, 2008). It has been examined that angiogenesis is a multistep process that is regulated by vascular endothelial growth factors and ANGPTL4 (Le Jan, et al., 2003). Studies highlighted a prominent role for ANGPTL4 in tumor angiogenesis and vascular permeability. This increase in ANGPTL4 expression enhanced endothelial cell migration and differentiation, both of which are important processes in angiogenesis, resulting in increased neovascularization (Padua, et al., 2008).

(Zhang, et al., 2012) identified that ANGPTL4 is a hypoxia-inducible factor 1 (HIF-1) target that contributes to vascular metastasis. HIF-1 is characterized as a transcription factor that occurs because of reduced oxygen availability and regulation of the genes that play roles in many factors of molecular biology like angiogenesis (Semenza, 2010).

(Le Jan, et al., 2003) indicated both the mRNA and protein ANGPTL4 levels become elevated because of hypoxia and, as a result exerts a proangiogenic effect. An elevated expression of ANGPTL4 was observed in breast cancer with the expression of cANGPTL4 fragment highly correlated to the expression of HIF-1α (Zhang, et al., 2012). These observations indicate that ANGPTL4 could be a key modulator in tumor angiogenesis in a hypoxic tumor microenvironment.

HIF-1 targets include numerous genes that play essential roles in promoting angiogenesis to increase oxygen delivery. Consequently, tumor cells metabolically shift from oxidative phosphorylation to glycolysis and lactic acid production to decrease oxygen demand. Thus, protecting cells from acidosis and influencing adaptive survival mechanisms (Semenza G. L., 2012). Hypoxia is one of the most common and important factors identified in the regulation of cancer progression and metastasis (Kaelin, 2005), (Semenza G. L., 2012).

I.9 TNBC and Tat-interactive protein p60

Tat-interactive protein p60 (Tip60) is a member of the histone acetyltransferases and is directly involved in genome maintenance, gene regulation, and DNA damage repair pathways. Tip60 is the acetyltransferase component of a multiprotein complex that includes 16 subunits and contains an ATPase, p400 which can sensitize cells to death, and helicases-Tip49a and Tip49b (Fuchs, et al., 2001), (Samuelson, et al., 2005). The Tip60 complex preferentially acetylates histone binding acetylation targets H2AK5, H3K14, and H4K5/8/12/16 (Kimura & Horikoshi, 1998). As a result of DNA damage, Tip60 is induced and then acetylates kinase activity at the sites of the damage (Legube, et al., 2002), (Sun, Jiang, & Price, 2010). Moreover, (Halkidou, et

al., 2003) reported that Tip60 predominantly accumulates in the nucleus of the tumor cell than a more diffused pattern which was observed in a benign state of prostate cancer.

Tip60 roles are not only limited to DNA repair but also in apoptosis. Researchers have evaluated the relationship between Tip60 and cancer, which showed that Tip60 functions as a tumor suppressor (Gorrini, et al., 2007), (Squatrito, Gorrini, & Amati, 2006). In human cancers, Tip60 mRNA or protein level was downregulated and associated with poor prognosis in these patients (Chevillard-Briet, et al., 2014), (Gorrini, et al., 2007), (Mattera, et al., 2009), (Zhao, Jin, & Gewirtz, 2012). It is also reported that in TNBC there are low expressions of TIP60 protein and RNA compared with luminal subtypes. (Idrissou, et al., 2020) reported successful inhibition of TIP60 activity in a TNBC cell line with chemical inhibitors. Studies on histone modifications in the appearance and progression mechanisms in TNBC would be essential in understanding the performances of TIP60 tumor biology (Stacy, et al., 2019).

Although the effect of histone alterations has not been fully understood, histone modification inhibitors such as histone deacetylase inhibitors have emerged as a promising class of multifunctional anticancer agents to potentially prevent TNBC growth and metastasis (Fedele, Orlando, & Cinieri, 2017). These inhibitors could also help to better understand the effect of histone modifications on the outcome and the metastasis of TNBC through gene expression regulation to define new biomarkers and targeted therapies (Fedele, Orlando, & Cinieri, 2017), (Tate, et al., 2012). Tip60 was chosen for this study because it is suggested to be an interactor or in the pathway with ANGPTL4 as such it would be interesting to identify and understand its role in TNBC overexpressing ANGPTL4 cells. Studying this protein could potentially be used to further understand if ANGPTL4 and TIP60 are working together to affect the tumorigenic ability of the TNBC cells.

I.10 TNBC and Aurora Kinase A

Abnormalities in genes controlling the cell cycle causes atypical cell proliferation, apoptosis, and transformation of malignant cells (Golias, Charalabopoulos, & Charalabopoulos, 2004), (Williams & Stoeber, 2012). Aurora kinases (AURKA) are a family of mitotic serine/threonine kinases involved in different processes of cell cycle regulation and are associated with several co-activators that drive localization and activation during cell division (Barr & Gergely, 2007), (Fu, Bian, Jiang, & Zhang, 2007), (Vader & Lens, 2008). AURKA is overexpressed in many types of human cancers, notably in the different subtypes of breast cancers (Dar, Belkhiri, & El-Rifai, 2009), (Sen, et al., 2002), (Tanaka, et al., 1999). This overexpression correlates with tumorigenesis and metastasis confirming the survival function of AURKA in cancer cells. AURKA overexpression may occur by gene amplification, transcription activation, or suppression of protein degradation. Multiple studies evaluated different cancers and, showed that AURKA transcription may be induced by HIF-1α (Klein, Flügel, & Kietzmann, 2008), (Lehman, et al., 2012).

However, the molecular and transcriptional regulation mechanisms of AURKA remain unknown, especially in TNBC. (Fanale, et al., 2013) reported that hypoxia directly links HIF-1 with AURKA expression in hormone-positive and HER2 overexpressing breast cancer cell lines and to a lesser extent TNBC. This highlights a possible pathophysiological role of this pathway in tumors and confirms that HIF-1 has an important role in signaling to the AURKA promoter. Since AURKA is a key regulator of the chromosomal segregation process in mammalian cells these results could provide innovative approaches for the development of possible therapeutic strategies against TNBC (Lee, et al., 2019). AURKA was chosen for this study because it is suggested to be an interactor with TIP60 or in the pathway with ANGPTL4. Therefore, it would

be noteworthy to identify its role in TNBC overexpressing ANGPTL4 cells. Studying this protein could potentially be used to further understand if its interaction with TIP60 and ANGPTL4 would be a driver for the tumor progression of the TNBC cells.

1.11 Controversial Role of ANGPTL4 in Cancer

ANGPTL4's function remains debatable because of its multifaceted roles and, therefore further clarification is needed in tumorigenesis. However, several research groups have shown promising results suggesting that ANGPTL4 could be a potential therapeutic target in the battle against TNBC (Huang, et al., 2011), (Kim, et al., 2011), (Padua, et al., 2008), (Zhu, et al., 2011). It was reported that the immunosuppression of cANGPTL4 by neutralizing antibodies results in tumor regression, reduced vascular disruption, and metastasis (Huang, et al., 2011), (Zhu, et al., 2011). Moreover, the inhibition of cANGPTL4 could potentially aid in chemosensitivity to cancer cells (Kim, et al., 2011). Given the implications that ANGPTL4 may have on cancer progression, a report suggested that ANGPTL4 mRNA could be used as a diagnostic marker to distinguish primary and metastatic cells in renal carcinoma (Verine, et al., 2010). A few other studies have also described ANGPTL4 expression level in primary and invasive tumors, that showed high ANGPTL4 mRNA and protein expression in human tumor biopsies often correlated with a poor prognosis and disease outcome (Hu, et al., 2016), (Kim, et al., 2011). (Zhu, et al., 2011) and colleagues have shown that increased cANGPTL4 protein levels could be detected in the serum of tumor-bearing mice. cANGPTL4 was also found to be secreted by hypoxic cancer cells, lending further confidence to the potential use of ANGPTL4 as a biomarker (Kim, et al., 2011).

Conversely, multiple studies reported that enhanced ANGPTL4 expression has inhibited tumor progression, invasion, and metastasis of tumor cells (Cazes, et al., 2006), (Galaup, et al., 2006), (Ito, et al., 2003), (Le Jan, et al., 2003). Consequently, ANGPTL4 has been reported in promoting cancer cell's tumorigenesis while also inhibiting tumor progression in different cancers (Ito, et al., 2003), (Le Jan, et al., 2003), (Ma, et al., 2015). Thus, ANGPTL4's role in cancer progression needs to be further understood and more importantly its role in TNBC.

1.12 Research Aims

ANGPTL4 was shown to drive the invasion and metastatic capability of TNBC across the lung and brain parenchyma, this led to the **central hypothesis** that ANGPTL4 primes TNBC cells to disseminate and metastasize to the liver. ANGPTL4 is known as a secreted protein in the plasma membrane and serves a multifaceted role in cancer progression. Thus, the first aim of this study is to evaluate the role of intracellular ANGPTL4 in TNBC cells. Also, to investigate the localization and interaction of TIP60 and AURKA with ANGPTL4 experimentally. Since, TIP60 and AURKA are predicted to be interactors in the ANGPTL4 pathway. The second aim is to assess the tumorigenic ability of exogenous ANGPTL4 in TNBC cells using a murine model.

Research aim 1: The first aim was to assess the mechanism of intracellular ANGPTL4 in TNBC cells and examine the localization and compartmentalization of TIP60 and AURKA in the ANGPTL4 pathway *in vitro*.

Research aim 2: The second aim was to evaluate the role of tumor-derived ANGPTL4 overexpression in TNBC cells as a driver of metastases *in vivo*.

II. CHARACTERIZATION OF ANGPTL4 OVEREXPRESSION IN TNBC *IN VITRO*

II.1 Introduction

In this study the role of intracellular ANGPTL4 in TNBC cells and its ability to drive proliferation was evaluated. ANGPTL4 has been established as an enhancer of tumor progression in TNBC and a driver of lung, liver, and brain metastasis. Studies have reported that intracellular ANGPTL4 primes TNBC to extravasate and colonize the lung and brain parenchyma via TGF-B pathway (Gong, et al., 2019), (Padua, et al., 2008). In addition, ANGPTL4 promotes TNBC cells to escape anoikis. The breast cancer cells can grow in the absence of EMT attachment and with signaling from pro-survival proteins they gain anchorage independence where the cells continue to grow, migrate, and survive in the vasculature (Zhu, et al., 2011). In addition, TNBC is known to be enriched for stem cells, the CD44 high and CD24 low phenotype, this means that the cancer stem cells have self-renewal, aggressive and increased metastatic ability (Idowu, et al., 2012), (Ricardo, et al., 2011). A study also reported that ANGPTL4 suppression inhibited the proliferation and migration of cancer cells in vitro, therefore solidifying its important in cancer progression (Huang, et al., 2011). Collectively, these studies indicate ANGPTL4 as a driver of metastatsis by enhancing tumor growth, cell motility and invasion (Tan, Teo, Sng, Zhu, & Tan, 2012).

In this study, a lentiviral transduction was performed to overexpress ANGPTL4 in TNBC cell line and the overexpression was confirmed using confocal microscopy, ELISA and immunoblot analysis. The Human Protein Atlas predicted that ANGPTL4 is in the nucleoplasm and possibly in the vesicles, however the nuclear and chromatin localization of ANGPTL4 in TNBC has not been shown. In addition, TIP60 and AURKA are suggested to be interactors in the

ANGPTL4 pathway, as such ANGPTL4, TIP60 and AURKA localization were evaluated using immunofluorescence microscopy and western blot analysis.

TNBC has the highest percentage of cancer stem cell population and is predicted to be more invasive and metastatic that the other breast cancer subtypes. To evaluate the ability of TNBC ANGPTL4 cells to form mammospheres and their ability to have an enriched stem cell population. A mammosphere assay was done with immunoblot analysis to confirm the stem cell phenotype by using CD44 and CD24 markers.

In this study, Oil Red O staining was performed to evaluate the lipogenic profile of the TNBC cell line that overexpressing ANGPTL4 mammospheres. Furthermore, soft agar colony formation assay was done to assess anchorage independence in addition to cell colony growth and size in the TNBC ANGPTL4 cell line.

II.2 MATERIALS AND METHODS

II.2.1 Cell Culture

MD Anderson metastatic breast cancer 231 cells (MDA-MB-231), a TNBC cell line was cultured in Delbucco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12 HAM) containing 5% fetal bovine serum (FBS), and 10 μg/ml gentamicin (complete medium) under 5% CO_2 at 37°C. The MDA-MB-231 cells that overexpress mGFP tagged ANGPTL4 and mGFP only were cultured in Delbucco's Modified Eagle Medium Nutrient Mixture F-12 (HAM) containing 5% FBS, 10 μg/ml gentamicin and 2 μg/ml puromycin at 37 °C under 5% CO_2.

II.2.2 Lentiviral mediated ANGPTL4 overexpression

The MDA-MB-231 cell line was purchased from Sigma-Aldrich and, the lentiviral particles for the transduction was obtained from Origene. $1x10^5$ MDA-MB-231 were seeded in a 24 well plate and incubated for 24 hours. Then, the cells were transduced with the respective plasmids for 16 hours with 8 ug/ml of polybrene. Then, the virus-containing medium was removed and replaced with fresh complete medium. Selection with puromycin at 2 μg/ml was initiated 24 hours after transduction and continued for two weeks. Single cells were propagated for three weeks. Colonies with robust GFP expression was determined by fluorescence microscopy and fluorescence activated cell sorting (FACS) were used in all subsequent experiments.

II.2.3 Subcellular Protein Extraction

Using MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP cell lines, they were lysed, and cell fractions were isolated. This was done using a Subcellular Protein Fractionation kit (Thermo Fisher Scientific) for cultured cells according to the manufacturer's guidelines.

II.2.4 Western Blotting

ANGPTL4 overexpression in the MDA-MB-231 cell line was confirmed with SDS-Page and immunoblot analysis with an ANGPTL4 antibody(). MDA-MB-231 mGFP and ANGPTL4 mGFP cells were rinsed twice with ice-cold PBS and attached cells were lysed in ice-cold Pierce Immunoprecipitation buffer containing Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) according to the manufacturer's instructions. The lysates were transferred to pre-cooled eppendorf tubes and spun at 13,000xg for 10 minutes at 4 °C. The supernatants were quantified for protein concentration and 25ug of protein lysates were resolved by gel electrophoresis and subjected to immunoblot analysis with antibodies specific for the indicated protein.

II.2.5 Enzyme Linked Immunosorbent Assay

MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP cells were seeded in 100mm x 20mm plates for 24 hours. Then, the cells were rinsed three times with Phosphate Buffered Saline (PBS) and grown in phenol red-free FBS in DMEM/F12 medium containing 0.02% of gentamicin for 48 hours. The amount of secreted ANGPLT4 in cell culture medium of the respective cell lines were determined using a sandwich human ANGPTL4 ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

II.2.6 Confocal Immunofluorescence Microscopy

MDA-MB-231 mGFP and ANGPTL4 mGFP cells were seeded at a density of 75,000 cells per well on glass coverslips inside a 24 well plate and grown for 48 hours. For GFP expression the cells were fixed at room temperature with 4% Paraformaldehyde (PFA) in the

dark, rinsed thrice with ice cold PBS and mounted with SlowFade™ Diamond Antifade Mountant with DAPI (Invitrogen™).

To stain for intracellular proteins, PFA was removed, and the cells on the coverslips were permeabilized for 10 minutes in immunofluorescence (IF) buffer consisting of 2% BSA/PBS with 0.2% saponin. The cells were rinsed in 2% BSA/PBS, incubated with primary antibodies overnight. Next day the coverslips were incubated with secondary antibodies for 1 hour at room temperature and rinsed three times (5 min each) in 2% BSA/PBS after each antibody incubation **(**Bailey**,** et al., 2014).The coverslips were rinsed once with PBS and mounted on glass microscope slides with SlowFade™ Diamond Antifade Mountant with DAPI (Invitrogen™).
All images were captured with a Leica confocal microscope and merged fluorescence pictures were generated using Fiji-ImageJ.

II.2.7 Mammosphere Assay

The MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP cells were cultured for 7 days in Mammocult medium (STEMCELL™ Technologies) at 37 °C under 5% CO_2 on ultra-low attachment plates or flasks. The colonies were counted and imaged with an inverted light microscope after 7 days. The mammospheres were used for immunoblotting and Oil Red O staining. The p-value was calculated using student's t test using graph pad software, USA.

II.2.8 Soft Agar Colony Formation Assay

1.25 x 10^3 MDA-MB-231 mGFP or ANGPTL4 mGFP cells were suspended in complete medium with added 2ug/ml puromycin mixed with 3% noble agarose. The cells were seeded on

the semisolid layer of complete medium and 0.6% noble agarose in six well plates at 37 °C under 5% CO_2. Complete medium supplemented with 2ug/ml puromycin was added twice a week to prevent drying-out of the agarose and to preserve moisture. The colonies were counted and imaged using an inverted light Nikon Ti2 microscope after 7 days. The p-value was calculated using student's t test using graph pad software, USA.

II.2.9 Oil Red O staining

Mammospheres were stained with Oil Red O stain (60% 0.5% Oil Red O Solution in 40% ddH_20) according to the manufacturer's instructions. Fluorescent and brightfield mammosphere colonies were counted and photographed with an inverted light microscope after 7 days.

II.3 RESULTS

II.3.1 Generation and evaluation of ANGPTL4 expression in TNBC ANGPTL4 mGFP overexpression cell line. MDA-MB-231 cells were transduced with either the plasmid containing the empty vector or with the pLenti-C-mGFP-P2A-Puro plasmid with the ANGPTL4 gene. ANGPTL4 mGFP was designated as the MDA-MB-231 ANGPTL4 overexpression cell line and MDA-MB-231 overexpression empty vector control cell line referenced as MDA-MB-231 mGFP (**Figure 2.1A**). Confocal microscopy showed the expression of mGFP expression in the control and ANGPTL4 mGFP cell lines were used with DAPI to determine staining of the nucleus (**Figure 2.1B).** The genetically engineered cell lines mGFP and ANGPTL4 mGFP cells with strong homogenous mGFP expression was determined by fluorescence microscopy and FACs was then used for later experiments (Simeon J. (2017) Honors Thesis).

Western blot analysis of ANGPTL4 expression in MDA-MB-231 overexpression cell lines. Total protein was extracted from the cell lines and immunoblotted with antibodies. This result confirmed the expression of ANGPTL4 in the ANGPLT4 mGFP cell line and the lack of expression in the MDA-MB-231 cell line transduced with an empty vector (**Figure 2.1C, Lane 2**).

ELISA Quantification of ANGPTL4 secretion by the transduced MDA-MB-231 overexpression cell lines. The amount of ANGPTL4 secreted in culture medium by MDA-MB-231 overexpression empty vector control and ANGPTL4 overexpression cell lines was assessed by an ELISA assay. As shown in **Figure 2.1D,** the MDA-MB-231 ANGPTL4 overexpression cell line secreted 0.87735 ng/ml of ANGPLT4 in comparison to the MDA-MB-231 overexpression empty vector control cell line secreting 0.022 ng/ml, n=3. This confirmed ANGPTL4 overexpression in the respective cell line. The error bars indicate the standard deviation.

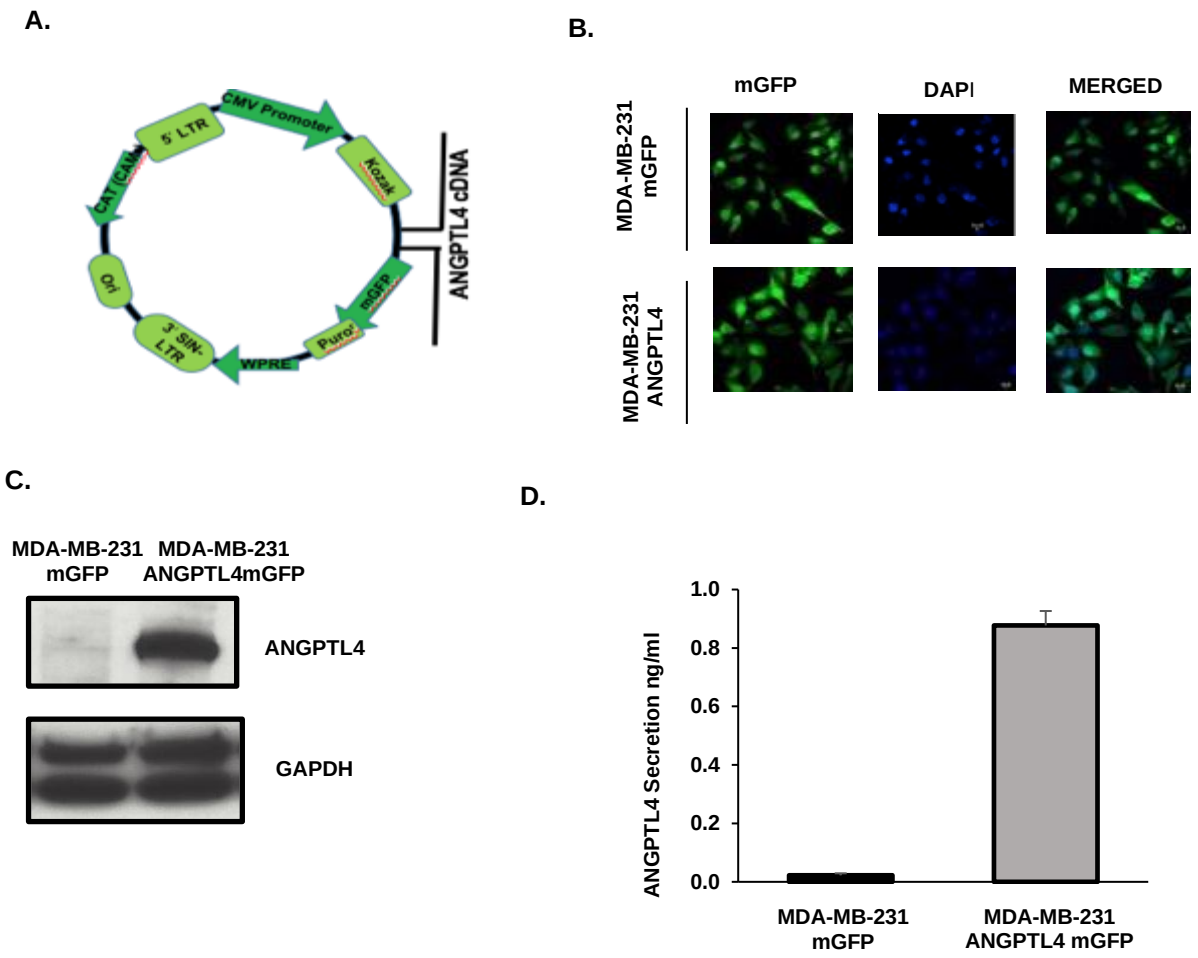


Figure 2.1: Lentiviral-mediated transduction of MDA-MD-231 overexpressing ANGPTL4 confirmation. Map of lentiviral overexpression cloning vector, pLenti-C-mGFP-P2A-Puro plasmid (A). Confocal images of the cell lines expressing mGFP in the cells mounted on coverslips with DAPI for nuclear staining(B). Western blot analysis of ANGPTL4 expression with a commercially available ANGPTL4 antibody and GAPDH as the loading control(C). The amount of ANGPTL4 secreted by the cells measured by the ELISA kit (D) confirming that the ANGPTL4 mGFP cell line is overexpressing ANGPTL4.
***Note**: The lentiviral overexpression map image was adapted from Origene and reprinted from Honors Thesis (Simeon, 2017).The Characterization of Angiopoietin-like 4 dependent Triple Negative Breast Cancer Cells Extravasation using an *in vitro* model of the Human Blood Brain Barrier. Honors College, Fulbright College, University of Arkansas.
***Note:** This figure was reprinted from (Simeon, Thrush, & Bailey, Angiopoietin-like protein 4 is a chromatin-bound protein that enhances mammosphere formation in vitro and experimental triple-negative breast cancer brain and liver metastases in vivo, 2021). *Journal of Carcinogenesis*.doi:10.4103/jcar. JCar20_

II.3.2 The nuclear localization of chromatin bound ANGPTL4 and its association to Tip60 and AURKA

Subcellular fractions were accessed for intracellular localization of ANGPTL4 in TNBC cells. ANGPTL4 is predicted to be in the nucleoplasm and potentially in the vesicles as seen in the Human Protein Atlas database. So, to determine the intracellular localization of ANGPTL4 in the MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP cells, subcellular fractionation and intracellular staining were done. Subsequently, the cells from the respective cell lines were imaged with confocal immunofluorescence microscopy. The nuclear localization of ANGPTL4 was confirmed **(Fig 2.2A)**. TIP60 and AURKA are suggested to be interactors in the ANGPTL4 pathway as such, intracellular staining was conducted to evaluate the localization of TIP60 and AURKA. TIP60 and AURKA was shown to colocalize in the nucleus of the MDA-MB-231 ANGPTL4 cell line and not in the MDA-MB-231 mGFP cell line **(Fig. 2.2A)**. ANGPTL4 being identified as a nuclear protein, was assessed in the chromatin lysate by immunoblot analysis to see if it also localizes in the chromatin. ANGPTL4 was present in the chromatin lysate of the MDA-MB-231 ANGPTL4 mGFP cell line and thus ANGPTL4 can be identified as a chromatin bound protein **(Fig 2.2B)**. Tip60 and AURKA was also evaluated by immunoblot analysis to see if they were localized in the chromatin fractions. TIP 60 and AURKA was shown to localized in the chromatin of the MDA-MB-231 ANGPTL4 mGFP cells **(Fig 2.3B)**.

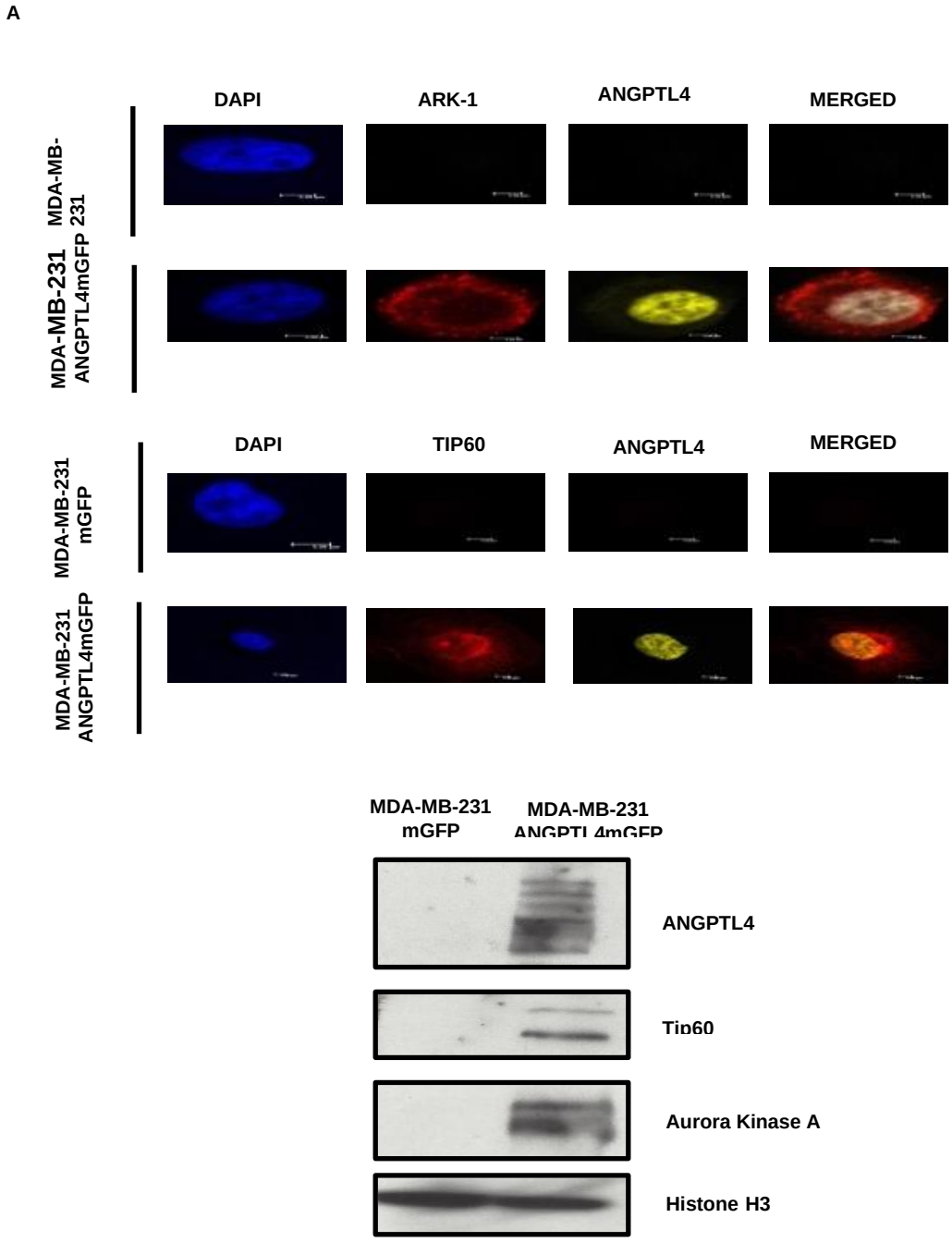


Figure 2.2: Chromatin bound ANGPTL4, TIP60 and AURKA. Immunoflourscense microscopy images of nuclear ANGPTL4, Tip60 and AURKA(A). Immunoblots depicting the presence of TIP60, AURKA and ANGPTL4 in chromatin fractions(B).
***Note**: This figure was reprinted from Simeon J, Thrush J, Bailey TA. (Simeon, Thrush, & Bailey, Angiopoietin-like protein 4 is a chromatin-bound protein that enhances mammosphere formation in vitro and experimental triple-negative breast cancer brain and liver metastases in vivo, 2021)*Journal of Carcinogenesis.*doi:10.4103/jcar. JCar20_20

II.3.3 MDA-MB-231 overexpressing ANGPTL4 cells promotes increased stem cell formation

The MDA-MB-231 mGFP and ANGPTL4 mGFP cell lines were evaluated for stem cell formation in mammocult media for 7 days. The number and the size of mammospheres quantified were analysed with brightfield and flourscence microscopy. The number of MDA-MB-231 ANGPTL4 mGFP mammospheres were significantly greater than the mGFP mammospheres. The average number of mGFP and ANGPTL4 mGFP mammospheres that is ≥ 200 µm is 0.75 um and 4.0 um, N=16 respectively. The quantity of mammospheres formed by the ANGPTL4 mGFP is 63, while the mGFP cell line had 12, N=3. The error bars depict standard deviation **(Fig 2.3A)**. In terms of morphology, MDA-MB-231 mGFP cells formed small compacted aggregrates compared to the larger loosely compacted aggregrates formed by the MDA-MB-231 ANGPTL4 mGFP cells**(Fig. 2.3B)**. Immunoblot analysis was done to evaluate the presence of the CD44 and CD24 markers in the adherent and stem cell population of MDA-MB-231 mGFP and ANGPTL4 mGFP cell lines. Mammospheres derived from the mGFP and ANGPTL4 mGFP cell lines expressed smaller amounts of the CD24 marker compared to the adherent cell lines. CD44 marker being expressed by both the adherent and mammospheres mGFP and ANGPTL4 mGFP cell lines**(Fig 2.3C)**. Oil Red O staining was used to analyze the presence of triglycerides in the mGFP and the ANGPTL4 mGFP cell lines. The ANGPTL4 overexpressing cell lines retained more Oil Red O staining than the cell line expressing endogenous levels of ANGPTL4 suggesting that there is more triglycerides in the ANGPTL4 mGFP cell line **(Figure 2.3D)**.

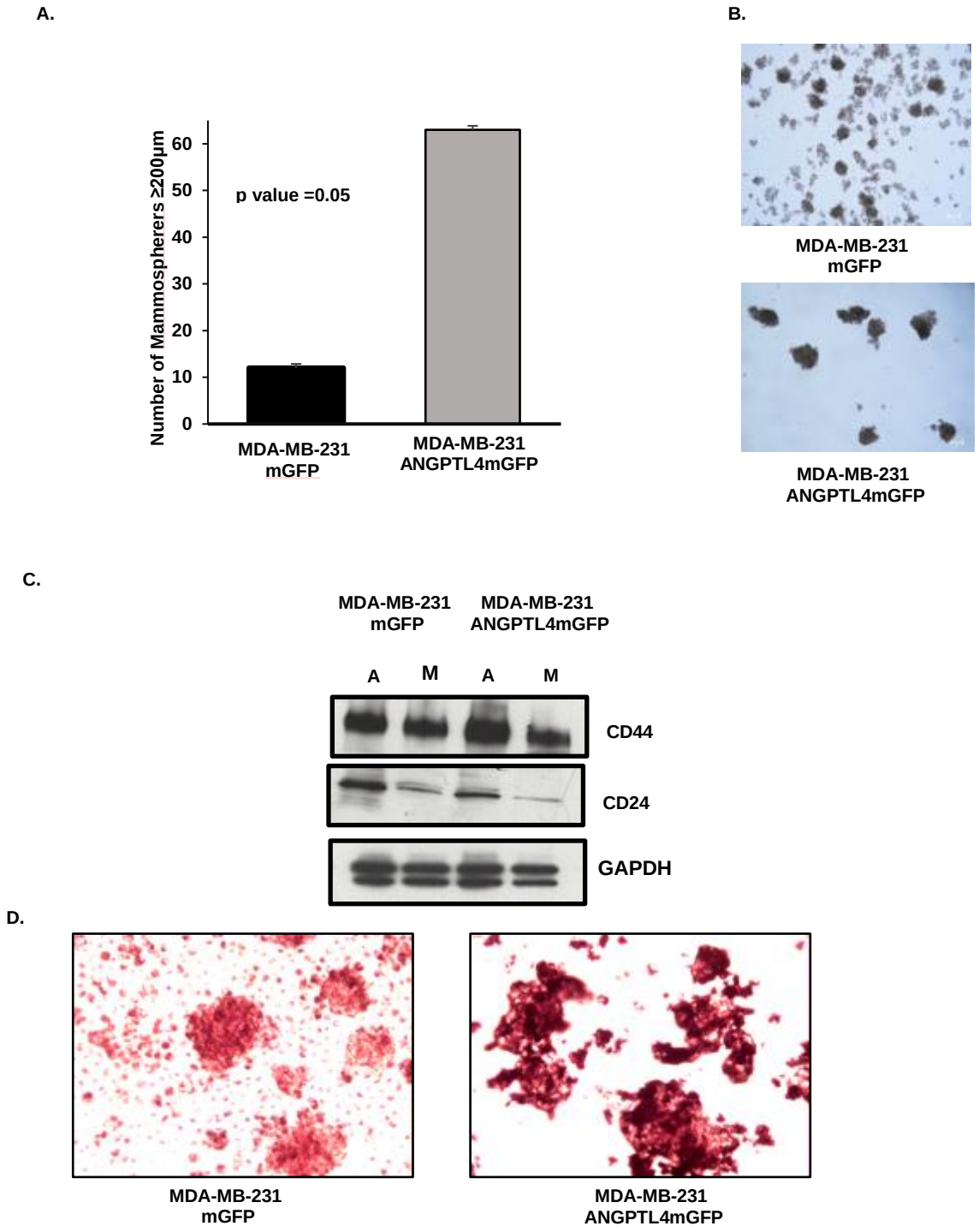


Figure 2.3: The formation of mammospheres *in vitro* from MDA-MB-231 mGFP and ANGPTL4 mGFP cell lines. Graph showing the number of mammospheres ≥200 µm formed from mGFP and ANGPTL4 mGFP cells lines, respectively (A). Brightfield images of primary mammospheres formed in mammocult suspension resulting from the mGFP cell line and the ANGPTL4 mGFP cell lines (B). Western blot analysis was performed to evaluate for the presence of CD24 and CD44 stem cell markers in adherent (A) and mammospheres (M) cells

Figure 2.3: (Continued) derived from MDA-MB-231 mGFP and ANGPTL4 mGFP cells (C). Mammospheres formed from mGFP and ANGPTL4 mGFP cells stained with Oil Red O to analyze the presence of triglycerides(D).

***Note**: This figure was reprinted from (Simeon, Thrush, & Bailey, Angiopoietin-like protein 4 is a chromatin-bound protein that enhances mammosphere formation in vitro and experimental triple-negative breast cancer brain and liver metastases in vivo, 2021) *Journal of Carcinogenesis.*doi:10.4103/jcar. JCar20_20.

II.3.4 Angiopoietin like protein 4 promotes anchorage independent growth

MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP cells were grown in media containing 0.3% agarose to produce a semi-solid medium. The ANGPTL4 overexpression cell line propagated more colonies than the mGFP cell line **(Figure 2.4A)**. The average number of colonies ≥200 um derived from the MDA-MB-231 mGFP and ANGPTL4 mGFP cells were 11 and 54 respectively, N=3. The combined average number of mGFP and ANGPTL4 mGFP colonies ≥200um was 0.305, N=36 and 5, N=36 repectively. Error bars depict standard error. In addition, brightfield and fluorescence microscopy shows that ANGPTL4 cells formed larger loosely aggregated cells like the morphology of the MDA-MB-231 ANGPTL4 mGFP mammospheres **(Figure 2.4B).**

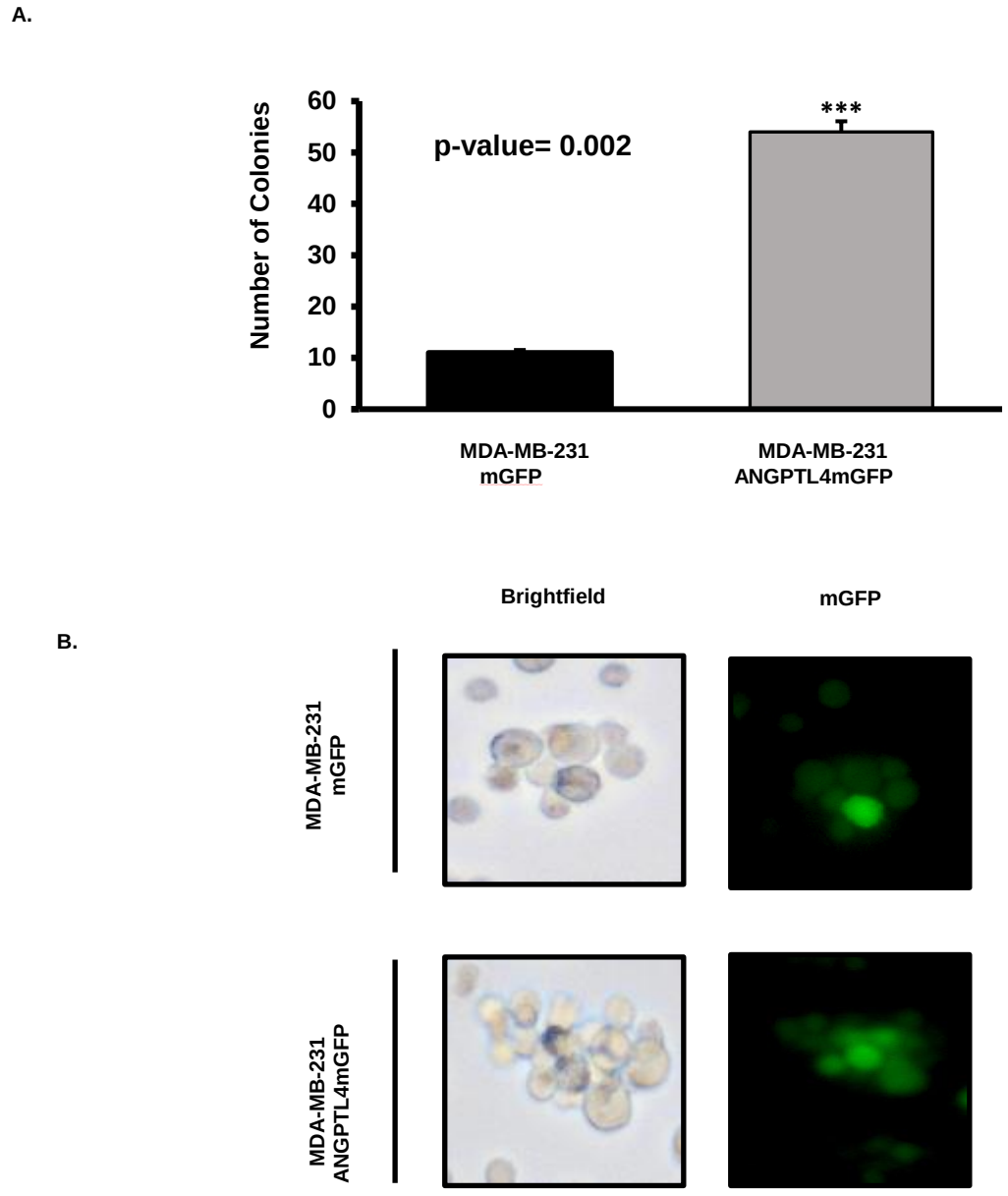


Figure 2.4: MDA-MB-231 mGFP and ANGPTL4 mGFP cells showed anchorage independent growth. The number(A) and morphology (B) of colonies formed from mGFP and ANGPTL4 mGFP cells in 0.3% of agarose contaning medium shown using microscopy.
***Note**: This figure was reprinted from (Simeon, Thrush, & Bailey, Angiopoietin-like protein 4 is a chromatin-bound protein that enhances mammosphere formation in vitro and experimental triple-negative breast cancer brain and liver metastases in vivo, 2021) *Journal of Carcinogenesis.*doi:10.4103/jcar. JCar20_20.

II.3.5 Discussion

To validate the role of intracellular ANGPTL4 expression in TNBC cell line, immunofluorescence microscopy, immunoblot analysis, mammosphere and soft agar colony formation assay can be used to determine the tumorigenic, anchorage-independence and invasive ability of the ANGPTL4 primed MDA-MB-231 cell line. ANGPTL4 overexpression was assessed and confirmed using immunoblot analysis, fluorescent microscopy, and an ELISA assay for use in the following experiments.

Subcellular Fractionation, immunofluorescence microscopy and immunoblot analysis were performed to evaluate the localization of ANGPTL4, TIP60 and AURKA. According to the Human Atlas Protein database ANGPTL4 is predicted to be localized in the nucleosome and possibly the vesicles, however the localization of ANGPTL4 has yet to be shown in TNBC cells. ANGPTL4 was shown to localize in the nucleus and chromatin of MDA-MB-231 ANGPTL4 mGFP cells. This means that ANGPTL4 is a chromatin bound protein and could potentially associate with DNA machinery in the chromatin. In addition, TIP60 and AURKA are possible interactors in the ANGPTL4 pathway as such their localization was evaluated as well. TIP60 and AURKA colocalized with ANGPTL4 in the nucleus and they were also present in the chromatin of the ANGPTL4 overexpressing cells.

(McGuire, et al., 2019) evaluated that low Tip60 expression increases the risk of breast cancer reccurrence. In this study, TIP60 enhances of the tumorigenic capability of ANGPTL4 mGFP cells due to the increased levels in ANGPTL4 overexpressing cell line. Tip60 is a known to be involved in DNA damage response and transcriptional regulation (Gao, et al., 2014), (van Beekum, et al., 2008), (Sapountzi, Logan, & Robson, 2006). Tip60 is necessary for DNA repair via acetylation of the histones. In addition, Tip60 interacts with PARR, and it induces the

regulation of PPAR gamma activity, which stimulates lipid metabolism which aids in cancer progression (La Paglia, et al., 2017), (Li, et al., 2007), (van Beekum, et al., 2008). Therefore, ANGPTL4 and tip60 may be working together to alter the transcriptional regulation of genes associated with lipid metabolism or aid in DNA repair.

In this study, a mammosphere assay was performed to determine the ability of ANGPTL4 mGFP cells to form stem-cell enriched mammospheres. The mammospheres derived from the ANGPTL4 mGFP cells were significantly greater in the size and number compared to the mammospheres derived from the mGFP cell line. Additionally, immunoblot analysis showed that the mammospheres consisted of low levels of the CD24 and proportionate levels of the CD44 stem cell markers compared to the adherent mGFP and ANGPTL4 mGFP cell lines. TNBC is enriched with cancer stem cells compared to other breast cancer subtypes and shows CD44 high and CD24 low phenotypes with self-renewal ability, enhances invasion and metastatic abilities (Ma, et al., 2014), (Honeth, et al., 2008).

Oil Red O staining analyzed the presence of tricycerides in the mammospheres derived from the mGFP and ANGPTLL4 mGFP cells. The ANGPTL4 mGFP had increased staining intensity compared to the mGFP mammosphere cell line suggesting that there are elevated lipids in the ANGPTL4 mGFP mammosphere derived cells. Obesity is a risk factor for breast cancer and under obese conditions ANGPTL4 as a driver of cancer progression (Dietze, Chavez, & Seewaldt, 2018), (Kolb, et al., 2019), (Sun, et al., 2017). A study reported that patients with an upregulation of ANGPTL4 in uveal melanoma derived multicellular spheroids endured a metabolic shift to suggesting that ANGPTL4 could potentially play a role in lipid metabolism (Ness, et al., 2017).

Anchorage independent growth of the mGFP and the ANGPTL4 mGFP cell lines were evaluated using a soft agar colony formation assay. The ANGPTL4 mGFP cell lines propagated larger and a greater number of colonies compared to the mGFP cell line. This suggests that ANGPTL4 promotes anchorage independent growth of TNBC cells. (Zhu, et al., 2011) reported that ANGPTL4 drives anchorage-independence and as a result promotes the ability of cancer cells to evade anoikis and become resistance through activation of ROS in skin carcinoma cells.

II.3.6 Conclusion

Cancer stems cells are known to make tumors more aggressive through self-renewal, migration, and therapy resistance. The MDA-MB-231 ANGPTL4 mGFP cell line had an increased number and growth of mammospheres formed in comparison to the mGFP cell line. Also, the morphology of the mammospheres between the two cell lines were different. The ANGPTL4 mGFP cells had bigger aggregates compared to the mGFP cell line. Therefore, that data suggests that ANGPTL4 is a driver of mammosphere formation in the MDA-MB-231 cell line. ANGPTL4 enhanced the anchorage independent growth of the MDA-MB-231 cell lines compared to the mGFP cell line. The ANGPTL4 mGFP cells showed increased colony growth and size similar to the mammospheres formed.

In addition, the results suggested that ANGPTL4 is localized in the nucleus of TNBC cells and a chromatin bound protein. TIP60 and AURKA predictive interactors in the ANGPTL4 pathway were localized in the nucleus and chromatin of the ANGPTL4 cell line. TNBC cell line overexpressing ANGPTL4 was shown to retain more of the Oil Red O staining than the mGFP cell line expressing normal levels of ANGPTL4. This indicated that ANGPTL4 could potentially alter the lipid profile of the cells. DNA damage consists of alterations in chromatin structure and TIP60 is a chromatin-modifying protein and key component of repair. It also plays an important role in antiproliferative responses to DNA damage and elicits such control via acetylation of histones in the of DNA repair occurs. Additionally, TIP60 has a fundamental role in lipogenesis, and proper modulation might be a promising strategy for the alleviation of cancer related obesity and its associated metabolic diseases (Li, et al., 2018).

As stated, aberrant expression of AURKA may lead to genetic instability and cause development of many cancers. It's role in promoting tumor progression, such as the activation

of epithelial–mesenchymal transition reprograms cancer cells stemness. Therefore, AURKA could be a potential attractive target for cancer therapeutics in conjuncture with breast cancer ANGPTL4 stemness phenotype. In conclusion, ANGPTL4 promotes tumor growth, formation of large cellular colonies and tumor stemness of TNBC cells *in vitro.*

III. ANGPTL4 ENHANCES TUMOR GROWTH AND LIVER METASTASES *IN VIVO*

III.1 Introduction

After evaluating the role ANGPTL4 using *in vitro* assays, the overexpression of ANGPTL4 in TNBC was evaluated via *in vivo* studies. Scientists have used *in vivo* models as important research tools to study cancer initiation, invasion, and metastasis in a physiologically relevant system in breast cancer research. This represents an essential step between *in vitro* systems and clinical studies. In vitro studies may not account for all the interactions between cells and biochemical processes that occur in the complexity of organ systems and thus the use of animals in *in vivo* studies addresses many of the disadvantages of only using *in vitro* studies (Tang, n.d.). *In vivo* studies also provide data that is important for validation of the function or proof of concept and for evaluation of efficacy of a gene or drug in a complex model (Vandamme, 2014). Moreover, advances in gene editing have helped scientists replicate human diseases in animals with better accuracy (Lee, et al., 2020).

TNBC risk of distant recurrence peaks between 1 to 3 years after surgery and the time from recurrence to death is 9 months, significantly shorter than other types of breast cancer. Triple Negative Breast Cancer is associated with a higher rate of liver metastasis and, TNBC liver metastasis is fatal to patients who have not undergone treatment, usually having 4-6 months of survival before death (Kennecke, et al., 2010). To date, there are not many breast cancer mouse models that are developed focused on the formation of liver metastases only. (Rikhi, et al., 2016) established a murine model of direct injection of TNBC MDA-MB-231 cell line in the frontal lobe of the liver, which the researchers were able to evaluate invasion and metastasis to the lungs. (Lim, et al., 2020) established a liver-metastasis model of injecting TNBC from a patient and implanting it on the liver lobe by doing a surgical hepatic incision, simulating liver

metastasis. Using this model, they found lymph-node metastasis following liver metastasis growth. In this study, a nude mice model was used to study liver metastasis. Nude mice are athymic, so it makes them accepting of foreign cells which makes them great for cancer research, understanding the mechanism of malignant tumors and testing potentially treatments (Wettersten, Ganti, & Weiss, 2014), (Ito, et al., 2002).

ANGPTL4 is a factor that has been shown to aid in tumor progression, metastasis, and invasion in a few human cancers. For these in vivo studies, the TNBC cell lines with the endogenous and overexpression of ANGPTL4 were injected subcutaneously in athymic mice and, the result of tumor growth and metastases was evaluated after a four-to-six-week period. The primary tumors and major organs were harvested for further analysis. Tissue resection and staining was done to determine the presence of ANGPTL4 and TIP60, in addition to Oil Red O staining of the liver and tumor tissue.

III.2 MATERIALS AND METHODS

III.2.1 Mouse human tumor xenograft model

All animal work was done in accordance with a protocol approved by the Institutional Care and Animal Use Committee at the University of Arkansas. Immunocompromised female mice age matched at four weeks old were used for xenografting studies. The mice were kept at the Central Laboratory Animal Facilities (CLAF), where they acclimatized for one week prior the experiment. The athymic mice were subcutaneously injected in the right flank with either the MDA-MB-231 cell line or the ANGPTL4 mGFP cell line or PBS. The allotted number of mice were held in a cage of dimensions 11.5”x 7.5’x5’ and was monitored once a day for physical and/or neurological distress post injection until euthanasia. Tumor areas were measured every three days using an electric caliper and were quantitated with the formula length multiplied by the width (LXW). The mice were anesthetized four to six weeks post subcutaneous injection with the cells, with isoflurane mixed with compressed air/oxygen. Primary tumors and organs were harvested for tissue immunofluorescence imaging to identify GFP-positive tumors and metastatic lesions. Two separate studies were conducted in which eight animals were used per cell line in study one, and in study two six animals were used per cell line.

III.2.2 Whole Tissue Immunofluorescence Microscopy

Primary tumors and liver tissue was sectioned at 6.0 μm and stained for intracellular proteins. Slices were fixed with 4% PFA, then the tissue slices were permeabilized for 20 minutes in immunofluorescence (IF) buffer consisting of 2% BSA/PBS with 0.2% saponin. The tissue slices were rinsed in 2% BSA/PBS, incubated with primary antibodies overnight and secondary antibodies for 1 h at room temperature with three rinses (5 min each) in 2% BSA/PBS after each

antibody incubation. After, the tissue slices were then rinsed thrice with PBS and mounted with SlowFade™ Diamond Antifade Mountant with DAPI (Invitrogen™).

All images were captured with a Nikon epifluorescence microscope in conjunction with NIS Elements software to obtain fluorescent images at the indicated magnifications. Merged fluorescence pictures were generated using Fiji-ImageJ.

III.2.3 Oil Red O staining

Primary tumors and liver tissue were stained with Oil Red O stain (60% 0.5% Oil Red O Solution in 40% ddH_20) according to the manufacturer's instructions. Tumor and liver tissue images were captured using a Nikon epifluorescence microscope in conjunction with NIS Elements software to obtain brightfield and fluorescent images at the indicated magnifications. Merged fluorescence pictures were generated using Fiji-ImageJ.

III.3 RESULTS

III.3.1 ANGPTL4 drives primary tumor size and promotes TNBC metastasis to the liver

To evaluate tumorigenicity and the metastatic ability of MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP overexpressing cell lines TNBC cells were injected subcutaneously in the right flank of the athymic female mice for a 4 to 6-week period two separate studies. The first study was conducted to evaluate liver metastasis in a 4-week period. A second study was designed to evaluate if the results were reproducible. The mice were evaluated over a 6-week period. The mice injected with the ANGPTL4 mGFP cells showed a greater mean primary tumor size in comparison to the mice that were injected with mGFP cells**.** Photographs showing tumor growth 4 weeks post injection of mice injected with mGFP and ANGPTL4 mGFP cells. In study 1, The mean primary tumor area derived from mGFP and ANGPTL4 mGFP cells were 3.14 mm^2 and 20.32 mm^2 respectively **(Figure 3.1 A and B).** In study 2, the mean area of the primary tumor area derived from the mGFP and ANGPTL4 mGFP cells is 0 mm^2 and 26.06 mm^2 respectively, N=6. There was no area calculated for the primary tumor formed from the injected mGFP cells because it was diminutive. (data not shown) **(Figure 3.1 C and D).**

The weights of the mice injected with MDA-MB-231 mGFP and ANGPTL4 mGFP were analyzed, however, there was not any significant difference in between the two sets of mice in both studies conducted. The mean weight of the mice xenograft injected with mGFP cells and ANGPTL4 mGFP cells was 25 and 24 g respectively in study 1 **(Figure 3.2A).** In study 2, the mean weight of the mice xenograft injected with mGFP cells and ANGPTL4 mGFP cells was 23 g and 22 g respectively **(Figure 3.2B).** The brains, spleen, lung, kidneys, intestines, and livers of

all mice were evaluated for mGFP-positive metastatic lesions. The organs containing no metastases were not shown.

A comparison of the livers showed that seven out of the eight mice injected with ANGPTL4 mGFP cell line developed liver metastases, compared to one out of the eight mice injected with the MDA-MB-231 mGFP cell line developing metastasis **(Figure 3.2C and D).** Additionally, there was no significant difference in the liver weights measured from the mice injected with the MDA-MB-231 mGFP and MDA-MB-231 ANGPTL4 mGFP **(Figure 3.2E).** The mean liver weight of the mice injected with the mGFP cells and the ANGPTL4 cells was 1276.15 mg and 1227.4 mg respectively. No metastatic lesions in the spleen, brains, spleen, intestines, and lungs were reported.

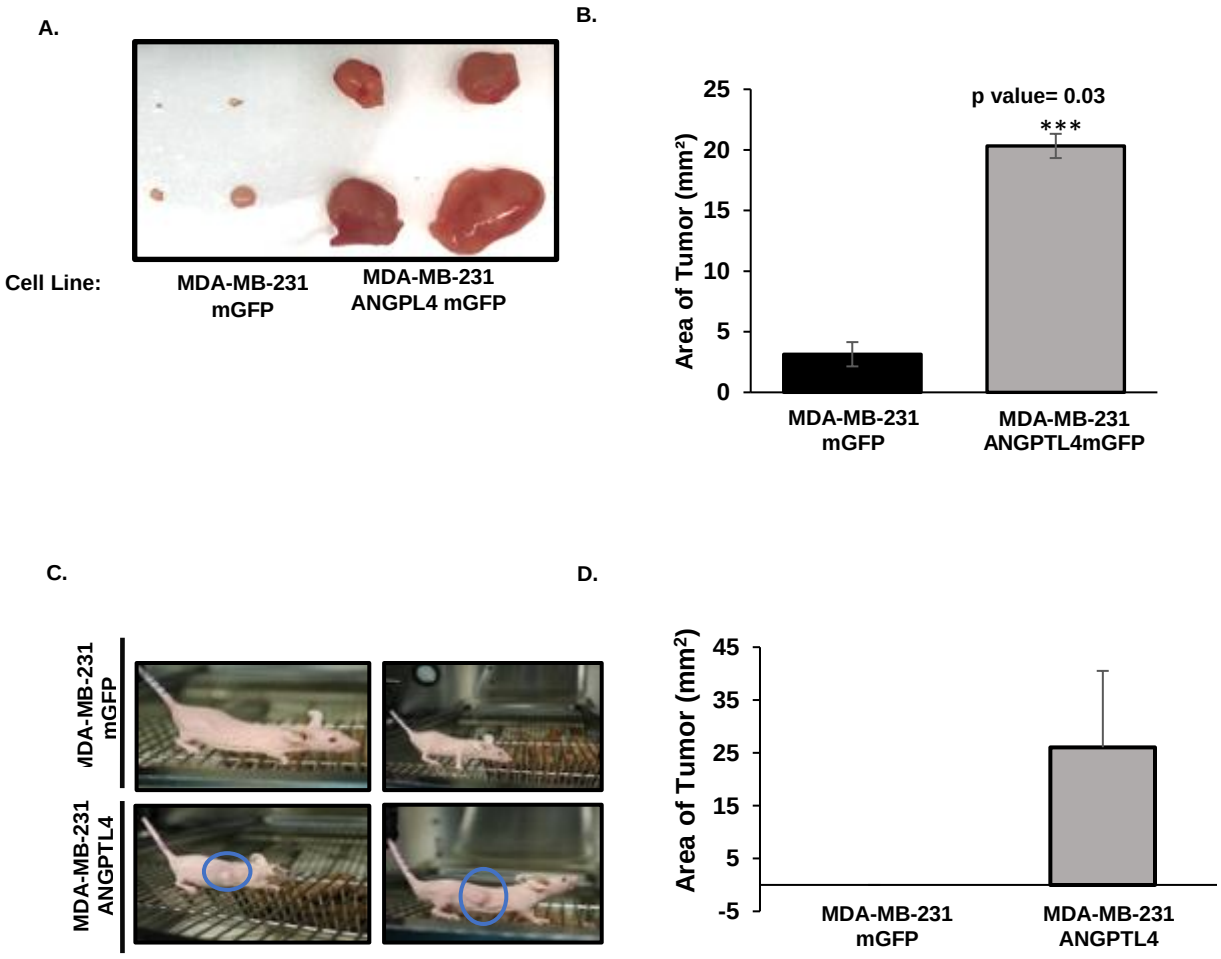
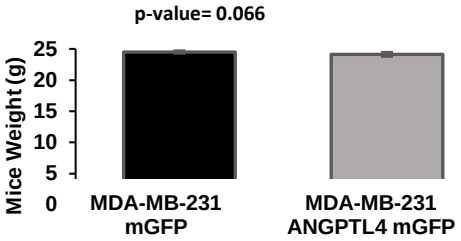


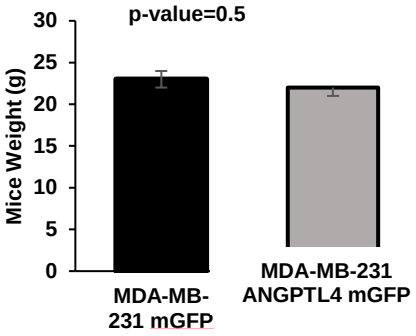
Figure 3.1: ANGPTL4 promotes tumor proliferation *in vivo*. Primary tumors were removed from mice 4 weeks post subcutaneous injection with MDA-MB-231 mGFP and ANGPTL4 mGFP cells. Primary tumor photographs (A) and the mean tumor area (LxW) (B). In study 2, the images of primary tumors were taken 4 weeks post subcutaneous injection with MDA-MB-231 mGFP and ANGPTL4 mGFP cells. Photographs of mice with primary tumors (C) and the mean tumor area (LxW) (B)

*__Note__: This figure was reprinted from Simeon J, Thrush J, Bailey TA. (In Press). Angiopoietin-like protein 4 is a chromatin-bound protein that enhances mammosphere formation *in vitro* and experimental triple-negative breast cancer brand liver metastases *in vivo. Journal of Carcinogenesis*.doi:10.4103/jcar. JCar20_20.

A.



B



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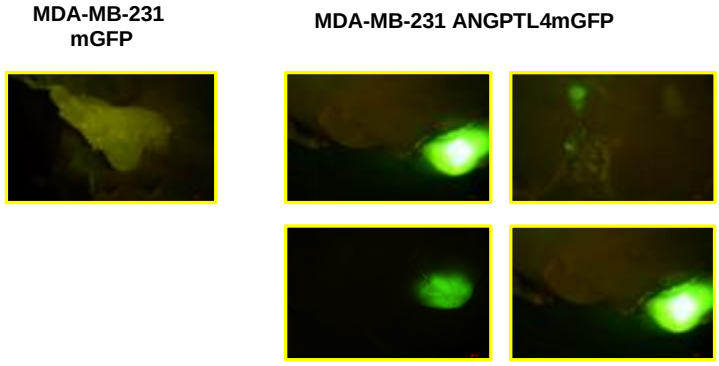

D.

Organ	MDA-MB-231 mGFP	MDA-MB-231 ANGPTL4 mGFP
Liver	1 of 8	7 of 8
Lungs	0 of 8	0 of 8
Brain	0 of 8	0 of 8
Spleen	0 of 8	0 of 8
Kidneys	0 of 8	0 of 8

E.

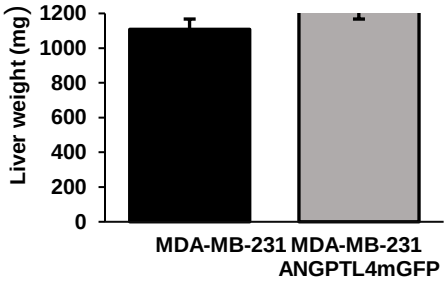

Figure 3.2: Metastatic liver trophism derived from MDA-MB-231 mGFP cells and ANGPTL4 mGFP cells injected in a mouse xenograft. Mean weight of the mice collected before euthanasia in both studies (A and B). Micrographs showing the number of metastatic lesions on the livers of mice subcutaneously injected with MDA-MB-231 ANGPTL4 mGFP cells and the mGFP cells(C). Table showing the number of metastases on organs removed from the mice injected

Figure 3.2: (Continued) with mGFP cells and ANGPTL4 mGFP cells(D). Mean liver weight of the mice collected after euthanasia (E).***Note**: This figure was reprinted from Simeon J, Thrush J, Bailey TA. (In Press). Angiopoietin-like protein 4 is a chromatin-bound protein that enhances mammosphere formation *in vitro* and experimental triple-negative breast cancer brain and liver metastases *in vivo. Journal of Carcinogenesis*.doi:10.4103/jcar. JCar20_20.

III.3.3 Immunofluorescence microscopy and Oil Red O Staining of tissue resected from the mGFP and ANGPTL4 mGFP injected mice

GFP+ TNBC liver metastases were excised from liver tissue and assessed for the presence of nuclear ANGPTL4, Tip60 and AURKA. No AURKA was detected (data not shown). ANGPTL4 was expressed in the nucleus of the liver tissue around the metastatic lesion and primary tumor, immunofluorescence staining was done with ANGPTL4 antibody and imaged with immunofluorescence microscopy. Experiments *in vitro* showed that ANGPTL4 colocalized with Tip60, therefore, immunofluorescence analysis was done to evaluate if TIP60 is associated with ANGPTL4 in the liver tissue and primary tumors. The slices were mounted with DAPI (nuclear stain), and images were taken with a Nikon epifluorescence wide-field microscope. TIP60 and ANGPTL4 was shown to compartmentalize in the nucleus in both tumor and liver tissue. **(Figure 3.3A)**

Oil Red O staining of the primary tumor (xenografts) and liver tissue derived from the mGFP and ANGPTL4 mGFP cell line evaluated for the presence of triglycerides was comparable between the mice. The Oil Red O staining was proportional between the mGFP and ANGPTL4 mGFP Liver and tumor tissue. **(Figure 3.4B)** Tissue slices were stained with 60% 0.5% Oil Red O Solution in 40% ddH_20, counterstained with hematoxylin and imaged using a Nikon epifluorescence wide-field microscope at indicated magnifications.

A.

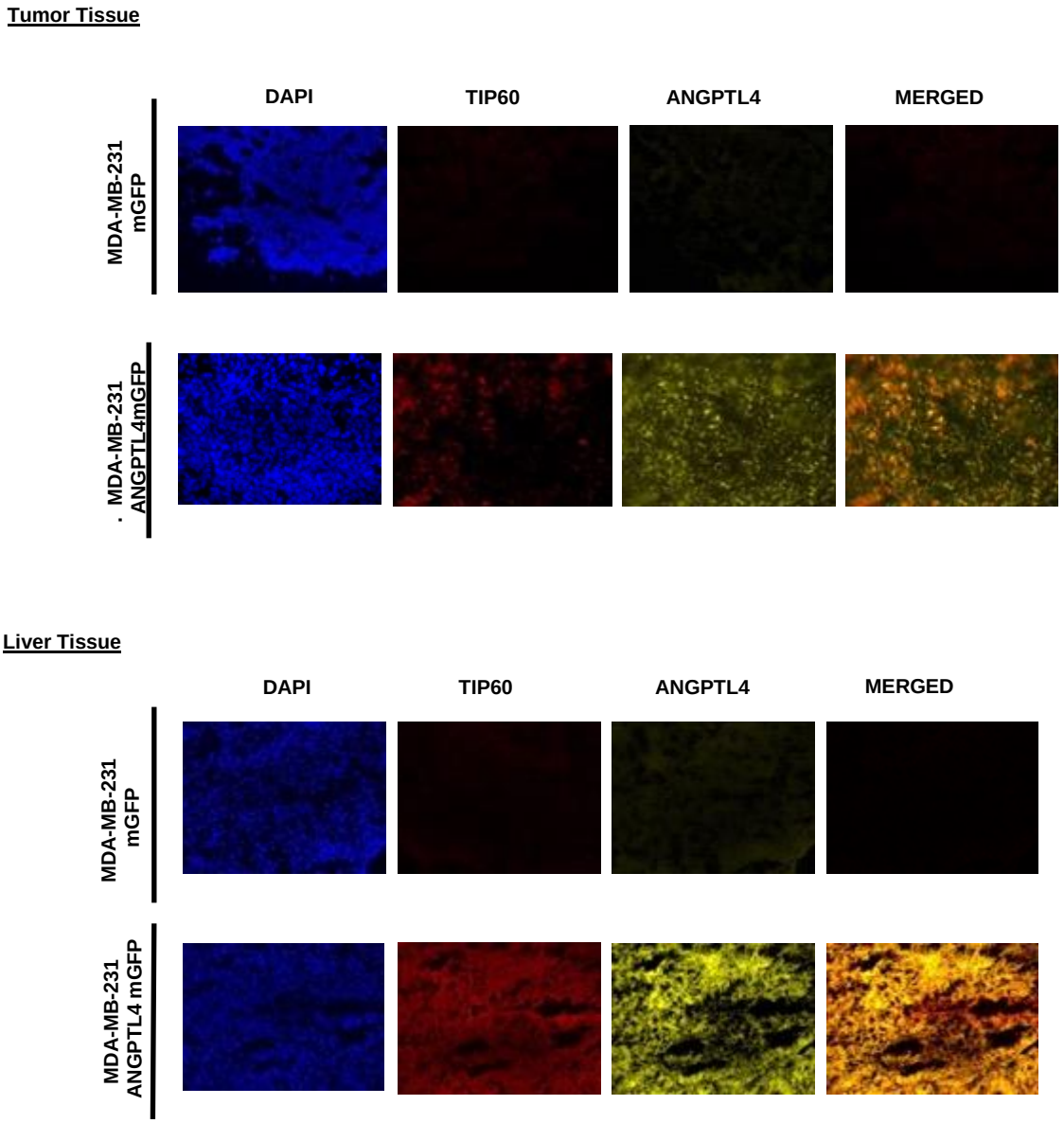

Figure 3.5: Immunoflouresence microscopy showing the colocalization of ANGPTL4 and Tip60(A). Oil Red O staining of the primary tumors and liver tissue from the mice injected with mGFP and ANGPTL4 mGFP cells(B).

B.

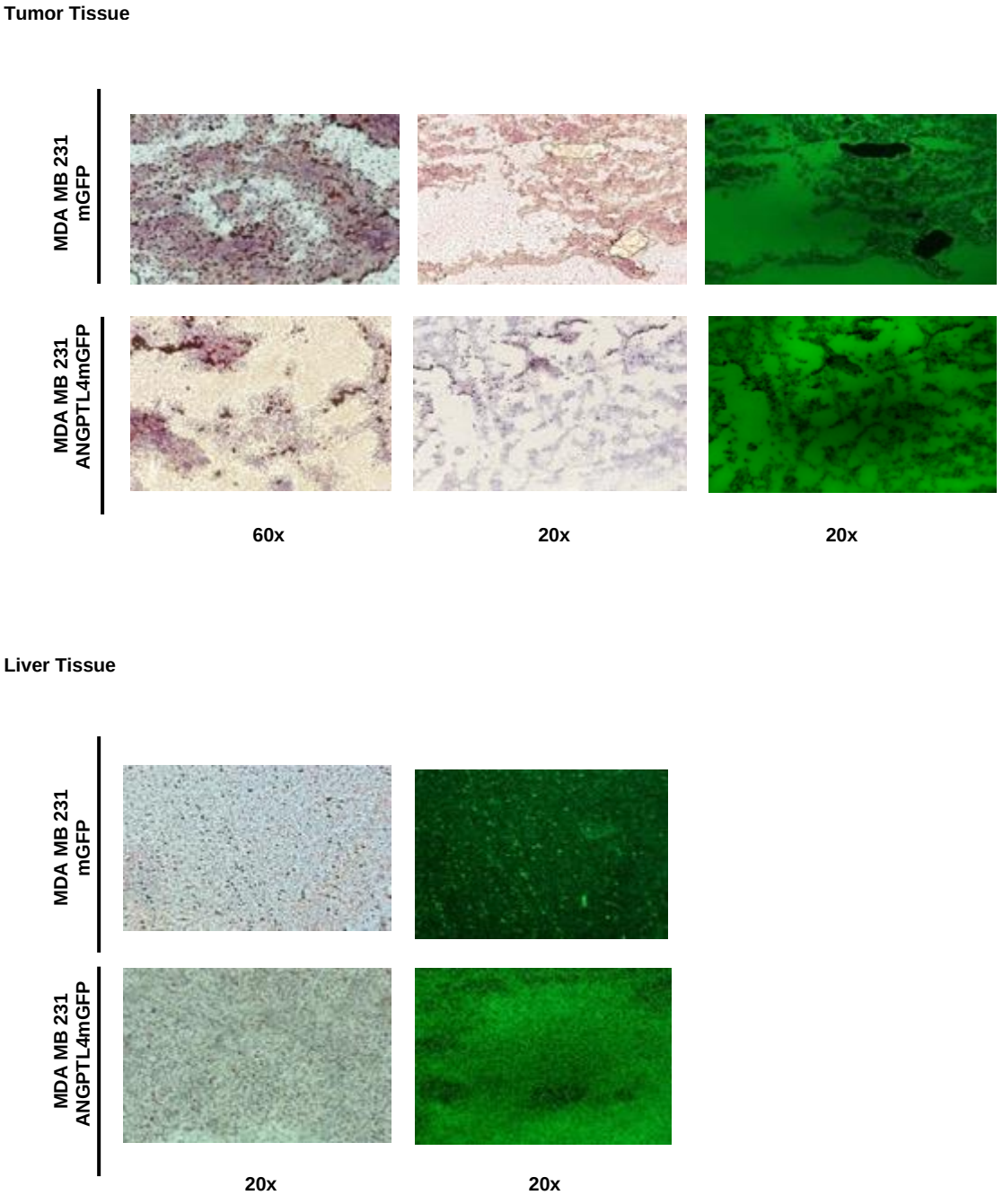

Figure 3.5:(Continued) Immunoflouresence microscopy showing the colocalization of ANGPTL4 and Tip60(A). Oil Red O staining of the primary tumors and liver tissue from the mice injected with mGFP and ANGPTL4 mGFP cells(B).

III.6 Discussion

The liver is a site of breast cancer metastasis, along with bone and lung (Berman, Thukral, Hwang, Solin, & Vapiwala, 2013), (Hess, et al., 2006). Liver metastasis in TNBC patients results in a poor prognosis (Bonotto, et al., 2014), (Gerratana, et al., 2015) and as median survival of breast cancer patients with liver metastasis ranges from 4-6 months if left untreated. Thus, a clinical need for a liver TNBC murine model is necessary to investigate the mechanisms used by metastatic tumor cells and what can be used as a potential therapeutic option.

Currently, there a few mouse models to evaluate breast cancer liver metastasis with varying results that pose their own challenges. The intrasplenic injection delivers tumor cells via the splenic vein (Soares, et al., 2014). The intrasplenic injection minimizes the development of simultaneous multi-distal metastasis which allows for the study of specific liver metastases. However, the intrasplenic model requires the removal of the spleen splenic tumors, this procedure that impacts immune function.

The most commonly used model of breast cancer metastasis relies on the intracardiac injection which occurs by injecting tumor cells directly into the circulation via the tail vein or left ventricle of the heart (Minn, et al., Genes that mediate breast cancer metastasis to lung, 2005), (Kang, et al., 2003). Injection of the tumor cells result in bone, brain, lung, and liver metastasis, including other organs (Basse, Hokland, Heron, & Hokland, 1988). Due to the multi distal organ metastases, these mice frequently need to be euthanized before evaluating specific liver metastasis preventing the ability to fully investigate metastatic growth within the liver.

Orthotopic injections of include the injection of tumor cells in the mammary fat pad of immune competent hosts to assess subsequent metastasis (Aslakson & Miller, 1992), (Dexter, et al., 1978). But the rate of liver metastasis from using this model, like the subcutaneous injection

is very low compared to other metastatic sites such as the lung. The orthotopic and portal vein injection results in liver metastasis after lung metastasis is established, complicating the need of liver-specific metastasis (Aslakson & Miller, 1992), (Doornebal, et al., 2013).

Another murine model for cancer liver metastasis utilizes spontaneous metastasis in genetically engineered mice. To date, reports of spontaneous murine models of breast cancer metastasis that readily spread to the liver are not common and if they do develop it is in a low percentage of mice (Derksen, et al., 2006), (Fantozzi & Christofori, 2006).

The portal vein injection model was developed to address the need for improved liver metastasis models, that delivers tumor cells directly to the liver. This model delivers tumor cells to the liver without complications of concurrent metastases in other organs or removal of the spleen. Additionally, it represents an important tool to study breast cancer metastasis to the liver and may be applicable to other cancers that frequently metastasize to the liver including colorectal and pancreatic cancer.

The subcutaneous model used for this study is known as a spontaneous model for metastasis. This model has its own advantages and disadvantages. A few strengths for using this model are the metastatic disease development from tumor, site mimics human disease progression and low cost. However, it's only applicable to certain cell lines, asynchronous metastatic development occurs, and the mouse microenvironment (Gómez-Cuadrado, Tracey, Ma, Qian, & Brunton, 2017). (Munoz, et al., 2006) showed the subcutaneous model using MDA-MB-231 cells usually metastasize to the liver, lung and lymph nodes.

To validate the role of ANGPTL4 in TNBC *in vivo,* subcutaneous injection was conducted to determine tumorigenesis, invasiveness, and metastatic capability of ANGPTL4 overexpressing TNBC cells.

ANGPTL4 drives tumor growth and TNBC liver metastasis. The MDA-MB-231 mGFP and ANGPTL4 mGFP cells were injected subcutaneously into the murine model and after a 4–6-week period the primary tumors (xenografts) and livers were resected. The mice injected with the ANGPTL4 cell line had greater tumor area and number compared to the mGFP cell line. This showed that ANGPTL4 is a driver of proliferation of the TNBC cells. ANGPTL4 enhances proliferation of many human cancers including breast, bone, thyroid (Munoz, et al., 2006), (Yang, et al., 2020), (Zhang, et al., 2012).

ANGPTL4 enhances liver metastasis of TNBC cells. Majority of the ANGPTL4 mice developed liver metastasis, whereas only one mouse developed liver metastasis when injected with mGFP cell line. ANGPTL4 is promotes cancer cell invasion and metastasis. ANGPTL4 has been shown to prime breast cancer cells to disrupts the endothelial cell to cell junction of the lung and brain parenchyma to invade and colonize these distal organs (Gong, et al., 2019), (Padua, et al., 2008).

Additionally, TIP60 is localized with ANGPTL4 in the nucleus of the primary tumors (xenografts) and the liver tissue resected around the metastatic lesion in the ANGPTL4 mGFP mice. TIP60 regulates gene transcription and DNA damage response. Tip60 can also function in cancer cell progression and metastatic ability of the cancer cells because of the epithelial and mesenchymal transitions the cancer cells undergo to invading tissue.

III.7 Conclusion

ANGPTL4 serves an important role in tumor cell motility, invasion, cell migration and metastasis. It has also been reported to inhibit metastases in lung and gastric cancers by preventing tumor progression and invasiveness (Chen, et al., 2018), (Tan, Teo, Sng, Zhu, & Tan, 2012). Conversely, ANGPTL4 expression seems to fluctuate in different human cancers such oral, hepatocellular, colorectal, and renal cancer. Therefore, since ANGPTL4 doesn't appear to have a consistent role in its expression in tumors, there is currently no effective targeted therapy against ANGPTL4 or standard molecular mechanism in which it aids tumor progression and metastases in all human cancers.

In this study, the role of ANGPTL4 is evaluated in TNBC. The findings from this study demonstrate that the overexpression of ANGPTL4 in TNBC cells is strongly associated with increase growth, primary tumor formation and metastatic liver trophism *in vivo*.

Furthermore, the role of TIP60 and ANGPTL4 in the MDA-MB-231 cell line was also assessed. The epithelial to mesenchymal transition is controlled by epigenetic modifications such as histone acetylation. As mentioned, TIP60, a histone acetyltransferase that functions in DNA repair relates to these transitions in cancer progression to tumour dissemination and high relapse in breast cancer. Here, it is shown that ANGPTL4 is localized with TIP60 in the primary tumor (xenograft) and liver tissue resected from the mice derived from the ANGPTL4 overexpression cell line.

In this study, it has been demonstrated that ANGPTL4 promotes tumor size and metastasis of TNBC cells.

IV. FUTURE DIRECTIONS

TNBC liver metastasis standard treatment is chemotherapy with a high chance of recurrence and increased incidences of death. Therefore, it is imperative to evaluate efficient treatment options that may lengthen and increase the quality of life of TNBC metastatic patients. One feasible area for favorable treatment strategy is exploiting the immune system to control metastatic cancer in TNBC. Tumor-associated antigens can be recognized by the immune system and evoke an immune response which may result in tumor cell death due to a T-cell tumor-specific response (García-Teijido, Cabal, Fernández, & Pérez, Tumor-Infiltrating Lymphocytes in Triple Negative Breast Cancer: The Future of Immune Targeting, 2016). However, tumor cell variants can evade the immune system by the activation of immune suppressive pathway and escape immune destruction. As mentioned, TNBC is associated with high levels of tumor infiltrating leukocytes and PD-L1 expression, supporting the investigation of immune checkpoint inhibitors (Pelekanou, et al., 2018). As a result, developing new and precise molecular biomarkers during prognosis would help to differentiate TNBC metastatic tumors because of its heterogenous nature. Cancer biomarkers can monitor an individual's risk of developing cancer in a specific tissue such as the liver and a result develop an efficient alternative for treatment. In addition, morphological and functional imaging technologies can also be used during *in vivo* experiments which can track measurements of the metastatic biological processes.

The mouse model in this study of that can be used to evaluate effective therapies for TNBC liver metastasis. This is important because patients with TNBC liver metastasis have poor prognosis, and a high mortality rate. One advantage is that this mouse model is athymic as such it expresses predominately macrophages. The dynamic function of macrophages was evaluated as M1 macrophages being tumoricidal, whereas M2 macrophages promotes cancer (Mills, 2012),

(Mills, Lenz, & Harris, A Breakthrough: Macrophage-Directed Cancer Immunotherapy, 2016). ANGPTL4 is significantly expressed in macrophages and has shown to drive the polarization of macrophages in colorectal cancer (Aryal, et al., 2016). Therefore, a future experiment would be to evaluate immunotherapies that causes M1 polarization in the tumor immune microenvironment of TNBC xenografts that overexpress ANGPTL4.

The current chemotherapy therapeutic strategies for the management of TNBC are platinum compounds and taxanes targeting DNA repair complexes, taxanes for p53 and anthracycline regimen for cell proliferation. TNBC is very aggressive and heterogenous subtype of breast cancer. Though, TNBC responds well to chemotherapy, incidences of recurrence are very high, and prognosis remains poor. (Verma, Provencher, & Dent, 2011) reported a treatment review of TNBC patients undergoing treatment and evaluated that the first line for metastatic treatment would be the standard chemotherapy drugs despite the current development of novel targeted therapies.

TNBC metastatic patients with visceral metastases have a relatively shorter overall survival have limited duration of response to successive lines of chemotherapy. So, it is imperative to select the agents most likely to result in a more meaningful treatment and possibly a longer overall survival. Therefore, because of TNBC biologically heterogenous nature, identifying molecular biomarkers to predict response to specific chemotherapy is requred to further improve treatment strategies with the current number of chemotherapy options and future combinations with targeted therapies so patients can have a better overall survival.

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VI. APPENDIX

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Dr. Tameka Bailey
From:	Jeff Wolchok
Date:	February 25, 2020
Subject:	IACUC Approval
Expiration Date:	February 24, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol #**20052**, *The Role of ANGPTL4 and Its Receptor Beta 1 Integrin in Liver Homing*.

In granting its approval, the IACUC has approved only the information provided. Should there by any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond February 24, 2021 you may submit a modification to extend the project up to three years, or submit a new protocol. The IACUC may not approve a study for more than three years at a time.

The following individuals are approved to work on this study: Dr. Tameka Bailey-Jennings, Jodi Simeon, and Chandra Wiley. Please submit personnel additions to this protocol via the modification form prior to their starting work.

The IACUC appreciates your cooperation in complying with University and federal guidelines involving the care and use of animals.

JCW/rek

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