Investigation of epigenetic programs promoting triple-negative breast cancer organotropism metastasis

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ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer characterized by negative expression of progesterone receptor, estrogen receptor and HER2. The high metastatic rate and lack of effective targeted therapies for TNBC results in a high mortality rate compared to other breast cancer subtypes.

In a previous study from the Ursini-Siegel lab, overexpression of the oncogenic signaling adapter protein p66ShcA was shown to be required for TNBC metastasis towards the liver and lung. However, p66ShcA overexpression alone is not sufficient to trigger metastatic events, suggesting the existence of additional pro-metastatic pathways. Since p66ShcA is transcriptionally upregulated at the epigenetic level, we predict that the additional pro-metastatic pathways are concurrently regulated through similar epigenetic reprogramming events.

To study organotropic metastasis, 4T1-TNBC murine cell lines, derived from the primary tumor, or from metastases towards the liver, lung or bone were used. ChIP-qPCR was performed on all cell lines to profile histone modifications at the p66ShcA promoter site, identifying H3K4me3 and H3K9ac as potential markers upregulating its gene transcription.

RNA-sequencing and DNA-methylation microarray analysis showed the involvement of DNA methylation in regulating TNBC organotropism metastasis and revealed several differentially expressed pathways that may be under regulation by DNA methylation. We further examined DNA-methylation as a vulnerability within the cell lines by treating the cells with decitabine, a DNA methyltransferase inhibitor. In-vitro and in-vivo growth curves identified cell line-specific resistance towards decitabine. Further analysis using Gene Ontology and qPCR on specific gene targets suggests the resistance may be caused by lower expression level of immune-related genes.

Overall, both histone modification and DNA-methylation were shown to affect TNBC organotropic metastasis. Our data suggests that combining decitabine with treatments upregulating anti-tumor immune responses may be an effective approach in targeting TNBC metastasis.

RÉSUMÉ

Le cancer du sein triple négatif (TNBC) est un sous-type agressif de cancer du sein caractérisé par une expression négative du récepteur de la progestérone, du récepteur des œstrogènes et de HER2. Le taux élevé de métastases et le manque de thérapies ciblées efficaces pour le TNBC entraînent un taux de mortalité élevé par rapport aux autres sous-types de cancer du sein.

Dans une étude précédente du laboratoire Ursini-Siegel, la surexpression de la protéine adaptatrice de signalisation oncogène p66ShcA s'est avérée nécessaire pour les métastases TNBC vers le foie et les poumons. Cependant, la surexpression de p66ShcA seule n'est pas suffisante pour déclencher des événements métastatiques, suggérant l'existence de voies pro-métastatiques supplémentaires. Étant donné que p66ShcA est régulé positivement de manière transcriptionnelle au niveau épigénétique, nous prévoyons que les voies pro-métastatiques supplémentaires sont régulées simultanément par des événements de reprogrammation épigénétique similaires.

Pour étudier les métastases organotropes, des lignées cellulaires murines 4T1-TNBC, issues de la tumeur primaire, ou de métastases vers le foie, les poumons ou les os ont été utilisées. ChIP-qPCR a été réalisée sur toutes les lignées cellulaires pour profiler les modifications des histones au site du promoteur p66ShcA, identifiant H3K4me3 et H3K9ac comme marqueurs potentiels régulant à la hausse la transcription du gène pro-métastatique.

L'analyse des microréseaux de séquençage d'ARN et de méthylation d'ADN a montré l'implication de la méthylation de l'ADN dans la régulation des métastases organotropes TNBC et a révélé plusieurs voies exprimées de manière différentielle qui peuvent être sous régulation par la méthylation de l'ADN. Nous avons en outre examiné la méthylation de l'ADN en tant que vulnérabilité au sein des lignées cellulaires en traitant les cellules avec de la décitabine, un inhibiteur de l'ADN méthyltransférase. Des analyses de croissance in vitro et in vivo ont été réalisées, identifiant la résistance spécifique de la lignée cellulaire à la décitabine. Une analyse plus approfondie utilisant Gene Ontology et qPCR sur des cibles génétiques spécifiques suggère que la résistance peut être causée par un niveau d'expression inférieur des gènes liés au système immunitaire.

Dans l'ensemble, il a été démontré que la modification des histones et la méthylation de l'ADN affectent les métastases organotropes du TNBC. Nos données suggèrent que la combinaison de la décitabine avec des traitements régulant positivement la réponse immunitaire peut être une approche efficace pour cibler les métastases TNBC.

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CONTRIBUTION OF AUTHORS

The project was under the supervision and guidance of Dr. Michael Witcher and Dr. Ursini-Siegel. For experiments, all cell lines used in the project were provided by Dr. Peter Siegel. Mice in-vivo experiment was performed and analyzed by Young Im. DNA-methylation microarray was analyzed with the help of Dr. Kathleen Klein and Benjamin Lebeau. RNA-sequencing was analyzed with the help of Benjamin Lebeau as well. The rest of the experiments and analysis were conducted by Xiaoting You.

The thesis was edited following the suggestions of Dr. Michael Witcher and Dr. Ursini-Siegel.

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1. INTRODUCTION

Breast cancer is the leading cause of both cancer incidence and cancer mortality in women, accounting for 1 in 4 cancer cases and 1 in 6 cancer deaths worldwide (Sung et al., 2021). 2.3 million new breast cancer cases were estimated to occur in 2020 globally, with 684 thousand new deaths (Sung et al., 2021). The global breast cancer burden in women has been increasing since 1980, due to growth and aging in the female population (Harbeck et al., 2019). Despite current efforts in breast cancer prevention and treatment, breast cancer remains a problem affecting many people.

Breast cancer is highly heterogenous and can be categorized into different subtypes. Triple-negative breast cancer is the most aggressive subtype and lacks the expression of several actionable drug targets found in other subtypes. Because of its unique molecular profile and inherent genomic instability (Guo & Wang, 2021), effective treatments targeting triple-negative breast cancer are limited and more studies are essential.

This thesis will focus on investigating epigenetic modifications involved in triple-negative breast cancer (TNBC) metastasis. Drug treatments targeting epigenetic machineries will be used to test their effects on preventing TNBC growth and metastasis, with the overarching goal to develop new treatment strategies.

1.1 Triple-Negative Breast Cancer

1.1.1 Breast cancer classification

The development of gene expression profiling allows more accurate classification of breast cancer into five intrinsic subtypes with distinct clinical outcomes: luminal A, luminal B, HER2 amplification, normal-like and basal-like (T. Sørlie et al., 2001). Molecular screening techniques such as genomic sequencing are expensive and time-consuming, therefore, immunohistochemistry (IHC) against key protein targets was used as a surrogate classification method (Tang & Tse, 2016). Differential expression of the progesterone receptor (PR), estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2) and Ki-67 protein, an indicator of proliferation, are commonly used for IHC classification (Glass et al., 2007). Previous studies

have demonstrated that IHC classification correlates well with molecular classification using genomic sequencing (Lacroix et al., 2001; Onitilo et al., 2009). The use of a classification system can provide information for predicting breast cancer progression and treatment effectiveness in a clinical setting.

The five most commonly recognized molecular subtypes of breast cancer are: luminal A (ER+/PR+, HER2-, Ki67-), luminal B (ER+/PR+, HER2-, Ki67+, or ER+/PR+, HER2+, Ki67+), HER2 amplification (ER-, PR-, HER2+), normal-like (similar to luminal A with different gene expression profile) and basal-like (ER-, PR-, HER2-) (Dai et al., 2015). Luminal A subtype expresses higher level of ER-related genes and lower level of proliferation-related genes comparing to luminal B, and is associated with better prognosis (Therese Sørlie et al., 2001). The distinct molecular profile allows development of unique treatment strategies targeting each subtype. Luminal A tumors can be treated using hormonal therapy targeting ER, whereas luminal B tumors benefit from a combination of chemotherapy and hormonal therapy due to its increased proliferation ability (Brenton et al., 2005). HER2 amplification subtype and some luminal B tumors over-expressing HER2 can be treated using anti-HER2 monoclonal antibody Herceptin and are sensitive towards anthracycline and taxane-based neoadjuvant chemotherapy (Brenton et al., 2005). Oppositely, very few treatment options are available for the basal-like subtype, causing it to have poorer prognosis comparing to the other subtypes (Cheang et al., 2009).

1.1.2 Triple-Negative Breast Cancer Characteristics

Triple-negative breast cancer (TNBC) is characterized by negative expression of progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor-2 (HER-2). Most TNBC tumors are basal-like. Gene profiling analysis shows the genomic overlap between TNBC and basal-like breast cancer can be as high as 60-90% (Prat et al., 2014). Other breast cancer subtypes may also be identified as TNBC, but at a much lower percentage: around 10.2% of TNBC belong in the HER2 amplification subtype, 4.6% are normal-like, 3.5% are luminal B and 1.1% are luminal A (Ahn et al., 2016).

TNBC is highly heterogenous and can be further classified into 4 subtypes: LAR, expressing luminal androgen receptor and cell surface mucin; MES, mesenchymal subtype expressing growth factor receptor; BLIS, basal-like immunosuppressed subtype; and BLIA, basal-like immune-activated subtype (Burstein et al., 2015; Yin et al., 2020). Different TNBC

subtypes also contain different percentage of molecular groups. BLIS and BLIA subtypes contain only the basal-like breast cancer cases. Half of the MES cases are basal-like, while the other half are either normal-like or luminal B group. Lastly, most of the LAR subtypes belong in the HER2 amplification group, with around 14.3% of cases are luminal B (Ahn et al., 2016).

More than 170,000 TNBC cases are estimated to be diagnosed each year worldwide, accounting for 15-20% of all breast cancer cases (Morris et al., 2007). Epidemiologic studies showed that TNBC is most prevalent in women younger than 40 years old and in women of African-American ethnicity (Millikan et al., 2008).

TNBC has higher recurrence and invasion rates compared to other breast cancer subtypes. Approximately 46% of TNBC patients experienced distant metastasis, primarily towards the brain, liver, and lung (Xiao et al., 2018). 33.9% of patients with TNBC experienced recurrence after surgery, comparing to 20% of recurrence among non-TNBC patients (Dent et al., 2007).

The high invasion and recurrence rate of TNBC also cause the patients to have a worse survival rate and a shorter survival time comparing to patients with non-TNBC. The difference in survival rate is especially significant at higher stage of metastatic cancer. For stage IV invasive breast cancer, where the cancer has spread from the primary site to distant organs, the five year survival rate of hormone receptor positive/HER2 positive subtype is around 45%, while the survival rate of TNBC subtype is only around 11% (Howlader et al., 2018). This striking difference is mainly due to the difficulty in controlling metastatic TNBC.

Overall, TNBC is an aggressive breast cancer subtype with a low survival rate and high metastatic rate.

1.1.3 Current Treatment Strategies

Another factor contributing to the high mortality rate in TNBC is the difficulties in developing effective targeted treatment regimens. Due to its negative expression of PR, ER and HER-2, TNBC is unresponsive towards hormonal therapy or trastuzumab therapy targeting HER-2 overexpression (Yin et al., 2020). Therefore, chemotherapy and radiotherapy are the main treatment options for TNBC.

The recommended standard chemotherapy for TNBC is anthracycline-based or taxane-based, or a combination of both (Yagata et al., 2011). Through a retrospective study, Liedtke et al. reported that the pathological complete response rate of TNBC patients was 20% for anthracycline-based treatment, 12% for taxane-based treatment, 28% for anthracycline and taxane combined treatment and 14% for other treatments (Liedtke et al., 2008). TNBC was reported by several clinical trials to show similar response rate as other subtypes of breast cancer under conventional chemotherapy treatment. For example, a randomized clinical trial on vecacizumab treatment has shown no difference between the level of efficacy in TNBC patients comparing to patients with other breast cancer subtypes (Miles et al., 2008; Miller et al., 2007). Similar comparison results were observed in trials using combination of gemcitabine/paclitaxel (Albain et al., 2008), capecitabine/docetaxel (O'Shaughnessy et al., 2002), and gemcitabine/vinorelbine (Martín et al., 2007). However, although TNBC patients show similar efficacy for many conventional chemotherapy treatments as non-TNBC patients, a huge problem regarding TNBC is its early onset of metastasis and high relapse rate. Once relapse occurred, the patient should be considered as resistant towards taxane and anthracyclines, making future treatment using conventional chemotherapy difficult.

Another commonly used treatment option for TNBC is radiotherapy. In a meta-analysis conducted using the Surveillance, Epidemiology, and End Results database, Yao et al. confirmed that TNBC patients receiving radiotherapy have survival advantage comparing to patients without radiotherapy (Yao et al., 2019). Especially following modified radical mastectomy (MRM), TNBC patients were shown to benefit from dose escalation of whole breast radiotherapy (Abdulkarim et al., 2011). However, a meta-analysis on prognosis of patients with different breast cancer subtypes following MRM has also shown that the decrease in local recurrence is less significant in TNBC patients comparing to luminal subtype patients, suggesting that TNBC may be more resistant towards radiotherapy (Kyndi et al., 2008).

One issue regarding chemotherapy and radiotherapy is the serious side effects, which may include nauseous and vomiting, appetite loss, hair loss, anorexia, weight loss and tiredness (Nurgali et al., 2018). Another issue is that not all TNBC patients can be successfully treated with chemotherapy and radiotherapy. Choosing the effective treatment regimen is very crucial due to the high heterogeneity nature of TNBC, which can be further classified into four subtypes

based on gene expression profiling and clinical data (Burstein et al., 2015). The four subtypes have distinct molecular profiles and prognoses, causing differential sensitivity towards chemotherapy and radiotherapy treatment. To counter this problem, the national comprehensive cancer network guideline suggests that a combination of conventional chemotherapy regimens, such as taxane, adriamycin, cyclophosphamide, and paclitaxel should be used (Lee et al., 2020; Yin et al., 2020).

Moreover, much of the recent literature has shown that neoadjuvant chemotherapy can increase the overall survival rate and disease-free survival time of patients with TNBC (Biswas et al., 2017; Chen et al., 2017; Gamucci et al., 2018). Approximately 28.2% of patients showed pathological complete response after receiving neoadjuvant chemotherapy, defined as disappearance of all invasive cancer in the breast. (Gamucci et al., 2018).

One type of neoadjuvant treatment utilizes platinum salts, which are DNA damaging agents that can cause single strand or double strand breaks of DNA (Lee et al., 2020). In tumor cells that contain an impaired DNA repair function, platinum salts can cause accumulating DNA damage which leads to tumor cell death (Byrski et al., 2012; Byrski et al., 2010). Around 10% to 20% of TNBC cases contain BRCA1/2 mutation, which make them good target for platinum salts treatment (Gonzalez-Angulo et al., 2011; Hartman et al., 2012; Xie et al., 2017). A meta-analysis of nine randomized controlled trials showed that using platinum salts as neoadjuvant chemotherapy significantly increased the pathological complete response rate in TNBC patients from 37.0% to 52.1% (Poggio et al., 2018).

Another type of TNBC treatment recently under investigation is poly (ADP)-ribose polymerase (PARP) inhibitors. The rationale behind using PARP inhibitors as TNBC treatment is that a high percentage of TNBC cases carries mutation in DNA repair pathway. Approximately 70% of BRCA1/2 mutated breast cancer cases are TNBC and about 15% of total TNBC cases contain mutation on BRCA1/2 (Tung et al., 2016; Winter et al., 2016). Cancer cells carrying deleterious mutation in BRCA1/2 are defective in the homologous recombination pathway responsible for double-strand DNA repair (Miki et al., 1994; Wooster et al., 1995). Consequently, these cells are highly dependent on other DNA repair mechanisms and disruption of other mechanisms can lead to severe accumulation of DNA damage in the cancer cell, leading to apoptosis. PARP are a family of enzymes playing a key role in DNA single-strand break

repair through the excision repair pathway (Chambon et al., 1963; Leppard et al., 2003). PARP enzymes can recognize DNA damage and catalyze PARylation to recruit repairing factors onto the DNA damage site (Amé et al., 2004). Inhibiting the action of PARP enzymes in cells with BRCA1/2 mutations induces synthetic lethality and can promote cell death. Currently, two PARP inhibitors, olaparib and talazoparib, have been approved by the FDA as treatment for BRCA1/2 mutated TNBC (Litton et al., 2018; Robson et al., 2017). Other PARP inhibitors, such as rucaparib, niraparib and veliparib, are being investigated in clinical trials as potential TNBC treatment options (Barchiesi et al., 2021).

Immunotherapy is also an emerging direction of TNBC treatment. A large phase I clinical trial demonstrated that anti-PD-L1 treatment is effective in about 10% of TNBC patients (Nanda et al., 2016). Although the effective percentage is relatively low, the reported effect is long-lasting and safe. Therefore, the current challenge lies in how to increase the response rate of TNBC patients towards anti-PD-L1 treatment. Anti-CTLA-4 antibody is another immunotherapy regimen currently under investigation as a potential TNBC treatment. Ipilimumab, an anti-CTLA-4 antibody, was approved by the FDA as a treatment for advanced melanoma and is currently under investigation as a TNBC treatment (Yin et al., 2020). Combination therapy with MUC1 mRNA nano vaccine and anti-CTLA-4 monoclonal antibody was shown to significantly increase the anti-tumor immune response in mice comparing to using the individual treatments alone. However, the adverse reactions caused by the combination therapy is also significantly higher than using individual treatment (Liu et al., 2018). Therefore, optimizing the combination therapy to minimize harm towards the patients, while increasing cytotoxic effects to tumor cells remains to be a large concern for anti-CTLA-4 antibody treatment.

There are also ongoing clinical trials targeting specific receptors of the four different TNBC subtypes. For example, preclinical studies have been conducted on LAR subtype cell line MDA-MB-453 using androgen receptor antagonism (flutamide) and demonstrated inhibition on tumor proliferation (Doane et al., 2006). Phase II study with androgen receptor inhibitor, enzalutamide, also showed 25% clinical benefit rate in LAR subtype patients (Traina et al., 2018). A high proportion of MES subtype contain PI3K pathway alterations, such as PTEN loss or mutations activating PIK3CA, AKT1 or mTOR (Zhao et al., 2020). The PI3K signaling pathway is responsible for regulating cell growth and survival, and activation of the pathway can

lead to tumor progression and chemotherapy resistance (Pascual & Turner, 2019; Wein & Loi, 2017). Several clinical trials have produced success in using AKT inhibitor as neoadjuvant treatment: adding capivasertib, an AKT inhibitor, to paclitaxel increased progression-free survival from 3.7 to 9.3 months in patients with an altered PIK3CA tumor (Schmid et al., 2020). BLIS subtype correlates high rate of containing BRCA1/2 mutation and homologous recombination deficiency, making it a good target for platinum salts treatment (Zhao et al., 2020). Lastly, the BLIA subtype can be targeted using immunotherapies due to its activated immune response (Jiang et al., 2019). Subtype-specific treatments for TNBC is a promising direction, but more experimental data and clinical trials are needed before they can be widely used as treatment regimens.

Overall, with the establishment of TNBC subtypes, many treatment regimens and combinations are currently under testing as potential TNBC treatment. However, due to insufficient clinical data and safety data, the current major treatment option for TNBC remains to be chemotherapy and radiotherapy. More knowledge regarding TNBC would be helpful in discovering new therapy options.

1.2 Breast Cancer Metastasis

1.2.1 Metastasis Process

Breast cancer metastasis initiates with tumor cell invasion. For tumor cells to break away from the primary tumor, they must first alter their cell-to-cell adhesion, which is typically mediated through E-cadherin (Li & Feng, 2011). Epithelial-to-mesenchymal transition (EMT) is one mechanism proposed for breast cancer metastasis. Downregulation of E-cadherin is associated with disruption of the epithelial cell adhesion, while upregulation of N-cadherin facilitates the adhesion of tumor cells to stromal cells, supporting tumor cell invasion into the stroma (Cavallaro & Christofori, 2004). The downregulation of E-cadherin is also associated with activating downstream signaling pathways involved in promoting tumor cell migration and invasion (Makrilia et al., 2009).

Moreover, cell adhesion with extracellular matrix (ECM) is also altered or degraded to allow cell migration through the local tissue (Lingling Wang et al., 2021). Adhesion of the tumor

cells with the ECM is mediated through integrins (Mego et al., 2010) and the degradation of ECM is carried out by the metalloproteinases and the urokinase plasminogen activator (Danø et al., 2005; Egeblad & Werb, 2002). These steps allow tumor cell to penetrate tissue boundary and prepare for intravasation into blood circulation.

Tumor cells can secrete angiogenic factors to initiate vessel growth from pre-existing vessels or to recruit migrating endothelial cells (Asahara et al., 2000; Rafii, 2000). Tumor vessels provide breast cancer cells with nutrients and access to the systemic circulation. These blood vessels are in close proximity with tumor cells and have increased permeability compared to normal blood vessels, due to presence of abnormal openings throughout the vessel wall and defected endothelial cells (Hashizume et al., 2000).

Within the circulation system, tumor cells may migrate as an individual cell by activating the EMT cascade or may migrate as a cluster of cells (Friedl & Gilmour, 2009). Tumor cells can express chemokine receptors on their surface, which will bind to specific chemokines released at target organs, guiding the migrating tumor towards a site fitting for its extravasation and colonization (Müller et al., 2001).

After migrating through the circulation, breast cancer cells now face the challenge of extravasation. Depending on the organ, the vessel wall may be easily permeable or may prevent tumor cells from easily penetrating. Platelets and white blood cells can aid extravasation by forming complex with tumor cells, bringing them into the metastatic tissue (Yadav et al., 2015).

1.2.2 Immune System in Metastasis

Depending on the stage of breast cancer and the type of immune factors involved, the immune system can have a pro-tumorigenic effect or an anti-tumorigenic effect. The immune system also plays an important role in tumor metastasis and there are many immunotherapies proposed as treatment option for metastatic breast cancer.

One way the immune system can aid in breast cancer metastasis is by activating angiogenesis. One key player in promoting tumor angiogenesis is tumor-associated-macrophages (TAMs) (Lin & Pollard, 2007). TAMs associated with metastatic breast cancer were reported to express increased level of transcription factor hypoxia-inducible factor 2α (HIF-2α) compared to normal tissue-resident macrophages in breast (Leek et al., 2002). HIF-2α from the macrophages

and HIF-1α from the tumor cells (Talks et al., 2000) can collectively upregulate the expression of vascular endothelial growth factor (VEGF), which is pro-angiogenic (Loboda et al., 2012). Additionally, TAMs can also release enzymes degrading the ECM, such as collagenase and metalloproteases, which further facilitate tumor cell migration through the tissue (Leek et al., 2002).

Cytokines in the tumor microenvironment can also support tumor development and metastasis, by activating phenotypic transition within TAMs from M1 macrophages to M2 macrophages with IL-10 and TGF-β (Tower et al., 2019). M1 macrophages have anti-tumor function and can secrete elevated level of cytokines to illicit inflammation response (Chávez-Galán et al., 2015). Conversely, M2 macrophages are pro-tumorigenic and can exert their functions through suppressing matrix fibrosis and altering matrix protein composition to facilitate tumor migration (Pesce et al., 2009). They can also secrete factors such as IL-10, IL-12, VEGF and prostaglandin E2 to promote metastasis and angiogenesis, or to repress anti-tumor adaptive immunity (Schmieder et al., 2012). TNF-α can promote metastasis by activating tumor cell EMT through the NK-κB/Twist1 signalling pathway (Li et al., 2012). Blocking IL-8/CXCR1 signaling was also shown to significantly reduce the breast cancer stem cell population and systemic metastasis (Ginestier et al., 2010).

Moreover, fibroblasts present within the tumor microenvironment can produce and respond to cytokines and work cooperatively with immune cells. Cancer associated fibroblasts (CAF) can secrete nutrient-rich ECM proteins to support tumor cell growth and can produce ECM-degrading proteases to create a pro-metastatic tumor microenvironment (Boire et al., 2005; Sternlicht et al., 1999). CAF can also produce pro-inflammatory cytokines, which can cause cytokine imbalance to initiate angiogenesis and repress anti-tumor immune processes.

On the other hand, the immune system can also inhibit tumor growth and metastasis. CD8+ cytotoxic T lymphocyte plays a key role in eliminating tumor cells by targeting tumor cell-specific antigens and initiate direct cytotoxic killing of tumor cells (Mittendorf et al., 2012). Previous studies have shown that CD8+ T lymphocyte infiltration is associated with better patient prognosis and outcome, especially in basal-like triple-negative breast cancer (Liu et al., 2012; Mahmoud et al., 2011). Moreover, CD8+ T lymphocytes can differentiate into tissue-resident memory T cells, which express high levels of immune effector proteins and are

associated with even better prognosis comparing to regular CD8+ T lymphocytes (Savas et al., 2018). During acute inflammation, T helper cell type 1 produce cytokines such as IL-2 and IFNγ to repress tumor proliferation, activate macrophages, and promote antigen presentation to cytotoxic T lymphocytes to eliminate tumor cells (Tower et al., 2019).

In addition to T cells, many other immune cells also participate in tumor elimination. As mentioned before, M1 macrophages can release cytokines to activate anti-tumor inflammation. They are also capable of identifying tumor cells and releasing cytotoxic molecules, such as ROS and NO, to directly kill tumor cells (Bernsmeier et al., 2020) Reduced natural killer cell activity was shown to be associated with higher breast cancer stage and increased tumor progression (Mamessier et al., 2011). Dendritic cells were also reported to play a role in antigen processing and presenting to T lymphocytes to initiate anti-tumor immune response (Treilleux et al., 2004).

Overall, as concluded in **Figure. i**, there are both pro-tumorigenic and anti-tumorigenic immune cells within the tumor microenvironment. They can release cytokines or molecules to directly interact with tumor cells, cause changes to the ECM, or communicate with other immune cells.

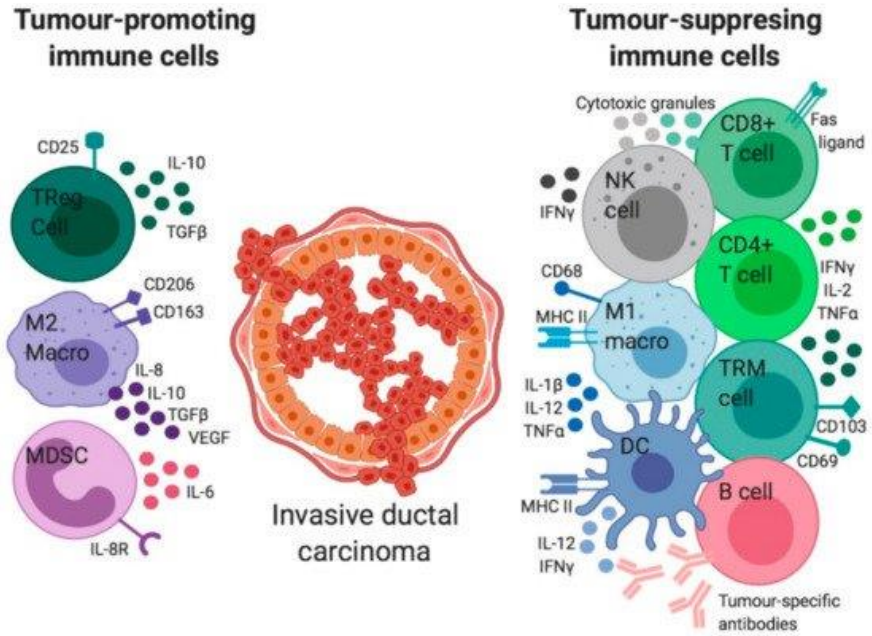

Figure. i. The immune microenvironment of invasive ductal carcinoma. Obtained from (Tower et al., 2019)**.**

There are pro-tumorigenic and anti-tumorigenic immune cells in the microenvironment. The anti-tumorigenic immune cells can secrete cytokines to inhibit tumor progression or initiate anti-tumor immune response. Natural killer cells and CD8+ cytotoxic T lymphocytes can also secrete cytotoxic granules to direct tumor killing. Conversely, the tumor-promoting immune cells can release cytokines to promote EMT, angiogenesis, tumor growth and inhibit anti-tumor immune response (Tower et al., 2019).

1.2.3 Role of p66ShcA in Tumor Metastasis

There are currently four known members within the Src homology and collagen domain (Shc) adaptor proteins family: ShcA (also termed Shc1), ShcB, ShcC, and ShcD. All members share a distinct domain organization of an N-terminus phosphotyrosine binding (PTB) domain, a C-terminus Src homology 2 (SH2) domain, and a central region containing tyrosine phosphorylation sites (Jones et al., 2007; Migliaccio et al., 1997; Natalicchio et al., 2011; Pelicci et al., 1992). The unique structure of Shc proteins allow them to interact with activated cell surface receptors such as G-protein coupled receptors (Chen et al., 1996), growth factor receptors (Pelicci et al., 1992) and cytokine receptors (Ravichandran & Burakoff, 1994). Upon binding, some isoforms of Shc proteins are phosphorylated at the tyrosine site and can interact with other mediators to activate downstream signaling cascades involved in cell proliferation, survival, migration and differentiation (Ahmed & Prigent, 2017). Among the four members, ShcB and ShcC expressions are nervous system-specific and function as mediators for neuronal signaling pathways (Nakamura et al., 1998). ShcD appears to be predominantly expressed in brain and skeletal muscle (Jones et al., 2007). Whereas ShcA is the most well-characterized and is ubiquitously expressed within many tissues (Pelicci et al., 1996; Pelicci et al., 1992).

The ShcA gene locus encodes for three isoforms: p66ShcA, p52ShcA, p46ShcA, which are named after their migration ability in SDS-PAGE (66kDa, 52kDa, and 46kDa) (Ventura et al., 2002a). Utilizing alternative promoter site, the ShcA gene locus gives rise to two transcripts: the p66ShcA transcript and the p52/46ShcA transcript. p52ShcA and p46ShcA can then be translated using different ATG codon as translation start site (Fig. ii) (Migliaccio et al., 1997; Ventura et al., 2002a). The three isoforms share identical PTB domain, collagen-homologous (CH) domain, and SH2 domain, while p66Shc contains an additional CH domain named CH2 (Fig. ii) (Migliaccio et al., 1997).

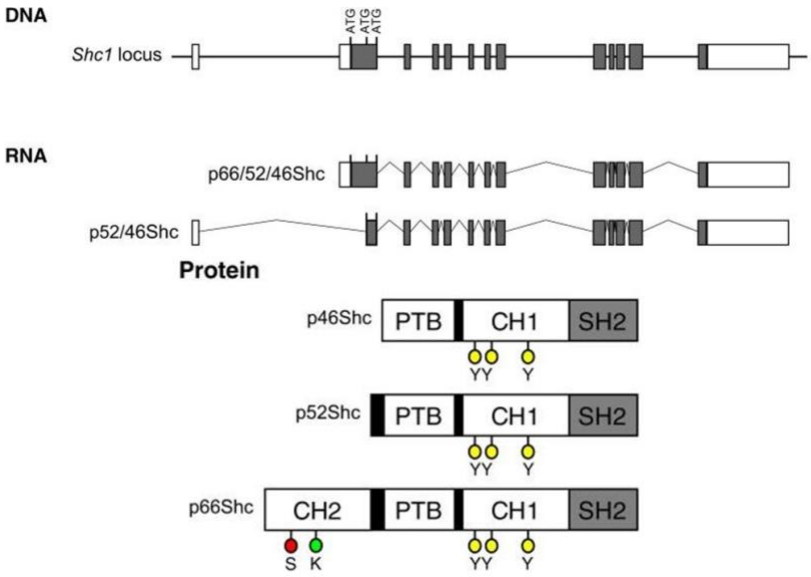

Figure. ii. Structure of ShcA locus, alternative transcripts, and protein isoforms. Adapted from (Wright et al., 2018).

The structure of ShcA gene locus, the two transcripts derived from alternative promoter site and the three protein isoforms derived from alternative ATG site are shown. The gray boxes on DNA/RNA sequence represent coding regions, while the white boxes represent noncoding regions. Amino acids important for ShcA protein functions are labeled on the protein structure: serine (red circles, S), lysine (green circles, K), and tyrosine (yellow circles, Y).

The p46/p52ShcA isoforms are ubiquitously expressed across cell types, whereas p66ShcA is only expressed in certain cell types with a highly variable expression level (Ventura et al., 2002a). The p45/p52ShcA isoforms are involved in conducting mitogenic signal transduction from tyrosine kinase to Ras that regulates cell proliferation (Pelicci et al., 1992). The PTB and SH2 domains allow ShcA proteins to interact with phosphotyrosine motifs of activated growth factor receptors to facilitate signal transduction (van der Geer et al., 1995). ShcA proteins also have a CH1 domain, which contains three key tyrosine phosphorylation sites (**Fig. ii.**) critical for tumor cell communication with the microenvironment (Ursini-Siegel et al., 2008). In addition to the conserved domains, the p66ShcA isoform has an additional cytochrome c binding domain and an N-terminus CH2 domain, which contains a serine residue (S36) that can be phosphorylated under oxidative stress stimuli (Migliaccio et al., 1999; Orsini et al., 2004). Upon phosphorylation, the prolyl isomerase Pin1 binds to the pS36 site, causing p66ShcA to

translocate into the mitochondria, where it can cause cell death and apoptosis through producing reactive oxygen species (ROS) (Gertz et al., 2008; Giorgio et al., 2005; Pinton et al., 2007).

Apart from its role in oxidative stress, p66ShcA has also been shown to be involved in tumor progression and metastasis. In lung cancer, loss of p66ShcA expression can cause hyperactivation of Ras signaling, leading to cancer cell proliferation and metastasis. p66ShcA expression was also shown to induce anoikis within detached lung cancer cells, preventing them from metastasizing (Ma et al., 2010). Overall, p66ShcA has anti-tumorigenic and anti-metastasis functions within lung cancer.

Conversely, p66ShcA promotes tumor metastasis within other types of cancer, such as breast cancer (Jackson et al., 2000), ovarian cancer (Muniyan et al., 2015) and prostate cancer (Lebiedzinska-Arciszewska et al., 2015; Veeramani et al., 2005). The opposing effects of p66ShcA on tumor progression in different cancer type may be caused by differences in tumor microenvironment or activation of variable mechanisms depending on the tumor tissue type (Hudson et al., 2018; Lewis et al., 2020; X. Li et al., 2014). Phosphorylation of p66ShcA isoform was shown to increase significantly within selected MDA-MB-231 breast cancer cell lines displaying site-preferential metastasis towards the lung comparing to parental cell line (Jackson et al., 2000). Similar trend was observed in 4T1 mouse TNBC cell lines, where p66ShcA is expressed at a higher level in selected cell lines preferentially metastasize towards the lung (Lewis et al., 2020). Moreover, p66ShcA protein expression was found to be increased within breast cancer specimens containing lymph node metastases, and the increased p66ShcA expression level correlates with an increased number of metastasis-positive nodes (Jackson et al., 2000).

Many articles have reported mechanisms through which p66ShcA can promote tumor metastasis. p66ShcA protein was shown to potentiate ARF6 activation, a member of the Ras superfamily of small monomeric GTPases that regulates actin cytoskeleton, intracellular trafficking and tumor migration (D'Souza-Schorey & Chavrier, 2006; Haines et al., 2014). The ShcA proteins were also shown to be recruited towards the focal adhesion complex through interacting with the focal adhesion kinase (FAK) (Wu et al.). The FAK/p66ShcA complex can activate the Rho guanyl exchange factors, leading to RhoA activation (Ma et al., 2007; Wu et al.), essential for actin cytoskeleton organization and regulating cell motility (Lessey et al.,

2012). Moreover, p66ShcA was reported to promote breast cancer metastasis by activating the EMT process. Overexpression of p66ShcA significantly upregulates EMT-promoting transcription factors and reduces expression of genes associated with adhesion and epithelial profile. Breast tumor cells with p66ShcA overexpression show increased level of vimentin and decreased level of E-cadherin, fitting for the EMT model (Hudson et al., 2014).

Furthermore, in a recent study published by the Ursini-Siegel lab, p66ShcA protein with a phosphorylatable Serine36 site was shown to be required for potentiating breast cancer metastasis towards the lung. Phosphorylation of the Ser36 site on p66ShcA promotes tumor cell survival within the circulation and colonization within the lung. However, expression of p66ShcA alone is not sufficient to increase lung-metastatic potential, suggesting the existence of additional players in promoting lung-metastatic breast cancer (Lewis et al., 2020).

1.2.4 Metastasis Organotropism

Metastasis organotropism occurs when breast cancer preferentially metastasizes towards specific organs.

In 1889, Stephen Paget noted that the distribution of breast cancer metastasis was not random and came up with the "seed and soil" hypothesis, where tumor cells are the "seeds" and preferentially spread towards organs with a suitable environment (the "soil") (Paget, 1989). Green and Harvey later showed that the interaction between tumor cells and the blood vessel endothelium also plays a role in determining metastasis site, expanding Paget's hypothesis to include cell interaction and adhesion as well (Greene & Harvey, 1964). In opposition, there were people rejecting Paget's hypothesis and declared that the anatomical structure of the vascular and lymphatic system is the key in regulating the metastatic site (Ewing, 1942). This hypothesis is supported by evidence such as the liver being the major metastatic target of gastrointestinal cancer due to the portal venous system (Langley & Fidler, 2011). The current consensus is that both the "seed and soil" hypothesis and the anatomical structure hypothesis cooperatively determine the metastatic site, so there is not one hypothesis superior to the other.

Breast cancer subtype is one factor affecting organotropism metastasis in tumor cells. Subtypes with enriched HER2 level were shown to be more prone of having brain metastasis, while triple-negative breast cancer has a higher probability of developing lung metastasis (Wu et

al., 2017). Another study investigating archival tissues of 3,726 patients confirmed that HER-enriched tumors have higher probability of brain, liver, and lung metastasis; whereas the basal subtype is more likely to metastasize towards the brain and lung, with a significant lower rate towards the bone and liver (Kennecke et al., 2010).

The immune system can also affect tumor metastasis sites. One major player involved in promoting organ-specific metastasis is tissue-resident microphages. For example, Kupffer cells are liver-specific microphages that can phagocytose tumor cells (Kan et al., 1995) and initiate Fas-mediated apoptosis within cancer cells (Song et al., 2001). However, Kupffer cells can also facilitate liver-metastasis by arresting circulating tumor cells at the liver sinusoid and promote liver colonization (Bayón et al., 1996). Alveolar macrophages in lung have both pro-metastatic and anti-metastatic abilities as well. They can secrete inflammatory cytokines to initiate immune response and mediate macrophage-directed tumor killing (Almatroodi et al., 2014). On the other hand, the alveolar macrophages can upregulate the production of leukotriene B4, a leukotriene capable of inducing tumor cell proliferation and growth in lungs (Nosaka et al., 2018). The microphages can also bind to breast cancer cells by the α4-integrin-VCAM-1, guiding them inside the tissue. Moreover, microglia (brain microphages) (Pukrop et al., 2010) and osteoclasts (bone macrophages) (Lu et al., 2011) were also shown to display pro-metastatic properties other than their usual phagocytic and cytotoxic functions, suggesting that tissue-residing macrophages may transit between pro- and anti-metastatic properties depending on stimulation and cell signaling from the tumor cells.

The major cancer metastatic sites also have other distinct properties supporting organ-specific metastasis. In liver, hepatic stellate cells can release periostin, leading to fibrosis within the microenvironment to support metastatic pancreatic tumor growth (Nielsen et al., 2016). Human hepatic sinusoidal endothelial cells were shown to produce macrophage migration inhibitory factors to promote chemotaxis, EMT, proliferation and apoptotic resistance within colorectal cancer cells (Hu et al., 2015). Moreover, the hepatic blood vessels also play a role in regulating liver metastasis. The high level of angiopoietin-like 6 proteins within the hepatic blood vessels interacts with integrin/E-cadherin of circulating colorectal cancer cells, promoting colonization of the cancer cells into the liver tissue (Marchio et al., 2012). In addition to molecular features, the structure of the hepatic vessels is also unique. The vasculatures have

small windows and no sub-endothelial basement membrane, allowing transportation of bigger molecules across the vasculature wall (Reichen, 1999), supporting development of unique microenvironment and interactions.

Compared to liver-metastatic, more studies regarding organ-specific breast cancer metastasis were conducted in lung. One factor leading to lung-specific metastasis is interaction between lung vascular endothelial cells and tumor cells. To effectively facilitate gas exchange, the pulmonary capillaries are very thin, which also increases the possibility of physically trapping tumor cells within the narrowing blood vessels (Lu & Kang, 2007). Cancer cell extravasation and adhesion is regulated by chemokine receptors and ligands expressed on tumor cells and endothelial cells. Breast tumor has a high expression level of chemokine receptors CXC44 and CCR7, while their respective ligand CXCL12/SDF-1 and CCL21/6Ckine are expressed at a high level within the lung, mediating migration and adhesion of breast tumor cells towards the lung (Müller et al., 2001). $\alpha 6\beta 4$ integrin expressed on breast tumor cells was shown to bind to human CLCA2 protein expressed on endothelial cells of the pulmonary arteries, arterioles, and venules (Abdel-Ghany et al., 2001). In addition to proteins involved in adhesion, signature lung-metastatic genes can also target breast cancer to migrate towards the lung. Genes such as EGF receptor ligand epiregulin, the cyclooxygenase COX2 (Gupta et al., 2007) and cell adhesion molecules matrix metalloproteinases 1 and 2 (MMP-1 and MMP-2), SPARC, and Vcam1 (Minn et al., 2005) were found to be associated with lung-metastasis.

Similar to lung-metastasis, genomic profiling of bone-metastasis breast cancer cell lines has revealed many genes involved in bone-specific metastasis, such as CXCR4, MMP1, ADAMTS1, FGF5, CTGF, IL11, follistatin, and proteoglycan-1. Most of the genes encode cell surface or secretory proteins responsible for facilitating interactions with tumor cells, moderating the bone microenvironment and promoting angiogenesis or tumor invasion (Kang et al., 2003). One notable feature of bone-metastasis is that the gene signature profile of these metastases are very different compared to that of lung-metastasis, suggesting that different organ-specific metastasis requires regulation of different set of genes. Other than the genetic profile of tumor cells, bone resident cells can also secrete different factors to prepare an optimum environment for breast cancer colonization. Following bone resorption by osteoclasts, factors such as PTHrP and IGF1 are released from the bone, which can stimulate tumor proliferation, further bone

resorption and downstream expression of other factors involved in bone-specific metastasis (Hiraga et al., 2001). Furthermore, bone marrow-derived haematopoietic progenitor cells can express vascular endothelial growth factor receptors 1 (VEGFR1) that are recruited to form cellular clusters before the arrival of tumor cells. VEGFR1 also upregulates fibronectin in resident fibroblasts to set up a permissive niche to attract metastatic tumor cells (Kaplan et al., 2005). Bone colonization can also be induced by interaction of N-cadherin expressed on osteocytes and tumor E-cadherin. Moreover, the osteocytes were reported to produce factors such as CCL5, MMP and adenosine to promote tumor migration into the bone microenvironment (Sottnik et al., 2015).

The concept of organotropism metastasis was also demonstrated through selection of TNBC cells that preferentially metastasize towards specific organs. 4T1 is a murine breast cancer cell line that was isolated from a spontaneously arising breast tumor in a BALB/c mouse (Aslakson & Miller, 1992). 4T1 can be used as an animal model for stage IV human breast cancer and can metastasize towards the bone, liver, lung and brain once injected into the mammary fat pad of BALC/c mice (Aslakson & Miller, 1992; Eckhardt et al., 2005; Lelekakis et al., 1999). 4T1-derived cell lines demonstrating site-preferential metastasis towards the lung, liver and bones were developed by Dr. Peter Siege's lab (Tabariès et al., 2015). The parental 4T1 cells were subjected to two or three rounds of in vivo selection after being injected into BALB/c mice. For each round, cells metastasized towards a specific organ were explanted and re-injected into mice. After selection, cell lines that preferentially and aggressively metastasize towards the bone (Rose et al., 2007), the lung (Rose et al., 2010) and the liver (Tabariès et al., 2011) were established. Gene expression microarray on the cell lines showed 395 differentially expressed genes and the cell lines preferentially metastasizing towards the same organ are clustered closely together (Tabariès et al., 2015). The lung- and liver-metastatic cell lines were shown to recruit T lymphocytes and granulocytic infiltration, suggesting cells metastasizing towards different organs have differential immune response as well (Tabariès et al., 2015). Moreover, Lewis et al. demonstrated that these cell lines express differential level of p66ShcA, depending on their metastatic preferential site. The lung- and liver- metastatic cell lines express p66ShcA at a higher level comparing to the other cell lines (Lewis et al., 2020). Considering that p66ShcA expression is under epigenetic regulation, it is possible that there are other epigenetically regulated genes

and pathways involved in promoting TNBC organotropism metastasis, which is the main research target of this project.

Overall, depending on the site of metastasis and tumor type, varied molecular factors and mechanisms are involved in promoting organ-specific metastasis. By investigating the distinct mechanisms, it is possible to improve the existing treatment regimens targeting invasive breast cancer.

1.3 Epigenetic Regulation of Gene Transcription

1.3.1 Gene Transcription Processes

Gene transcription is a critical step in regulating gene expression during development. Gene transcription is carried out by RNA polymerase (RNA Pol), which binds to specific DNA promoters and transcribes RNA transcripts from the DNA sequence. Coding RNA transcripts then are transported into the cytoplasm to be translated and subsequently carry out essential cellular functions. Over fifty years ago, three forms of RNA polymerases were identified in eukaryotic organisms (Roeder & Rutter, 1969) and were later shown to exhibit different transcriptional roles (Sentenac, 1985). RNA Pol I transcribes the ribosomal RNA precursors within the nucleolous; RNA Pol II transcribes messenger RNAs encoding proteins, majority of microRNAs and small nuclear RNAs; and RNA Pol III transcribes transfer RNAs and the small ribosomal RNA (Cramer, 2019). In this thesis, RNA Pol II will be the focus of discussion.

The first step in gene transcription is the recruitment of the pre-initiation complex onto the DNA sequence core promoter site (Fuda et al., 2009). Other than the core promoter, regions proximal to the core promoter and more distant enhancer sites can also regulate gene transcription by binding to various transcription factors (Fuda et al., 2009). These transcription factors subsequently recruit coactivators that either phosphorylate RNA Pol II to enhance its activity or less commonly, recruit RNA Pol II to promoters. The human genome contains approximately three billion base pairs of DNA (McGinty & Tan, 2015). To store this large amount of information into the cell nucleus, DNA is organized as nucleosomes, structures with 145 to 147bp of DNA wrapped around histone protein octamers (Simpson, 1978). Nucleosomes compact DNA sequences and can make the promoter inaccessible for polymerases, hence inhibit

initiation of gene transcription (Lorch et al., 1987). An active promoter is located at an open-chromatin region, where the DNA is not compacted and there is no nucleosome blocking access.

One class of promoter for RNA Pol II is those harboring CpG islands, which are short DNA sequences capable of destabilizing nucleosomes and recruiting proteins involved in opening chromatin (Deaton & Bird, 2011). The function of CpG islands may be repressed by addition of DNA methylation, through the binding proteins with a methyl-CpG domain onto the methylated sequence (Schübeler, 2015). Another class of promoters for RNA Pol II contains a TATA box upstream of the transcription start site (Müller & Tora, 2014), where the TFIID complex binds, and acts to recruit RNA Pol II.

TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH are the six general transcription factors involved in facilitating RNA Pol II promoter recognition and transcription initiation (Reinberg et al., 1998; Roeder, 1996). The list of steps required for pre-initiation complex formation is shown in **Figure iii** (Sainsbury et al., 2015). TFIID contains a TATA-binding protein (TBP) subunit and TBP-associated factors (TAFs). TBP is responsible for recognizing and binding the upstream TATA-sequence (Burley & Roeder, 1996), whereas the TAFs can interact with activators or downstream promoter elements to further facilitate gene transcription (Burke & Kadonaga, 1997). TFIIA provides stabilization for the TFIID-TATA box complex by binding with the TBP subunit and upstream DNA sequences (Ranish & Hahn, 1991; Tan et al., 1996). This stabilization stimulates TFIID binding and is crucial when the TATA-box sequence is weak or when there is a mutation in the TBP subunit causing insufficient binding (Buratowski et al., 1989). The TBP-TATA box interaction creates a binding point for TFIIB, which recruits the TFIIF-RNA Pol II complex (Buratowski et al., 1989; Li et al., 1994). Like TFIIA, TFIIB was also shown to stabilize the interaction of TBP with the TATA-box sequence during weakened binding conditions (Imbalzano et al., 1994). Other than its role in directing RNA Pol II towards the transcription start site, TFIIF can also repress binding of Pol II to random sites on DNA (Roeder, 1996). The TFIIE factor then binds the complex through direct interaction with RNA Pol II and subsequently recruits TFIIH, thus completing the assembly of the pre-initiation complex at the transcription start site (Maxon et al., 1994).

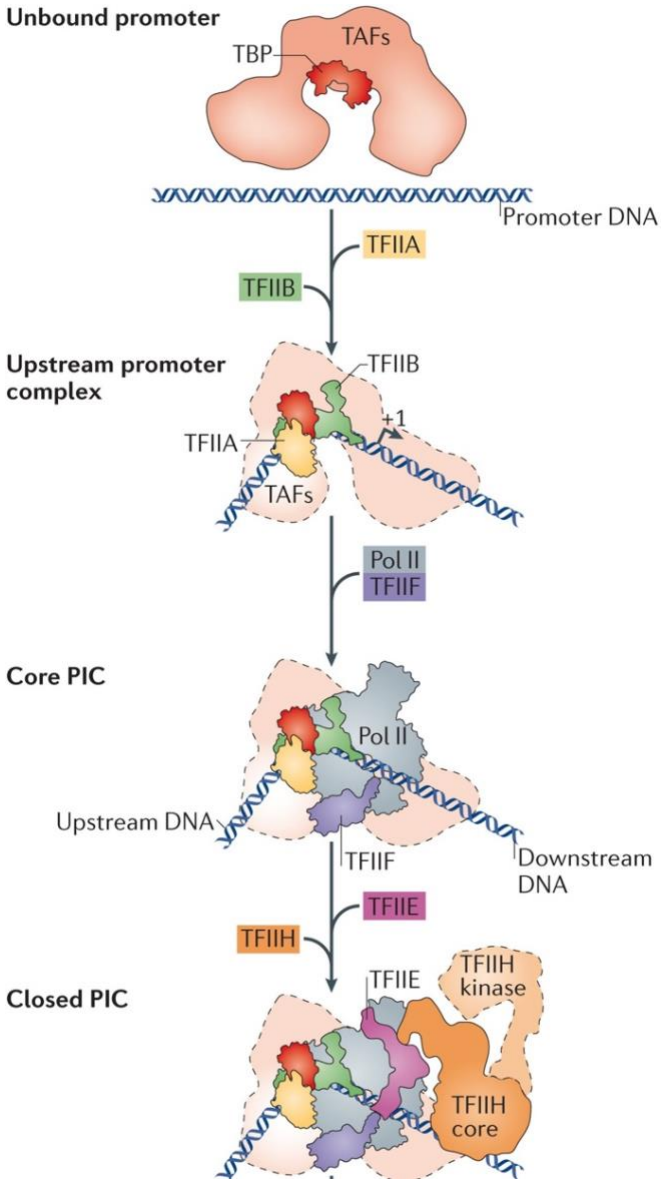

Fig. iii. Sequential assembly of the general transcription factors into the preinitiation complex. Adapted from (Sainsbury et al., 2015)

The stepwise recruitment of TFIID onto the TATA-box sequence, TFIIB, TFIIF/Pol II complex, TFIIE, and TFIIH to complete the assembly of the pre-initiation complex on the transcription promoter of DNA.

One important function of the pre-initiation complex is to open the promoter site to create a single-stranded region, which can be accessed by RNA Pol II to initiate transcription. The XPB and XPD subunits of TFIIH are ATPases with helicase activity (Guzder et al., 1994; Sung et al.,

1993). The XPB subunit is responsible for unwinding DNA at the promoter site to allow access of the RNA Pol II (Guzmán & Lis John, 1999), whereas the XPD subunit is involved with DNA repair pathways (Coin et al., 2007). The opened promoter region forms a transcription bubble allowing RNA Pol II to interact with the DNA sequence. The initial transcription bubble opened by TFIIH extends from -9 to -2 relative to transcription start site and expands downstream as nucleotide triphosphatases are added to synthesis RNA strand (Holstege et al., 1997).

The next step in gene transcription is the transition of RNA Pol II complex from initiation status to elongation. After synthesizing the first few phosphodiester bonds, RNA Pol II must be uncoupled from the promoter sequence and the pre-initiation factors to continue transcription downstream, which is a process named promoter clearance (Hsu Lilian & Lovett Susan, 2008). This process starts when the RNA-DNA hybrid in the initiation complex is synthesized beyond 8bp long. (Westover et al., 2004). As the RNA polymerase moves downstream of the DNA, the initiation transcription bubble continues to expand, and the upstream portion will eventually collapse (Holstege et al., 1997; Pal et al., 2005). The collapsed bubble now contains about 10bp of open DNA sequences, which is the typical size of a transcription elongation bubble. Moreover, transcription no longer requires assistance from the TFIIH factor after this step and most pre-initiation complex factors dissociate from Pol II (Pal et al., 2005).

During elongation, corresponding nucleoside triphosphate substrate enters the pre-insertion site, initiating conformation change of RNA Pol II into a catalytically active state (Vassylyev et al., 2007). A phosphodiester bond is then formed using two-metal-ion phosphoryl-transfer mechanism (Steitz & Steitz, 1993).

Certain DNA sequence can disturb the elongation process and cause transcription pausing, which may lead to polymerase backtracking, RNA dissociation and transcription termination (Landick, 2006). Polymerase pausing during initial elongation was shown to be a critical regulation step especially for genes involved in stimulus-controlled pathways (Kwak et al., 2013). RNA Pol II pausing is regulated and maintained by transcription factors, negative elongation factors (NELF) and DRB-sensitivity-inducing factors (DSIF) (Yamaguchi et al., 1999). To release RNA Pol II from the pausing position and continue transcription, positive transcription elongation factor-b (P-TEFb) is recruited to the promoter site. P-TEFb is a kinase composed of a catalytic subunit cyclin-dependent kinase 9 (CDK9) and regulatory subunit cyclin

T (Lis et al., 2000; Majello et al., 1999). P-TEFb can phosphorylate the carboxy-terminal domain (CTD) of Pol II, NELF, and DSIF (Marshall et al., 1996). Upon phosphorylation, NELF dissociates from Pol II (Fujinaga et al., 2004), whereas DSIF transits into a positive elongation factor (Yamada et al., 2006).

Finally, when RNA Pol II reaches the end of the gene sequence, transcription termination begins. A polyadenylation signal appears near the end of the DNA sequence, after being transcribed onto the nascent RNA strand by RNA Pol II, the poly(A) signal can recruit the 3′-end cleavage and polyadenylation (CPA) complex to the Pol II CTD subunit (Ahn et al., 2004). CPA releases the synthesized mRNA strand from the transcription complex into the cytoplasm, awaiting future translation (Proudfoot, 2016). One proposed mechanism of transcription termination mechanism involves Xrn2, a 5' to 3' exonuclease. The RNA Pol II continues to transcribe DNA, while the short RNA transcript produced are degraded by Xrn2. When Xrn2 catches up with Pol II, it stimulates the release of RNA Pol II from DNA sequence and can be recycled for future transcription (Connelly & Manley, 1988; Proudfoot, 1989). Another proposed mechanism for termination is facilitated by the association of CPA with the CTD domain of RNA Pol II. The association leads to dephosphorylation of Pol II and results in Pol II dissociation from the DNA sequence (Parua et al., 2018; Proudfoot, 2016).

1.3.2 Histone Acetylation

Epigenetics is defined as 'the study of heritable alterations in gene expression that are not caused by changes in the DNA sequence' (Waterland, 2006). All cells within the same organism carry the same DNA information, however, gene expression can differ significantly across cell types. The different gene expression pattern across cell types permits differentiation into specialized tissue. It is important that transcriptional profiles which maintain tissue identity are stable through mitosis. This heritable signature of gene transcription is strongly regulated by epigenetic mechanisms. There are three primary types of epigenetic regulation: 1. methylation of DNA on cytosines; 2. post-translational histone modification and chromatin remodeling; 3. Non-coding RNA (Gibney & Nolan, 2010). This thesis will mainly focus on the first two categories.

In eukaryotic cells, DNA wrap around histone proteins to form chromatin. The basic unit of chromatin is the nucleosome, where two of each core histones (H2A, H2B, H3 and H4) forms a histone octamer, wrapped by around 147bp of DNA (Alberts et al., 2003). Another type of

histone (H1) binds to DNA sequences between the nucleosomes (Gibney & Nolan, 2010). The core histones contain a central globular region with unstructured polypeptide N-terminal histone tail. The highly basic N-terminal tails protrude into the surrounding environment and can interact with neighbouring nucleosomes to regulate chromatin structure and gene transcription (Bannister & Kouzarides, 2011). Extension of the N-terminal tails into the environment makes them accessible to histone modification machineries, therefore most histone modifications occur on the histone tails (Berger, 2007). There are multiple types of post-translational modification that can occur on histone, including acetylation, methylation, phosphorylation, ubiquitylation and SUMOylation (Berger, 2007). Lysine is a key amino acid in histone modification and can be acetylated, methylated, ubiquitylated and SUMOylated (Jambhekar et al., 2019; Marmorstein & Zhou, 2014). Acetylation of the lysine residues generally leads to activate gene transcription and SUMOlyation is mostly associated with repression. On the other hand, methylation and ubiquitylation can have varying effects. Arginine residues can be methylated to activate gene transcription (Jambhekar et al., 2019). Another common histone modification is phosphorylation of the tyrosine, threonine and serine residues, which is also related with transcription activation (Ellenbroek & Youn, 2016).

The "histone code hypothesis" states that post-translational histone modifications regulate gene transcription through recruiting regulatory proteins and modifying the chromatin structure (Jenuwein & Allis, 2001). The enzymes responsible for adding histone modifications are often referred as "writers". Depending on the type of histone modification carried out, the "writers" can be classified as histone acetyltransferases, histone methyltransferases, or other types. Opposite to the function of "writers", "erasers" are responsible for removing specific histone markers, such as histone deacetylases and histone demethylases (Gillette & Hill, 2015). After establishment of the histone marker, proteins with domains that can recognize and interact with specific histone modifications are recruited. These proteins are often referred as "readers" and can regulate gene transcription through direct interaction with the transcription machinery or modifying the chromatin structure between an open euchromatin state and a closed heterochromatin state (Taverna et al., 2007). Euchromatin is defined as lightly packed chromatin, sparsely populated with nucleosomes and linker H1 histones. This allows access of the RNA Pol II transcription complex and trans-activating factors onto the DNA binding elements to upregulate gene expression. In contrast, heterochromatin is defined as chromatin enriched for

nucleosomes and the linker H1 protein, making DNA inaccessible to the transcription machinery (Kouzarides, 2007; Liu et al., 2005).

One well-studied histone modification is acetylation. Acetylation of lysine residues in the protruding N-terminal tails is recognized by proteins containing bromodomains (Taverna et al., 2007). Until 2017, 61 bromodomain modules and 42 bromodomain-containing proteins have been identified in human, which carry a wide variety of functions (Fujisawa & Filippakopoulos, 2017). Bromodomain-containing proteins may promote further chromatin structure remodeling by carrying out functions such as histone acetyltransferases (Ogryzko et al., 1996) or by facilitating binding of chromatin remodeling SWI/SNF complex (Hassan et al., 2001). They can also facilitate the assembly of pre-initiation transcription complex on the promoter site to promote gene transcription (Kasper et al., 2006; Kim et al., 1998). Moreover, some members are involved in histone eviction during transcription elongation (Carey et al., 2006).

Histone acetylation is carried out by histone acetyltransferases (HATs), which catalyze the transfer of an acetyl group from acetyl-CoA onto the lysine residue. Action of HATs can be reversed by histone deacetylases (HDACs) (Sterner David & Berger Shelley, 2000). There are three main types of acetyltransferases: the GNAT superfamily, the MYST family, and p300/CBP (Sterner David & Berger Shelley, 2000). The GNAT superfamily is characterized by four conserved motifs, C, D, A, and B (Neuwald & Landsman, 1997). Motifs A and B are involved in binding to different moieties of acetyl CoA, whereas motif D stabilizes the core structure of the protein (Srivastava et al., 2014). Furthermore, the GNAT acetyltransferases contain an Arg/Gln-X-X-Gly-X-Gly/Ala segment for acetyl CoA recognition (Wolf et al., 1998). The MYST family members are characterized by an acetyltransferase homology motif, which is similar to part of motif A of the GNAT superfamily (Neuwald & Landsman, 1997). Another well-studied acetyltransferase is p300/CBP. P300/CBP is a transcriptional co-activator possessing histone acetyltransferase ability and are expressed ubiquitously within higher eukaryotes, regulating cellular functions such as apoptosis, differentiation, and cell cycle (Giordano & Avantaggiati, 1999). P300/CBP contains at least four interaction domains, allowing interaction with various receptors or transcription factors, such as CREB, hormone receptors, c-Jun, and c-Myc (Yang et al., 1996). It can interact directly with transcription factors or modify the chromatin structure to promote gene transcription.

Histone acetylation is generally associated with upregulating gene expression. On a nucleosome, positively charged histone tails interact with the negatively charged DNA to organize and stabilize chromatin folding (Fletcher & Hansen, 1995). Upon acetylation, the lysine residues on the histone tails lose their positive charges and become neutralized, leading to weakened histone-DNA interaction and chromatin unfolding (Hong et al., 1993). Acetyl group added to the lysine residue also provides a binding site for bromodomains, which are present within many transcription factors and chromatin modifiers (Yang, 2004). By opening up the chromatin structure, RNA polymerase complex has an easier access to initiate transcription.

Two well-studied histone acetylation marks will be discussed: H3K27ac and H3K9ac. Acetylation of histone H3 on lysine 27 (H3K27ac) is carried out by p300/CBP (Tie et al., 2009). By comparing the enrichment data of H3K27ac with previously generated enhancer maps, Creyghton Menno et al. demonstrated that H3K27ac enrichment can be used to distinguish active enhancers from inactive enhancers. Moreover, the global H3K27ac pattern can be used to determine current and future cell development state (Creyghton Menno et al., 2010). Furthermore, H3K27ac is shown to be present next to proximal promoters or transcription start sites as well (Heintzman et al., 2009). The exact mechanisms exerted by promoter-specific H3K27ac remains unknown, but a recent article has suggested that it may play a role in associating with the transcription elongation machinery (Gao et al., 2020).

Acetylation of histone H3 on lysing 9 is carried out by GCN5 (GNAT superfamily) and PCAF (associated with p300/CBP) in mammals (Grant et al., 1999). GCN5 and PCAF are found within the SAGA complex and the ATAC complex in a mutually exclusive manner (Wang et al., 2008). Other than neutralizing lysine charges to unfold chromatin structure, reader proteins can also recognize modified histones and regulate gene transcription. The AF9 YEATS domain is shown to bind strongly with H3K9ac and upregulate gene expression (Li et al., 2016). Moreover, AF9 YEATS binding with H3K9ac recruits H3K79 methyltransferase DOT1L to the chromatin (Y. Li et al., 2014), which is responsible for removing regulatory proteins from the silenced chromatin (Fingerman et al., 2007).

1.3.3 Histone Methylation

Histone methylation involves the transfer of a methyl group onto the basic lysine, arginine and histidine residues of histone (Byvoet et al., 1972). Lysines can be monomethylated,

dimethylated or trimethylated (Rice & Allis, 2001). Common lysine methylation sites include H3K4, H3K9, H3K27, H3K36 and H4K20 (Greer & Shi, 2012). Arginines can be monomethylated, symmetrically dimethylated or asymmetrically methylated on sites such as H3R2, H3R8, H3R17 and H4R3 (Bedford & Richard, 2005; Greer & Shi, 2012). Histidines were shown to be monomethylated, but the occurance is very rare and not well-studied (Gershey et al., 1969). Histone methylation is deposited by methyltransferases and removed by demethylases. Epigenetic readers that carry chromodomains recognize methylation and regulate gene expression (Jambhekar et al., 2019; Zhao & Garcia, 2015). The majority of histone lysine methyltransferases carry a catalytic SET domain for enzymatic activity (Rea et al., 2000), except for the H3K79 methyltransferase DOT1 and homologues (Feng et al., 2002). Most lysine methyltransferases show site-preference or site-specific methylation (Lin et al., 1996).

H3K9 and H3K27 methylations are generally associated with gene repression. H3K9 is shown to be able to accept multiple methylations, becoming H3K9me (monomethylation), H3K9me2 (dimethylation), and H3K9me3 (trimethylation) (Rice & Allis, 2001). Using antibodies targeting H3K9 methylation, Rice et al. showed that G9a regulates H3K9me and H3K9me2, whereas Suv39h1 and Suv39h2 regulate H3K9me3. Moreover, dual staining experiments demonstrated that H3K9me3 does not localize with H3K9me or H3K9me3, whereas H3K9me and H3K9me2 show overlapping regions. This discovery suggests that H3K9me3 has distinct functions comparing to the other two methylation patterns, whereas H3K9me and H3K9me2 share similar functions and regions. Indeed, H3K9me and H3K9me2 are present throughout the whole genome and are primarily enriched on transcriptional silent regions. H3K9me3 is mainly enriched on pericentric heterochromatin region (Rice et al., 2003), which is constitutive heterochromatin that participates in cell division (Fioriniello et al., 2020). H3K9me3 is recognized and bind by the N-terminal chromodomain of heterochromatin protein 1 family (HP1, CBX, and Swi6) (Bannister et al., 2001). HP1 proteins bind to H3K9me3 residues through their chromodomain, then dimerize, and condense the chromatin, repressing access of RNA Pol II to the DNA sequence (Hiragami-Hamada et al., 2016; Machida et al., 2018).

H3K27me3 is enriched on facultative heterochromatin, which are chromatin regions that can convert between the open chromatin state and the closed chromatin state, based on cell type and development stage (Heard, 2005; Trojer & Reinberg, 2007). H3K27 is tri-methylated by the

catalytic SET domain of Polycomb repressive complex 2 (PRC2) (Kuzmichev et al., 2002). Trimethylated H3K27 site provides a binding target for PRC1 (Kuzmichev et al., 2002), which represses gene transcription through oligomerization and chromatin condensation (Cao et al., 2002). H3K27me3 also occludes binding of RNA Pol II (Chopra et al., 2011).

Opposing of the activities of H3K9 and H3K27 methylation, H3K4 methylation enrichment is detected at all active genes (Bernstein et al., 2005). There are multiple methyltransferases able to methylate the H3K4 site in mammals, such as the MLL proteins MLL1-4, Set1A and Set1B of the COMPASS family of methyltransferases (Hughes et al., 2004; Shilatifard, 2006). The methyltransferases can methylate the H3K4 site to become H3K4me1, H3K4me2, and H3K4me3 (Shilatifard, 2008). Conversely, Set1 is the only methyltransferase regulating H3K4 methylation in yeast. The review suggested that the high number of methyltransferases involved in humans is due to the high level of complexity of methylation profiles in eukaryotes. Methylation of lysine residues can be repressive or activating, and need to be carefully regulated, so more methyltransferases are needed to properly facilitate the differential histone methylation pattern within different cells or organs (Shilatifard, 2008). H3K4me3 is mainly associated with the promoter site of active genes (Bernstein et al., 2005). The CHD1 protein, responsible for maintaining an open chromatin state, and proteins containing a plant homeodomain finger (PHD) were shown to interact directly with H3K4me3 to promote gene transcription (Shi et al., 2006; Sims et al., 2005). H3K4me3 is a binding target for the transcription factor TFIID via the PHD finger of TAF3, facilitating transcription initiation (Vermeulen et al., 2007). H3K4me1 was shown to be highly enriched at almost all enhancers and can be used as a predictor of active distal enhancer locations (Heintzman et al., 2007). H3K4me can also be detected near transcription start sites (Core et al., 2014). Some articles suggest that the ratio of H3K4me3 to H3K4me1 enrichment on the promoter site corresponds to the rate of RNA Pol II recruitment and gene transcription (Core et al., 2014). An alternative view suggests that the pattern of enrichment of the two histone modification markers affect gene transcription level: the gene is active when H3K4me1 flank H3K4me3 (Cheng et al., 2014).

1.3.4 DNA Methylation

DNA methylation is an epigenetic modification that involves the transfer of a methyl group onto the C5 position of cytosines in the DNA sequence (Moore et al., 2013). Modified

cytosines were first discovered by Rolin Hotchkiss using paper chromatography in 1948 and he hypothesized that the cytosines were modified through methylation (Hotchkiss, 1948). Then in 1979, Taylor & Jones reported that treating cells with DNA methylation inhibitor led to cell differentiation, establishing the involvement of DNA methylation in regulating gene expression (Taylor & Jones, 1979).

Throughout the human genome, methylated cytosines only account for less than 1% of total DNA bases. Brain and thymus have the highest amount of DNA methylation (around 1%), whereas placental and sperm DNA are the least methylated (0.76 and 0.84%) (Ehrlich et al., 1982). DNA methylation predominantly occurs on CpG sites, which are regions of DNA where a cytosine precedes a guanine base. Most CpG sites in human (around 70 to 80%) are methylated, except for the CpG sites occurring in CpG islands (Antequera & Bird, 1993; Bird et al., 1985; Bird, 1995).

DNA methylation is carried out by DNA methyltransferases (DNMTs) that facilitates transferring of a methyl group from S-adenyl methionine to the fifth carbon of a cytosine residue of DNA (Moore et al., 2013). There are four types of DNMTs, including DNMT1, DNMT3A, DNMT3B, and DNMT3L (Jin & Robertson, 2013). Among the four members, DNMT3L does not have any enzymatic activity, but interacts with the other DNMTs to facilitate DNA methylation (Kareta et al., 2006). DNMT1 is responsible for copying the DNA methylation pattern of the parental strand during DNA replication, whereas DNMT3A/3B are responsible for de novo methylation to establish new DNA methylation pattern (Okano et al., 1999; Okano et al., 1998).

DNA methylation is classically associated with transcriptional repression, but its enrichment at different genomic regions can have differential effects in regulating gene transcription (Moore et al., 2013). DNA methylation can occur in intergenic regions, which account for approximately 45% of the human genome and encode noncoding DNA elements such as LINES and SINES (Schulz et al., 2006). DNA methylation at the intergenic regions silence the transcription of viral and transposable elements, preventing them from inserting into genes to disrupt gene structure and cause mutations (Kuster et al., 1997; Wu et al., 1997). DNA methylation can also be enriched on gene bodies, which is the genomic DNA sequence downstream of the first exon. DNA methylation of the gene body regions in dividing cells was

shown to upregulate gene expression level (Aran et al., 2011; Hellman & Chess, 2007). However, DNA methylation of gene body regions in non-dividing or slow dividing cells, such as brain cells, did not show any increase in gene expression level (Aran et al., 2011). Lastly, DNA methylation can be enriched on CpG islands, which is the most studied type of DNA methylation enrichment.

CpG islands are around 1kb DNA regions with high frequency of CpG sites, defined with a GC composition greater than 50% and an observed/expected CpG ratio greater than 60% (Gardiner-Garden & Frommer, 1987). CpG islands are present near the transcription start site of around 40% of genes and are rarely methylated in normal cells (Larsen et al., 1992). DNA methylation at the promoter site represses gene transcription through various mechanisms. The presence of DNA methylation near promoter region can block the physical access of transcription factors to DNA sequence, hence repress gene transcription (Bird, 2002). DNA methylation can also be recognized by three proteins families: the MBD proteins, the UHRF proteins and the zinc-finger proteins (Moore et al., 2013). The majority of MBD proteins contain a methyl-CpG-binding domain (MBD) for interacting with the methylated CpG island (Nan et al., 1993) and a transcriptional repression domain (TRD) that can bind to various repressor complexes (Nan et al., 1998). Moreover, some MBD proteins can stabilize DNA methylation profile by recruiting DNMT1 via the TRD domain for DNA methylation maintenance (Kimura & Shiota, 2003). The UHRF proteins functions similarly as the MBD proteins in maintaining DNA methylation profile. They bind to the methylated DNA sequence through a SET- and RING-associated DNA-binding domain, then recruit DNMT1 to the DNA sequence (Hashimoto et al., 2008). Lastly, Kaiso, ZBTB4 and ZBTB38 are zinc-finger proteins that can bind to DNA methylation through their zinc-finger motif (Prokhortchouk et al., 2001). The proteins contain an N-terminus POZ domain that can facilitate protein heterodimerization, homodimerization and interaction with corepressor components, such as histone deacetylase and N-CoR nuclear corepressor (Park et al., 2005).

Tumor cells were reported to have an altered DNA methylation profile comparing to normal cells, which contains a global hypomethylation throughout the genome and hypermethylation at specific genomic regions, mainly occurring on CpG islands (Baylin et al., 1998; Goelz et al., 1985). Hypermethylation of CpG island promoters of several tumor

suppressor genes, such as pRb in retinoblastoma tumors (Stirzaker et al., 1997), APC in colorectal carcinoma and (Hiltunen et al., 1997) and BRCA1 in sporadic breast cancer (Dobrovic & Simpfendorfer, 1997), were shown to silence gene expression and promote tumor growth. Moreover, changes in methylation states of the CpG island shores, which are regions up to 2kb distant from the CpG islands, have also been shown to regulate gene expression (Irizarry et al., 2009). By examining the pattern of methylation on CpG islands and CpG island shores, Muse et al. hypothesized that the hypermethylation events during carcinogenesis may initiate at the CpG island shores and progress into the CpG islands (Muse et al., 2020).

1.3.5 Other Epigenetic Modifications Involved in Gene Transcription

Another type of epigenetic regulation is non-coding RNAs. These are simply defined as transcribed transcripts that are not translated into proteins. Regulatory non-coding RNAs can be categorized based on its size, into short chain non-coding RNAs (including siRNAs, miRNAs, and piRNAs) and long non-coding RNAs (lncRNAs) (Ponting et al., 2009; Wei et al., 2017).

siRNA is cut from long double stranded RNA precursors by the Dicer enzyme and normally ranges from 20 to 30 nucleotides in size (Zamore et al., 2000). siRNA was shown to facilitate transcriptional gene silencing by directing DNA methylation and histone methylation (Bayne & Allshire, 2005; Kawasaki & Taira, 2004).

miRNA is processed from hairpin-shaped double stranded RNA via Drosha and Dicer enzymes (Lee et al., 2003). miRNA can directly repress gene expression by binding to complementary mRNA target, leading to mRNA removal and degradation (Yekta et al., 2004). Moreover, miRNA can regulate the expression level of histone modification proteins or DNMTs, therefore regulating chromatin remodeling and gene transcription (Benetti et al., 2008; Tuddenham et al., 2006)

piRNAs associate with Piwi proteins to regulate gene transcription (Lau et al., 2006). The piRNA-Piwi complex was shown to recruit Suv39 and HP1a to the sequence to repress gene transcription (Huang et al., 2013). Suv39 factor is responsible for enrichment of H3K9me2 and H3K9me3, which can then be bound by HP1a proteins to condense chromatin and limit RNA Pol II access. Moreover, Piwi proteins MILI and MIWI2 were shown to regulate the DNMT3 DNA methyltransferases (Kuramochi-Miyagawa et al., 2008)

Unlike the previous three short-chain non-coding RNAs, lncRNAs are more than 200 nucleotides in length (Mercer et al., 2009). lncRNAs were shown to be required for H3K27 and H3K9 methylation and DNA methylation for certain genes (Regha et al., 2007). Xist RNA, one type of lncRNA responsible for X chromosome repression, was shown to recruit PRC2 to the X chromosome to promote H3K27me3 enrichment (Zhao et al., 2008).

1.4 Epigenetic and Cancer

1.4.1 Epigenetic Regulations in Primary Tumor Growth

One of the first epigenetic modifications discovered in human cancer comparing to regular tissue is global hypomethylation (Feinberg & Vogelstein, 1983). Hypomethylation can lead to chromatin structure disruption, expression of silenced intergenic regions, and expression of tumorigenic genes. Eden et al. demonstrated that introducing a hypomorphic allele of DNMT1 increases mitotic recombination and loss of heterozygosity in mice, which promote chromosomal rearrangement and lead to increased rate of tumor development (Eden et al., 2003). DNA methylation plays an important role in repressing expression of intergenic region genes. Alu and LINE-1 are both non-coding genomic repetitive sequences with transposition ability (Choi et al., 2007). Hypomethylation of Alu and LINE-1 have been discovered in multiple types of cancer, including colon cancer (Sunami et al., 2011), hepatocellular cancer (Lee et al., 2009), and breast cancer (van Hoesel et al., 2012). Hypomethylation allows expression of Alu and LINE-1, which can translocate to different genomic regions and disrupt genomic stability (Saito et al., 2010). Moreover, hypomethylation can lead to expression of previously repressed oncogenes. For example, in breast cancer, hypomethylation was shown to upregulate expression of interleukin-10 (Son et al., 2010), Jagged1 and Notch1 (Cao et al., 2015), and FEN1 (Singh et al., 2008), corresponding to increased tumor progression and burden.

Conversely, DNA hypermethylation at certain gene promoter was also shown to promote tumor growth and development. DNA hypermethylation was suggested to be an early event in tumorigenesis of invasive breast cancer and may also occur in nearby normal breast epithelial cells to prepare the tumor microenvironment for metastasis (Umbricht et al., 2001). The CpG islands at transcription promoters are the main sites of hypermethylation modification during

oncogenesis (Baylin et al., 1998). In breast cancer, DNA hypermethylation was reported to be present on more than 100 gene promoters, causing silence of tumor suppressor gene expression (Jovanovic et al., 2010). The reported genes have variable functions, including cell cycle regulation, hormone signaling, DNA repair, cell migration and apoptosis (Widschwendter & Jones, 2002). When silenced or mutated, the cells can then grow out of control and lead towards cancer development (Buchholz et al., 1999).

A few frequently hypermethylated tumor suppressor genes in breast cancer are described below (Yang et al., 2001). The p16 gene encodes for p16(INK4A), a cyclin-dependent kinase inhibitor that regulates cell transition between the G1 and S phase (Shapiro Geoffrey et al., 1998). Hypermethylation of p16 CpG islands promotes tumor cells to escape cell senescence and maintain cell division ability for an expanded period (Huschtscha et al., 1998). Genes encoding steroid receptors estrogen receptor (ER)α and progesterone receptor (PR) are also frequently methylated at the CpG islands in breast cancer (Issa et al., 1994; Lapidus et al., 1996). Methylation causes decreased or loss of estrogen and progesterone receptor protein expression within the tumor cells, resulting in resistance towards hormone and endocrine therapy. Another frequently methylated gene in breast cancer is BRCA1, which is involved in sensing and repairing DNA damages within a normal cell to prevent tumorigenesis (Thomas et al., 1997). In a meta-analysis involving 20 case-control studies and 30 clinical studies, hypermethylation of BRCA1 promoter was shown to be associated with increased risk of breast cancer, lymph node metastasis and higher histological grade (Zhang & Long, 2015). E-cadherin responsible for cell adhesion was also shown to be hypermethylated in about 50% of breast cancer cases (Graff et al., 1995). Previous study has shown that loss of expression of e-cadherin proteins is associated with increased proliferation and metastasis in breast cancer, leading to poor patient survival (Bringuier et al., 1993). Lastly, a few apoptosis regulator genes, such as BCL2, DAPK, and APC, were also shown to be hypermethylated within breast cancer (Jovanovic et al., 2010)

Histone modifications also play an important role in regulating gene expression and altering chromatin structure within cancer cells. Several studies have been conducted on comparing the global histone modification enrichment between breast cancer cells and normal cells. Zhao et al. discovered a decrease in H3K9me2 and H3K9me3 during malignant breast cancer transformation, caused by elevated expression of KDM3A/JMJD1A demethylase (Zhao et

al., 2016). Messier et al. discovers an increase in H3K4ac enrichment in breast cancer cells and an increase in H3K4me3 enrichment in metastatic triple negative breast cancer cells (Messier et al., 2016).

Gene site-specific histone modifications were also discovered for different cancer types. In a genome-wide H3K4me3 and H3K27me3 study comparing luminal, HER2, and basal breast cancer subtypes, Chen et al. discovered unique histone modification enrichment patterns within each subtype, which are associated with distinct signature gene expression and signaling pathways (Chen et al., 2016). Moreover, the balance of histone modification writer and eraser can also regulate tumor progression. For example, the histone demethylase LSD1 was found to be overexpressed in ER-negative breast cancer subtypes and was associated with more aggressive disease progression (Lim et al., 2010).

Overall, DNA methylation and histone modifications were shown to affect gene expression and tumor progression within tumor cells. Many studies also suggest that depending on cancer subtype and progression stage, differential epigenetic mechanisms may be involved.

1.4.2 Epigenetic Regulation in Tumor Metastasis

Other than promoting tumor growth and progression, epigenetic mechanisms are also involved in facilitating tumor metastasis. Several studies have been conducted to examine the role of DNA methylation, both global hypomethylation and promoter-specific hypermethylation, in promoting metastasis. It was previously observed that breast cancer with positive lymph node involvement is hypomethylated comparing to non-metastatic breast tumor (Soares et al., 1999). Fang et al. identified two distinct breast cancer clusters with differential global methylation profile. The cluster with global hypomethylation on breast CpG islands is associated with worse prognosis and higher relapse rate. Moreover, each cluster has a unique metastasis transcriptome and high expression of signature cancer metastatic genes are observed in the hypomethylation cluster (Fang et al., 2011).

Dumont et al. showed that cancer cells can undergo DNA methylation reprogramming and induce epithelial-mesenchymal transition (EMT). The EMT process is accompanied by hypermethylation of E-cadherin promoter, estrogen receptor promoter, and TWIST promoter. Moreover, the researchers verified that no hypermethylation was observed for other commonly

hypermethylated but EMT-unrelated genes in breast cancer, such as BRCA1, GATA3, TIMP3, and DKK3, showing the hypermethylation is not a random event (Dumont et al., 2008). In another study of EMT transition, TINAGL1 was confirmed to be hypermethylated in invasive human breast cancer samples (Carmona et al., 2014). High expression of TINAGL1 is associated with metastasis-free survival and knockdown of TINAGL1 was shown to increase metastatic potential of breast cancer cell line (Korpal et al., 2011). Conversely, DNA hypomethylation at certain genes can also increase the metastatic potential of cancer cells. Bloushtain-Qimron et al. discovered that several transcription factors, including HOXA10, TCF3, and FOXC1, are hypomethylated and can participate in promoting EMT and differentiating stem cells (Bloushtain-Qimron et al., 2008). After arriving at the secondary site, the promoter regions responsible for EMT may undergo partial demethylation, allowing the metastasized tumor cell to reverse the EMT process and interact with local tissue (Park et al., 2001).

Histone modifications are shown to regulate tumor metastasis as well. Starting with histone acetylation, higher enrichment level of H4K26ac is found to be associated with increased angiogenesis (Hałasa et al., 2019). H3K4ac was shown to be enriched at the vimentin promoter, which is the key intermediate filament expressed in mesenchymal cells (Messier et al., 2016). Snail1 can bind to the E-cadherin promoter and recruit a complex containing Histone deacetylase 1 (HDAC1), HDAC2, and SIN3A to repress E-cadherin production and initiate EMT (Cano et al., 2000). Snail2 and Slug can repress E-cadherin expression through similar mechanisms, but by recruiting a different complex containing cTBP and HDAC3 (Bolós et al., 2003). CDH1 is responsible for initiating the expression of E-cadherin and is frequently mutated or silenced in colorectal cancer, gastric cancer, and breast cancer (Adib et al., 2022). The CtBP complex, containing HDAC1/2 and histone methylases, may also interact with ZEB factors to repress CDH1 expression, further decreasing E-cadherin level within tumor cells (Shi et al., 2003).

Similar to histone acetylation modification, histone methylation is also involved with promoting EMT. Polycomb group protein complexes can recruit DNA methyltransferases to silence E-cadherin expression by H3K27me3 enrichment during tumor cell progression (Cao et al., 2008). G9a can repress expression of epithelial-related genes to facilitate TGF-β-induced EMT process by methylating the H3K9 site (Dong et al., 2012). SET8 is a member of the SET methyltransferase family and is responsible for H4K20me enrichment (Fang et al., 2002). SET8

was shown to interact with TWIST to facilitate TWIST-promoted EMT and tumor invasion (Yang et al., 2012). Furthermore, H3K4 demethylase JARID1B and LSD1 were shown to regulate angiogenesis and tumor metastatic potential by downregulating epithelial chemokine expression level (Li et al., 2011). Lower enrichment levels of arginine methylation H4R3me3, H4R3me2 and lysine methylation H3K4me2 are associated with poor prognosis and more aggressive breast cancer phenotype (Elsheikh et al., 2009).

1.4.3 Cancer Treatments Targeting Epigenetic Mechanisms

Unlike genetic mutations, epigenetic mechanisms are reversible and in theory, may be restored to normalcy, by using various treatments targeting the writers or erasers of epigenetic modifications. Genes repressed by DNA hypermethylation can be re-expressed by using drugs to inhibit activity of DNMTs. The current DNMT inhibitors can be divided into two categories: nucleoside analogues and non-nucleoside analogues. Nucleoside analogues contain a modified cytosine structure and can be incorporated into DNA sequence during S phase DNA replication. (Jones & Taylor, 1980). DNMT recognizes the cytosine analogue as natural cytosine base and will perform methylation through nucleophilic attack, establishing a covalent bond between carbon-6 of the cytosine analogue and DNMT. When methylating regular cytosine base, DNMT is displaced from DNA sequence by beta-elimination at carbon-5. However, cytosine analogues contain a nitrogen group at the site of carbon-5, which blocks beta-elimination reaction. Hence DNMT will be bound by the cytosine analogues and can no longer carry out methyltransferase function within the cell (Santi et al., 1984; Stresemann & Lyko, 2008). Non-nucleoside analogues do not possess a cytosine structure to incorporate into DNA sequence, instead they may directly bind to the catalytic sites of DNMTs (Brueckner & Lyko, 2004). 5-azacytidine (azacytidine) and 5-aza-2'-deoxycytidine (decitabine) are the first two DNMT inhibitors synthesized and both belong to the nucleoside analogue category (Čihák, 1974). The FDA have approved their usage as treatments for myelodysplastic syndrome (Kristensen et al., 2009). The two DNMT inhibitors work well to remove DNA methylation at a low dose, however when given at a higher dosage, the two drugs demonstrate cytotoxic effects towards normal blood cells (Kantarjian et al., 2003). Zebularine is a more recently synthesized member of the nucleoside analogue category that seems to be more selective for cancer cells and less toxic (Cheng et al., 2004). But more studies and clinical trials need to be carried out before the effectiveness and

safety of Zebularine can be determined. There are also a few non-nucleoside analogue DNMT inhibitors, including (-)-epigallocatechin-3-gallate (EGCG), hydralazine, and RG108 (Kristensen et al., 2009). The effectiveness of EGCG and hydralazine were compared with decitabine in a study, showing that decitabine is still the most effective (Chuang et al., 2005). Although RG108 may be less toxic and more selective, the hydrophobicity of RG108 makes it difficult to be used as a cancer treatment (Siedlecki et al., 2006).

One promising research direction of DNMT inhibitor is its ability in altering the immune response of cancer cells. H. Li et al. examined the effect of low dose azacytidine in 63 common epithelial cancer cell lines (breast, colorectal and ovarian cancer) and discovered that DNMT inhibitor can upregulate the expression of immunoregulatory pathways within all three cancers (14 to 30% of cell lines from each cancer group). The enriched pathways include interferon signaling, cytokine and chemokine production, antigen processing and antigen presentation (H. Li et al., 2014). This discovery was supported by Chiappinelli et al.'s study, where DNMT inhibitors can upregulate cytosolic sensing of double-stranded RNAs leading to augmented type I interferon signaling and apoptosis within ovarian cancer cell lines (Chiappinelli et al., 2015). Roulois et al. also demonstrated that low dosage treatment with azacytidine can cause viral mimicry within colorectal cancer initiating cells, by inducing formation of dsRNAs and activating the IRF7 signaling pathway (Roulois et al., 2015). Moreover, several studies have examined the potential of combining DNMT inhibitor with immunotherapy. Treatment using vorinostat (a histone deacetylase inhibitor) and azacytidine was shown to upregulate PD-L1 expression in MDA-MB-231 TNBC cell line, suggesting the potential of sensitizing the cells towards anti-PD-L1 treatment (Terranova-Barberio et al., 2017). Decitabine was also shown to upregulate PD-L1, PD-L2 and PD-1 protein expression in leukemia cells (Yang et al., 2014).

Studies were also conducted to examine the effect of targeting histone modification as cancer treatments. Gursoy-Yuzugullu et al. demonstrated that inhibiting H4K20 methyltransferase SETD8 and H3K9 methyltransferase G9a can increase sensitivity of cancer cells towards radiotherapy (Gursoy-Yuzugullu et al., 2017). Another study was conducted to examine the effects of trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC), in upregulating gene expression. The study showed that TSA can cooperate with histone acetylase to recruit transcription machinery onto tumor suppressor genes previously silenced by SET

proteins (Almeida et al., 2017). Many HDAC inhibitors like TSA have been discovered and synthesized. However, only five have been approved by FDA to date as treatment options for T-cell lymphoma and multiple myeloma (Bondarev et al., 2021). The five approved HDAC inhibitors are vorinostat, belinostat, romidepsin, panobinostat, and tucidinostat (Bondarev et al., 2021). Many clinical trials have been carried out testing the effect of HDAC inhibitors in other type of cancer. For example, combination treatment using aromatase inhibitor exemestane and HDAC inhibitor tucidinostat in advanced stage breast cancer was examined in a phase III clinical trial. The combination treatment increased objective responsive rate and patients deriving clinical benefits. However, quality of life and overall survival data were not available at the time of publishing. So whether HDAC inhibitor is an effective treatment option of breast cancer remains to be unanswered (Jiang et al., 2019). Similar to the result of this clinical trial, more studies need to be conducted before the HDAC inhibitors can be used to treat other cancer types. One major difficulty in synthesizing effective HDAC inhibitor is that most inhibitors do not have a global effect on HDAC and most HDAC inhibitors can only affect a small number of gene transcription (Chiba et al., 2004). Moreover, HDACs can interact with many factors and are involved in multiple pathways, so many HDAC inhibitors may show high toxicity and severe side effects (Kristensen et al., 2009).

Due to the complexity of epigenetic modifications, combination therapy using multiple epigenetic treatments or combining epigenetic treatments with other forms of therapies were studied. Combining low dosage of DNMT inhibitor azacytidine and HDAC inhibitor entinostat was shown to increase response rate in Non-small cell lung cancer patients (Juergens et al., 2011) and in metastatic colorectal cancer (Azad et al., 2017). Combination of azacytidine and HDAC inhibitor vorinostat was shown to increase sensitivity towards chemotherapy within large B cell lymphoma patients, who were previously unresponsive towards chemotherapy-only treatment (Pera et al., 2016). With these successful examples, combination therapy involving epigenetic drugs may be a promising direction for developing novel cancer treatments.

2. AIMS

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype with increased metastatic potential (Xiao et al., 2018) and high relapse rate (Dent et al., 2007). Due to the negative expression of ER, PR and HER2, the main treatment options for metastatic TNBC remain to be chemotherapy and radiotherapy, which often contain severe side effects (Yin et al., 2020). Because of the characteristics of TNBC, it is crucial to examine the mechanisms promoting TNBC metastasis and propose novel treatment regimens targeting metastatic TNBC.

Previous studies in breast cancer metastasis demonstrated that tumor cells spreading towards different organs contain distinct gene expression profile and unique interactions with the tumor microenvironment (Kang et al., 2003; Müller et al., 2001; Song et al., 2001), suggesting the importance in considering site-specific features when studying cancer metastasis. Moreover, epigenetic mechanisms, such as histone modifications and DNA methylation, are highly involved in breast cancer growth and metastasis (Carmona et al., 2014; Fardi et al., 2018). However, studies regarding epigenetic modifications promoting organotropism metastasis of breast cancer are lacking and may be a promising direction for further research.

In a previous study conducted by Lewis et al., p66ShcA was shown to be required for TNBC metastasis towards the lung. Moreover, the study showed that variable levels of p66ShcA are expressed across some of the cell lines used in this project (Lewis et al., 2020). This finding proposes that p66ShcA may play a role in promoting site-specific breast cancer metastasis. Lewis et al. also demonstrated that overexpressing of p66ShcA alone is not sufficient to initiate metastasis towards the lung (Lewis et al., 2020), suggesting the existence of additional pathways involved in promoting site-specific metastasis along with p66ShcA. Since histone acetylation and cytosine methylation were identified to be mechanisms regulating p66ShcA expression (Ventura et al., 2002b), we hypothesize that the additional regulatory pathways are concurrently regulated with similar epigenetic modifications as p66ShcA. Identifying the epigenetic mechanisms regulating organotropism metastasis in TNBC may provide directions for developing new treatment options targeting metastatic TNBC.

To investigate these hypothesis, TNBC variant cell lines demonstrating site-preferential metastasis were analyzed using RNA-sequencing, chromatin immunoprecipitation, and DNA-

methylation microarray. Following information gained from the bioinformatic analyses, in-vitro and in-vivo epigenetic treatments were carried out on the cell lines to explore targetable pathways and potential treatment regimens effective for metastatic TNBC.

3. MATERIALS AND METHODS

3.1 Cell Culture

The 4T1-derived primary tumor explanted cell lines (152 and 154) (Rose et al., 2010), 4T1-derived lung-metastatic cell lines (526, 533 and 537) (Rose et al., 2010), 4T1-derived liver-metastatic cell lines (2772 and 2776) (Tabariès et al., 2011) and 4T1-derived bone-metastatic cell lines (590 and 592) (Rose et al., 2007) were kindly provided by Dr. Peter Siegel. The cell lines were cultured as previously described (Rose et al., 2007)

3.2 RNA Extraction, Reverse Transcription and Real-time qPCR

Total RNA was extracted using Aurum Total RNA Mini Kit (BIO-RAD) following manufacturer instructions. Reverse transcription was carried out on extracted RNA samples using the following concentration: 5uL of 100ng/uL RNA, 4uL of 5X All-In-One RT MasterMix (abm) and 11uL nuclease-free water, and then reverse transcribed into cDNA using PCR machine. Real-time qPCR was carried out on QuantStudio 3 and 7500 Fast Real-Time PCR System (Applied Biosystems), using the following concentration: 1uL of 5uM forward and reverse primer, 5uL of GoTaq qPCR Master Mix (Promega), 2uL of nuclease-free water and 2uL of DNA. Technical duplicates or triplicates of each sample were performed on each plate. The primers used for qPCR are: p66ShcA: forward primer 5'-CTGAAACTGTCTGGGTCTGAG-3' and reverse primer 5'-TAGCCTGGTTGGACCTCT-3'; Tlr3: forward primer 5'-CTTTGTCTTCTGCACGAACCT -3' and reverse primer 5'- CCCGTTCCCAACTTTGTAGAT -3'; Tnfaip3: forward primer 5'-TAGTGATGGAGATCCCTGTGC-3' and reverse primer 5'-CGTGCTGAACAAGCTCAAAGT-3'; Adam8: forward primer 5'-CCAACGTGACACTGGAGAACT-3' and reverse primer 5'-GAACACAGGGCAGACACCTTA-3'; Ifih1: forward primer 5'-TCTGAATCCGGGAAGGTTATC-3' and reverse primer 5'-AGCTGGGTATCGCCACTTAAT-3'; Ptpn22: forward primer 5'-GACCCCTGGAAAAAGTTTCAC-3' and reverse primer 5'-GAACACGTTCTGTAGGCTTGC-3'; CX3CL1: forward primer 5'-ACGAAATGCGAAATCATGTGC-3' and reverse primer 5'-CTGTGTCGTCTCCAGGACAA-3'; Ddx58: forward primer 5'-ATTGCTGAGTGCAATCTCGTC-3' and reverse primer 5'-TGTTCGCCTTTTCTTTTTCAA-3'; Ifnb1: forward primer 5'-

TGTCCTCAACTGCTCTCCACT -3’ and reverse primer 5’- TCCAGGCGTAGCTGTTGTACT -3’;. The primers were designed using Primer3 website (https://primer3.ut.ee/)

3.3 Protein Extraction and Western Blotting

Cells were lysed using lysis buffer (20mM Tris pH7.5, 420mM NaCl, 1mM EDTA, 2mM MgCl2, 10% glycerol, 0.5% Triton and 0.5% NP40) supplemented with 1mM DTT, PSMF, NaF, P8340 and BGP. Lysis buffer was added to the cell pellet following this ratio: lysis buffer volume is twice of cell pellet volume. The mixture was left on ice for 15 minutes, then centrifuged at maximum speed for 15 minutes at 4 °C. Supernatant was collected and used for future western blotting. 20ug of protein was loaded per well and the gel was run on 120V until the dye front reached bottom of the gel. Membrane transfer was performed overnight at 34V, followed by 1 hr at 100V in 4°C cold room. Membrane was washed for 3 times for 5 minutes, 10 minutes and 15 minutes using TBST buffer. Membrane was then blocked using 5% milk in TBST or 3% BSA in TBST for 1 hr at room temperature, followed by primary antibody incubation at 4°C overnight shaking. Afterwards, the membrane was incubated with secondary antibody for 1hr at room temperature shaking. Western blotting result was then collected using Clarity Western Enhanced chemiluminescence kit (Bio-Rad). Primary antibodies used in this study were: anti-Shc1 (Milipore #06-203), anti-GAPDH and anti-CTCF.

3.4 Drug Treatment

Drugs used in this study are decitabine (MedChemexpress CO., Ltd # HY-A0004-10MG) and A-485 (Tocris Bioscience # 6387/5). Drugs were reconstituted, diluted and stored following manufacturer instructions.

3.5 Growth Analysis

800 cells were seeded into 12-well plates or 400 cells were seeded into 24-well plates in triplicates for each 4T1-variant cell line. Cells were seeded one day prior and treated with drug/DMSO control/DMSO-negative control at various concentrations on day 0. Cells were fixed at day 3 and at day 5 using 4% formaldehyde for 10 minutes and washed with PBS. Fixed

cells were than stained using 0.1% Crystal Violet and 10% ethanol in water for 30 minutes shaking, washed twice with water and left to dry overnight. Once dried, pictures of the well were taken using a plate scanner. 10% acetic acid solution was added and incubated for 20 minutes in room temperature shaking. 100uL solution was taken from each well in triplicates and loaded onto a 96-well plate to measure absorbance level at 590nm, which correlates with cell density.

3.6 RNA-Sequencing and Analysis

RNA-sequencing was performed on triplicates of each 4T1-variant cell lines. RNA was extracted using Aurum Total RNA Mini Kit (BIO-RAD) following manufacturer instructions. Collected RNA samples were sent for library preparation and sequencing at TCAG (The Centre for Applied Genomics, SickKids, Toronto). The samples were sequenced using NovaSeq 6000 on one lane of the S4 flow cell, generating 2 billion to 2.5 billion reads in total.

The reads were trimmed by Trimmomatic (Bolger et al., 2014), then aligned to reference mouse genome using STAR (Dobin et al., 2013). Number of reads mapping to each transcript annotated by Ensemble Gene ID was summarized using featureCounts (Liao et al., 2014). The count table generated was then normalized and analyzed using R package Deseq2 (Love et al., 2014). Differentially expressed gene analysis were performed between each metastatic site (lung, liver and bone) and the primary tumor explants. Only genes with an FDR-adjusted p-value < 0.05, log2 fold change > 1.0 or <-1.0, and average number of reads greater than 100 reads were deemed as significantly differential expressed genes. These genes were then entered into Gene Ontology (http://geneontology.org/) for pathway analysis, using the GO database, PANTHER database and Reactome database. Gene Set Enrichment Analysis (GSEA) was also used to generate GSEA enrichment plots of specific pathways.

3.7 DNA Extraction, DNA-methylation microarray and Analysis

DNA-methylation microarray was performed on triplicates of 4T1-derived primary tumor explanted cell lines (152 and 1541) and duplicates of the other 4T1-derived metastatic cell lines. Genomic DNA was extracted from the cells using PureLink Genomic DNA Mini Kit (Invitrogen) following instructions from manufacturer. DNA samples were sent to Genome

Quebec for bisulfite conversion, library preparation and microarray probing. Infinium mouse methylation beadchip (Illumina) was used for DNA-methylation microarray, which includes more than 285K methylation sites per sample at single-nucleotide resolutions. Sites include CpG islands, translation start sites, enhancer, imprinted loci, and other intergenic regions.

Table showing methylation enrichment level, heatmap for top variable CpG sites and PCA plot were generated by Dr. Kathleen Klein. Mouse mm10 genome was obtained from UCSC Genome Browser (https://genome.ucsc.edu/) and annotated with genomic regions (promoter, 5' UTR, exon, intron and distal intergenic) using the annotatePeak function in Rstudio (Yu et al., 2015). Methylation enrichment level at each microarray targeting site was then aligned onto the reference annotated mice genome and categorized into DNA methylation occurring at gene body region, gene promoter region and distal intergenic region. Heatmap and PCA plot were created using the Tidyverse package in Rstudio (Wickham et al., 2019). Similar to bioinformatic analysis performed for RNA-sequencing, differentially methylated gene list was generated by comparing DNA methylation enrichment across the cell types. Pathway analysis was then carried out on Gene Ontology (http://geneontology.org/), using the GO database, PANTHER database and Reactome database.

DNA-methylation and RNA-sequencing were also analyzed together to investigate genes that are differentially expressed across metastatic group that are under DNA-methylation regulation.

3.8 Chromatin Immunoprecipitation

The 4T1-variant cell lines were crosslinked using 1% formaldehyde in PBS for 10 minutes at room temperature, followed by 5 minutes incubation with 125mM glycine to stop the crosslinking process. The cells were then washed using PBS, collected in Eppendorf and centrifuged at 2500rpm for 5 minutes at 4°C. Supernatant was removed and cell pellet can be stored at -80°C.

Cell pellets were resuspended in ChIP-buffer (0.25% NP-40, 0.25% Triton, 0.25% sodium deoxycholate, 0.05% SDS, 50mM Tris pH8.0, 100mM NaCl, 5mM EDTA, 1mM PMSF,

2mM NaF and 1% P8340) and sonicated for 5 cycles at 20%, 5 cycles at 25% and 5 cycles at 30%, each cycle is 10 seconds long. Lysates were centrifuged at 13000rpm for 30 minutes at 4°C and supernatant is collected. The supernatant was diluted to protein concentration of 1ug/ml, 20uL of the diluted supernatant was stored at -20°C as input. 1ug of protein per sample was pre-cleared using 60uL of blocked Protein G Plus-Agarose Suspension (Milipore #IP04) for 3 hours at 4°C. Immunoprecipitation was carried out by adding 3uL of antibody and 100uL freshly blocked agarose G beads into each sample, shaking overnight at 4°C in the cold room. Antibodies used in this study include: anti-H3K9ac (Sigma-Aldrich #06-492), anti-H3K27ac (Sigma-Aldrich #07-360), H3K4me1 (abcam #ab8895), H3K4me3 (Sigma-Aldrich #07-473), H3K27me3 (Sigma-Aldrich #07-449) and H3K9me3 (Sigma-Aldrich #07-442)

Samples were then centrifuged at 4000rpm for 2 minutes at 4°C to pellet the agarose beads, which are linked with desired ChIP product. 4 consecutive washes with increasing salt concentration were carried out to remove unspecific binding from the agarose beads. Two more washes using TE buffer were performed to remove residual buffers from the beads. Elution buffer (1% SDS and 0.1M NaHCO3 in water) was added to each sample and the stored input samples, incubated for 15 minutes at 65°C to elute DNA and proteins from the agarose beads. The beads were then removed using centrifugation and the supernatants were left to reverse-crosslink at 65°C overnight.

After reverse-crosslinking, proteins and DNA were separated, and proteins were degraded using proteinase K treatment for 1 hr at 42°C. DNA was collected using DNA collection tube (Bio Basic #SD5005). DNA binding buffer (5M guanidine and 30% isopropanol in water) was added into each sample, then passed through DNA binding columns to allow retention of DNA on the column membrane. PE buffer (0.01M Tris pH7.5 and 80% ethanol in water) was used to wash the column twice. DNA was then eluted using 60uL of nuclease-free water and stored at -80°C. Real time qPCR was carried out on the samples following previously described qPCR protocol. The primer used for ChIP-qPCR is p66ShcA promoter: forward primer 5'-GTCTCTCTCCTTAGTTCCCCG-3' and reverse primer 5'-AGGAAACCTCTACTGGCTGAG-3'.

3.9 Tumour Growth Analysis in-vivo

In-vivo experiment was kindly performed by Young Im and Valérie Sabourin. The experiment was carried out using three 4T1-variant cell lines: primary explant 154, liver-metastatic 2776 and lung-metastatic 526). For each cell line, 50000 cells in 30uL PBS was injected into each side of mammary fat pad of balb/c mice. Treatment started when the tumor size reached between 150mm^3 to 200mm^3. 1mg/kg of decitabine, 2.5mg/kg of decitabine or saline were administered to mice by intraperitoneal injection. The following treatment schedule was used: daily treatment for 4-days, followed by a 3-day resting period. Size of breast tumor was measured every two days until day-8 by digital caliper using the following equation: $\text{volume} = \frac{4}{3} \times (3.14159) \times \frac{\text{Length}}{2} \times \left(\frac{\text{Width}}{2}\right)^2$ (Lewis et al., 2020).

4. RESULTS

4.1 Cell lines preferentially metastasize towards different sites express distinct genetic profile

4T1 is a highly tumorigenic and invasive mouse breast cancer cell line. It is triple-negative and can spontaneously metastasize towards distant organs such as liver, lung, bones, brain, and lymph nodes. 4T1 tumor growth and metastasis are very similar to breast cancer progression in humans, and can be used as a model for stage IV human breast cancer (Pulaski & Ostrand-Rosenberg, 2001). The 4T1 cells used for this project were generated by in-vivo selection of aggressive metastatic cells and direct explantation from primary tumors (Pulaski & Ostrand-Rosenberg, 2001; Rose et al., 2010; Rose et al., 2007; Tabariès et al., 2011; Tabariès et al., 2015). The metastatic cell lines display site-preferential metastasis towards either the bone, liver, or lung and are summarized in **Fig 1. A**.

To gain more knowledge about the cell lines, RNA-sequencing was carried out. Based on the heatmap generated using the top 100 variably expressed genes (**Fig 2. A)** and the PCA plot (**Fig 2. B**), liver-metastatic cell lines show highly conserved gene expression profile and cluster together. The lung-metastatic cell lines show variable gene expression profile, and one of them (537) display similar genetic makeup comparing to the primary explanted cell lines. To decrease inaccuracy in future studies, lung-metastatic cell line 537 will be excluded from most analyses. The other lung-metastatic cell lines (526 and 533) are quite different from each other, but still distinct from the primary explanted cell lines. Whereas both bone-metastatic cell lines cluster closely together with the primary cell lines. In fact, less than 100 significant differentially expressed genes were discovered by comparing primary tumor and the bone-metastatic cell lines (**Fig 3. A**), whereas 647 genes were discovered for lung-metastatic cell lines (**Fig 3. B**), and 722 genes were discovered for liver-metastatic cell lines (**Fig 3. C**).

4.2 Pathway analysis using differentially expressed genes indicates that immune-related pathways may play a regulatory role in determining organotropism metastasis

Due to the small number of differentially expressed genes in bone-metastatic cell lines, pathway analysis was not performed. A few interesting pathways came up when comparing liver/lung- metastatic cell lines with primary explants using Gene Ontology (**Fig 3. D-E).** Many pathways are associated with promoting metastasis. For example, upregulation of angiogenesis is observed in both lung- and liver-metastatic cell lines. Modification of integrin signaling pathway can alter cellular interaction with the ECM (Hamidi & Ivaska, 2018). A few pathways for the liver-metastatic cell lines seem to point towards EMT: downregulation of cadherin signaling pathway, mesenchymal cell differentiation and regulation of epithelial cell differentiation. Another category of pathways worth mentioning is the large number of immune-related pathways, such as positive regulation of neutrophil chemotaxis, regulation of response to cytokine stimulus and inflammation by chemokine and cytokine signaling.

GSEA pathway enrichment plots further demonstrate that pathways involved in promoting metastasis and immune system are differentially expressed between the metastatic cell lines and the primary explants (**Fig 4. A-D**). Cell-cell junction organization is downregulated, whereas cellular extravasation pathway is upregulated in the metastatic cell lines. The innate immune response pathway and cytokine signaling in immune system pathway are mainly downregulated within metastatic cell lines, with a small portion of genes appear to be upregulated.

qPCR on a few differentially expressed genes were run to validate the RNA-sequencing result and to gain more information regarding potential critical pathways (**Fig 5. A-F**). Tlr3, Tnfaip3, Adam8, Ifih1 and Ptpn22 are all genes involved in inflammation and regulating the immune system. Tlr3 (Toll-like receptor 3) can bind to viral double strand RNA and initiate downstream type I interferon signaling, activation of NF-kappaB complex and cytokine production (Alexopoulou et al., 2001). Tnfaip3 (tumor necrosis factor, alpha-induced protein 3) expression is elevated during inflammatory events and can form a negative feedback loop to regulate NF-kappaB signaling (Mérour et al., 2019). Adam 8 (ADAM metallopeptidase domain

8) displays proinflammatory function and promotes leukocyte recruitment (Dreymueller et al., 2017; Mérour et al., 2019). Ifih1 encodes MDA5 protein, which is responsible for detecting viral RNA and activating innate immune response by upregulating type I interferon-signaling and cytokine production (Rice et al., 2014). Ptpn22 encodes for a cytoplasmic tyrosine phosphate and is responsible for lymphocyte development, T-cell activation and autoimmune regulation (Brownlie et al., 2018). Tlr3, Tnfaip3, Adam8 and Ptpn22 are expressed at a lower level in all metastatic-cell lines (**Fig 5. A-C, E**), whereas Ifih1 is especially downregulated in the liver-metastatic cell lines (**Fig 5. D**), suggesting certain immune-related pathways may be involved in promoting metastasis in general and certain pathways are involved with site-specific metastasis.

Conversely, expression level of inflammatory chemokine CX3CL1 is upregulated in the metastatic cell lines (**Fig 5. F**). CX3CL1 was reported to display both pro-tumorigenic and anti-tumorigenic functions (Conroy & Lysaght, 2020). The upregulated expression level of CX3CL1 matches the previous pathway analyses (**Fig 3. D-E and Fig 4. C-D**), which also showed upregulation of chemokine and cytokine signalling pathways within the metastatic cell lines.

Overall, differentially expressed gene analysis and pathway analysis reveal that immune and inflammation-related pathways may play a role in promoting TNBC site-specific metastasis.

4.3 p66ShcA is differentially expressed across the cell lines and is regulated at the transcription level

In a previous study conducted by the Ursini-Siegel lab, p66ShcA expression was shown to be required for 4T1 cell lines to maintain their lung-metastatic potential, however, overexpression of p66ShcA alone is not sufficient to promote lung metastasis (Lewis et al., 2020). This discovery suggests that there may be additional genes or pathways other than p66ShcA that regulate organotropism metastasis of TNBC. Moreover, p66ShcA expression was shown to be regulated epigenetically at the transcription level (Ventura et al., 2002b), suggesting the additional pathways may be regulated through similar epigenetic mechanisms as p66ShcA.

To verify the hypothesis that there are additional regulatory pathways concurrently regulated with similar epigenetic modifications as p66ShcA in the cell lines, p66ShcA

expression level across the cell lines needs to be determined first. Moreover, whether p66ShcA expression is under transcription regulation or translation regulation should be verified. Western blot was carried out on the cell lines, identifying liver-metastatic cell lines and two of the lung-metastatic cell lines express high level of p66ShcA protein, the bone-metastatic cell lines express moderate level of p66ShcA, whereas the primary tumor explanted cell lines express low level of p66ShcA (**Fig 1. B**). qPCR using p66ShcA specific primers showed similar gene expression pattern as the western blot (**Fig 1. C**), confirming that p66ShcA expression is primarily regulated at the transcription level. Furthermore, p66ShcA is indeed expressed at various levels depending on the preferred metastatic site, confirming this would be a good model for studying epigenetic modifications involved in site-specific metastasis.

4.4 Enrichment level of activating histone modifications H3K9ac and H3K4me3 corresponds with increased expression level of p66ShcA

After establishing that p66ShcA expression is regulated at the transcription level in the cell lines, ChIP-qPCR was carried out on the p66ShcA isoform promoter site with antibodies targeting various histone modifications. Among the histone modification studied, H3K27ac, H3K9ac, H3K4me1 and H3K4me3 are generally associated with transcription activation, whereas H3K9me3 and H3K27me3 are associated with transcription repression. Based on previous qPCR and western blot performed on p66ShcA (**Fig 1. B-C),** we are expecting to see an enrichment of activating markers on liver cell lines and lung cell lines, which express high level of p66ShcA. The opposite enrichment trend is expected for the repressive markers. The expected enrichment pattern is observed for H3K9ac (**Fig 6. B**) and H3K4me3 (**Fig 6. D**), suggesting these two histone modification markers upregulate transcription level of p66ShcA in the liver- and lung-metastatic cell lines. No expected trend was observed in the two repressive markers (F**ig 6. E-F**).

4.5 DNA-methylation is involved in gene expression and pathway regulation within the cell lines

With the activating histone modification markers determined, the epigenetic mechanism responsible for repressing p66ShcA expression within the cell lines remains unknown. DNA-

methylation is a well-studied epigenetic modification that represses gene transcription when located on the promoter CpG site (Walsh et al., 1998), and upregulates gene transcription when presented on the gene body region downstream of the first exon (Aran et al., 2011; Hellman & Chess, 2007).

DNA-methylation microarray was used to measure the enrichment level of DNA-methylation on regions throughout the genome for the cell lines. Heatmap (**Fig 7. A**) and PCA plot (**Fig 7. B**) show a distinct DNA-methylation profile within each cell group, suggesting DNA-methylation may play a role in promoting site-specific metastasis.

To verify whether DNA-methylation is responsible for regulating gene transcription within the cell lines, DNA-methylation enrichment level for each available gene (at promoter site or on gene body) was plotted against RNA expression level from RNA-sequencing for each metastatic cell groups (**Fig 8**). In the lung- and liver-metastatic groups, DNA-methylation enrichment on the promoter sites correlates negatively with the RNA expression level, showing a repressive role of DNA-methylation on gene promoter. Conversely, a positive correlation is observed on gene body, showing an activating role of DNA-methylation when presented on gene body. No significant correlation is observed in the bone-metastatic cell lines, which is probably due to the low number of differentially expressed genes when compared with primary explanted cell lines. All correlation observed for the lung- and liver-metastatic cell lines are significant with a medium correlation r value.

Furthermore, pathway analysis was conducted using genes that are hypermethylated or hypomethylated in the lung- and liver-metastatic cell lines comparing to the primary tumor explanted cell lines. Overlapping pathways were observed between the pathway analyses conducted using DNA-methylation profile (**Fig 10**) and RNA expression profile (**Fig 3. D-E**). Angiogenesis, inflammation by chemokine and cytokine signaling, and a few pathways related with ECM interaction such as integrin signaling pathway and cadherin signaling pathway were observed before in RNA-sequencing pathway analysis. This observation suggests that these pathways may be under DNA-methylation regulation.

4.6 Decitabine treatment inhibiting DNMT showed cell line specific sensitivity

Since DNA-methylation regulates gene expression and pathways within the cell lines, we propose that inhibiting DNMT activity to alter DNA-methylation enrichment should disrupt cancer cell growth. The DNMT inhibitor used in this project is decitabine, a nucleoside analogue DNMT inhibitor that was approved by the FDA as treatment option for myelodysplastic syndrome (Čihák, 1974; Kristensen et al., 2009). Decitabine is structurally similar to cytidine and will be integrated into the DNA sequence during replication. Once integrated, DNMT will try to methylate decitabine and will be trapped and inhibited from future methylation function (Jones & Taylor, 1980). Because of its unique mechanism, decitabine is very effective against rapidly replicating cancer cells.

The cell lines were treated with 50nM, 100nM and 250nM of decitabine. Cell density was measured after 3 days of treatment and after 5 days of treatment. Three days after treatment start, the liver-metastatic cell lines and bone-metastatic cell lines already demonstrated sensitivity towards decitabine (**Fig 10. A**). Five days after treatment start, all of the liver-metastatic cell lines, bone-metastatic cell lines, one of the lung-metastatic cell lines (526) and one of the primary explanted cell lines (154) experienced significant growth defect even at the lowest decitabine concentration (**Fig 10. B-C**). This means that the sensitivity towards decitabine is not metastatic-group specific, but cell line specific.

To further understand this sensitivity and to examine the effect of decitabine in-vivo, three cell lines sensitive towards decitabine in-vitro (154-primary, 526-lung metastatic and 2776 liver-metastatic) were injected into mice mammary fat pads and treated with decitabine. The tumor volume was measured and graphed (**Fig 11**). 154 and 2776 displayed sensitivity towards decitabine in-vivo and the tumor volume grew in a lower rate comparing to the vehicle control. However, 526 lung-metastatic cell line showed resistance against decitabine treatment at both 1mg/kg and 2.5mg/kg dosage, which was not observed in-vitro.

4.7 Upregulation of interferon-beta expression may cause tumor cell sensitivity towards decitabine

Based on previous analysis, some immune-related genes and pathways may be regulated under DNA-methylation in these cell lines. qPCR was carried out on a few immune-related genes with/without decitabine treatment (**Fig. 12**). The gene expression level of Ifnb1 is significantly upregulated in all of the cell lines demonstrating sensitivity towards decitabine treatment (**Fig 12. C**). Whereas Ifih1 and Ddx58 expression level is upregulated in all of the sensitive cell lines, except in 154-primary explanted cell line. These findings suggest that cell line-specific sensitivity towards decitabine may be caused by interferon-beta signaling, however, the mechanism leading up to interferon-beta activation varies between the cell lines.

5. FIGURES

A

4T1- Variant	Cell Line Name	Generation Method
Primary tumor explant	4T1-152 4T1-154	Explanted directly from primary tumor
Preferential liver-metastatic	4T1-2772 4T1-2776 4T1-2792	In-vivo selection of breast cancer cells that aggressively grow in liver after three rounds of splenic injection
Preferential lung-metastatic	4T1-526 4T1-533 4T1-537	In-vivo selection of breast cancer cells that aggressively grow in lung after tail vein injection
Preferential bone-metastatic	4T1-590 4T1-592	In-vivo selection of breast cancer cells that aggressively grow in bones after two rounds of injection into the mammary fat pads

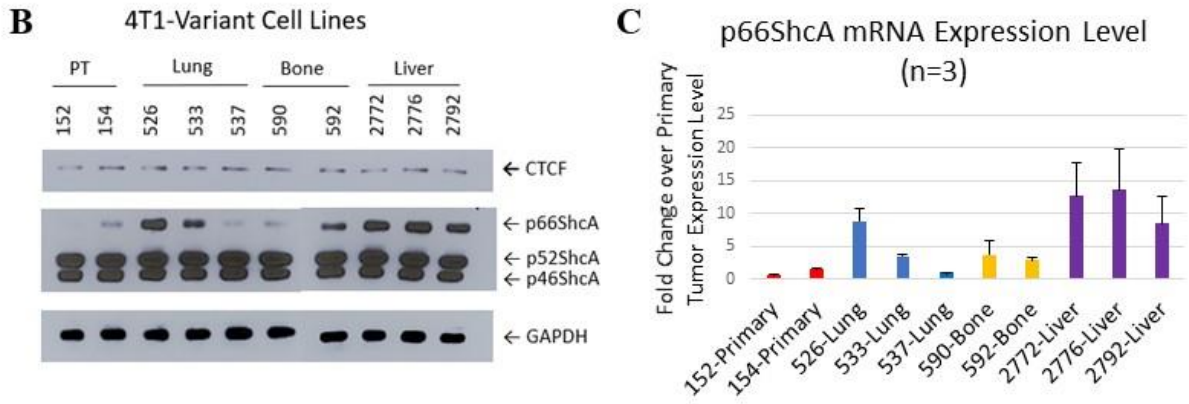

Figure 1. 4T1-derived cell lines demonstrate preferential metastatic sites and express p66ShcA at differential levels

A. The 4T1-variant cell lines used for this study were kindly generated and provided by Dr. Peter Siegel. The preferential metastatic site, cell line name and method of cell line generation are described in the table (Rose et al., 2010; Rose et al., 2007; Tabariès et al., 2011; Tabariès et al., 2015). **B.** Western blot was performed using CTCF, Shc1 (showing all three isoforms) and GAPDH antibody. Result shows differential expression level of p66ShcA across cell lines. **C.** qPCR was performed using primer specific for the p66ShcA isoform, the mRNA level shows similar trend as the protein level on western blot.

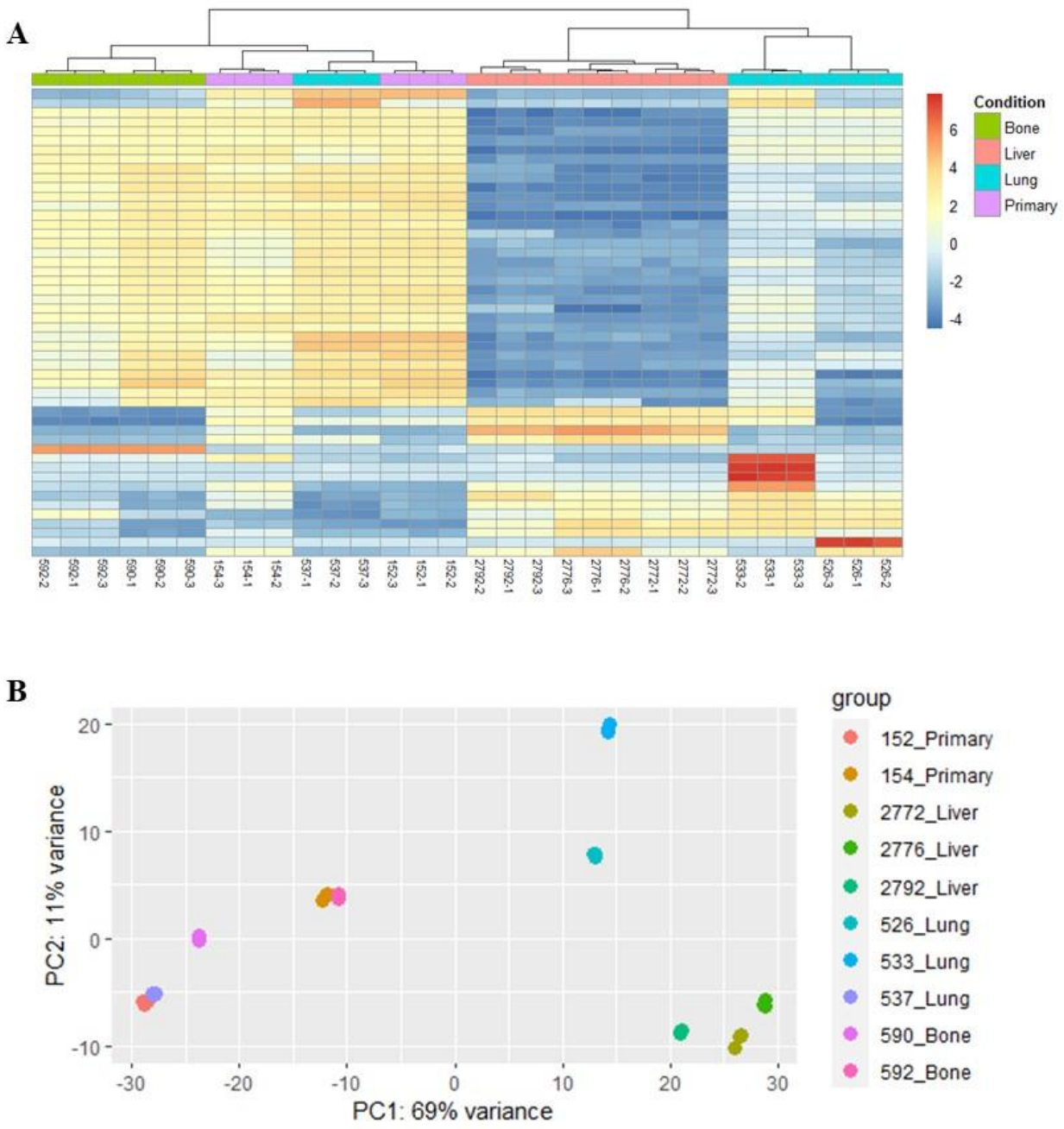

Figure 2. RNA-Sequencing of 4T1-variant cell lines shows clustering of cell groups
A. Heatmap of the top 50 differentially expressed genes generated using DESeq2. The liver-metastatic and bone-metastatic cell lines cluster together, whereas the lung-metastatic and primary explanted cell lines show variable gene expression profile. **B.** PCA plot was drawn using the first two principal components. Each point is one replicate of the corresponding cell line. Similar clustering profile as the heatmap in **(A)** can be observed.

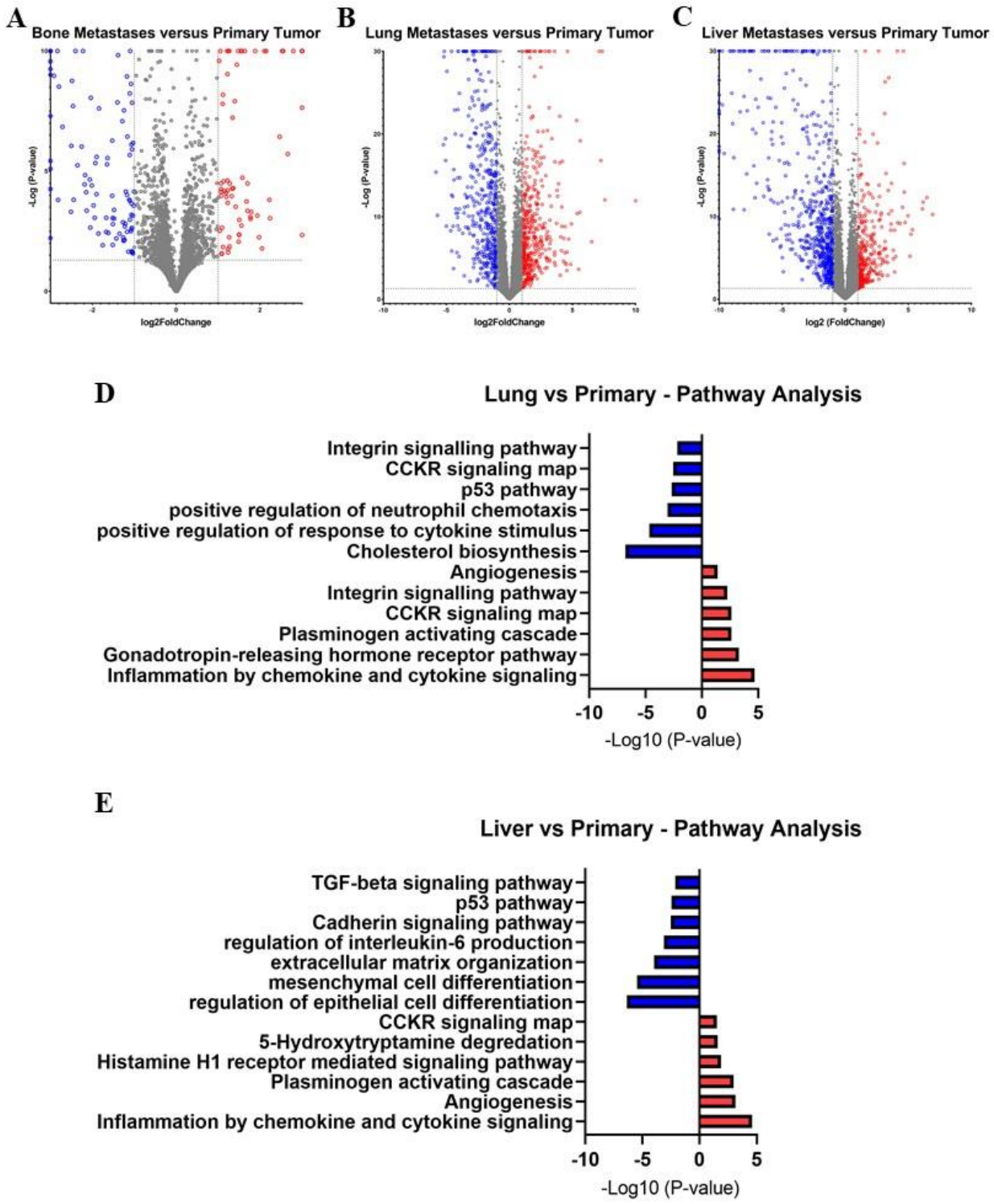

Figure 3. Differentially expressed gene analysis and pathway analysis reveal pathways that may be important for site-specific metastasis of TNBC

A-C. Volcano plots showcasing differentially expressed genes generated by comparing bone-metastatic **(A)**, or lung-metastatic **(B)** or liver-metastatic **(C)** cell lines with the primary explanted cell lines. 58 genes appear to be differentially expressed in bone-metastatic cell lines,

647 genes in lung-metastatic cell lines and 722 genes in liver-metastatic cell lines. The blue dots are genes downregulated in the metastatic group and the red dots are genes upregulated in the metastatic group. **D-F.** Pathway analyses were conducted with the differentially expressed genes using Gene Ontology. Pathways show a negative -Log(P-value) are downregulated in metastatic cells, whereas pathways show a positive -Log(P-value) are upregulated in metastatic cells. "Bone vs Primary" pathway analysis was not carried out, since the number of differentially expressed genes is too low for accurate analysis.

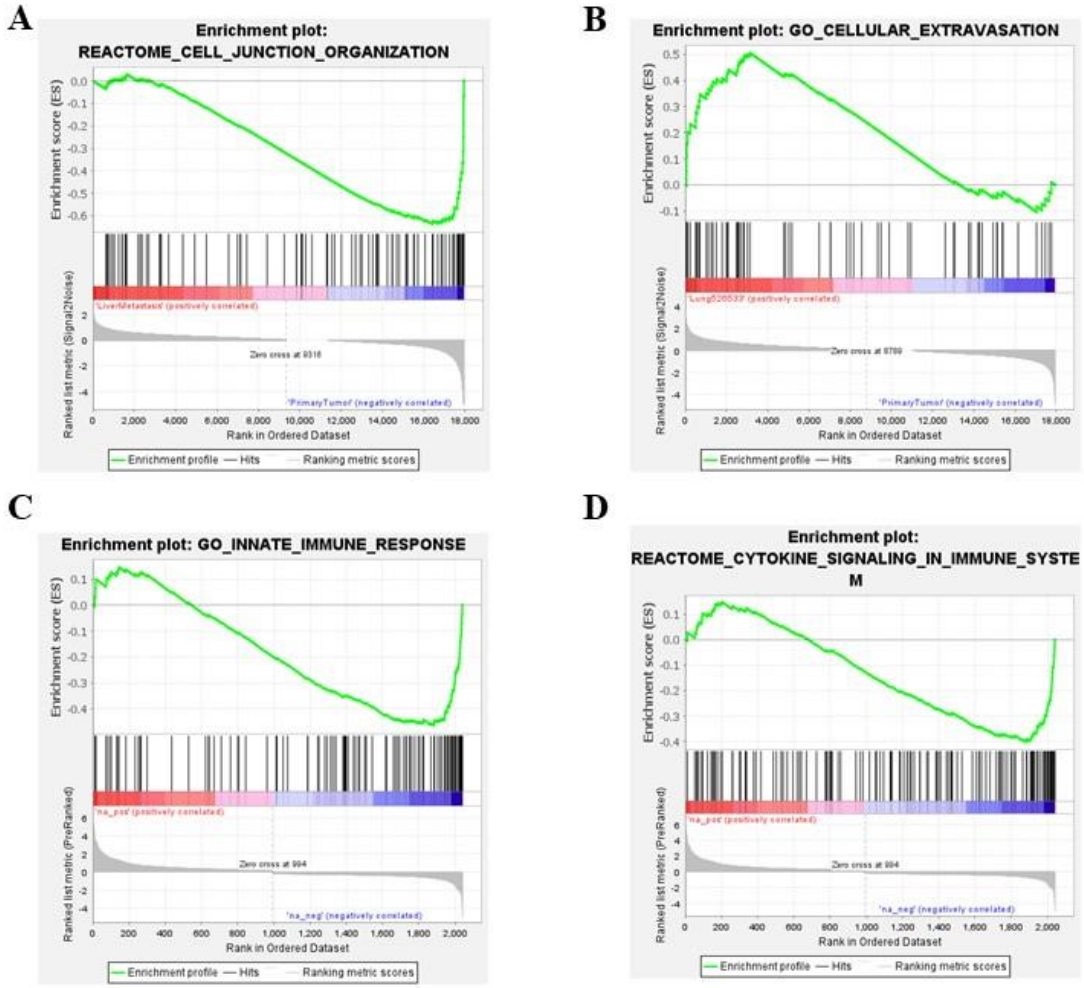

Figure 4. GSEA pathway enrichment plots further demonstrate the potential involvement of the pathways in promoting metastasis

A-D. Pathway enrichment plots were generated by comparing pathway enrichment level in metastatic cell lines with primary tumor explanted cell lines. **A.** Cell junction pathway is downregulated in metastatic cell lines **B.** Cellular extravasation pathway is upregulated in metastatic cell lines **C-D.** Pathways involved in immune system signaling are mainly downregulated in the metastatic cell lines, with a small portion of immune-related genes being upregulated.

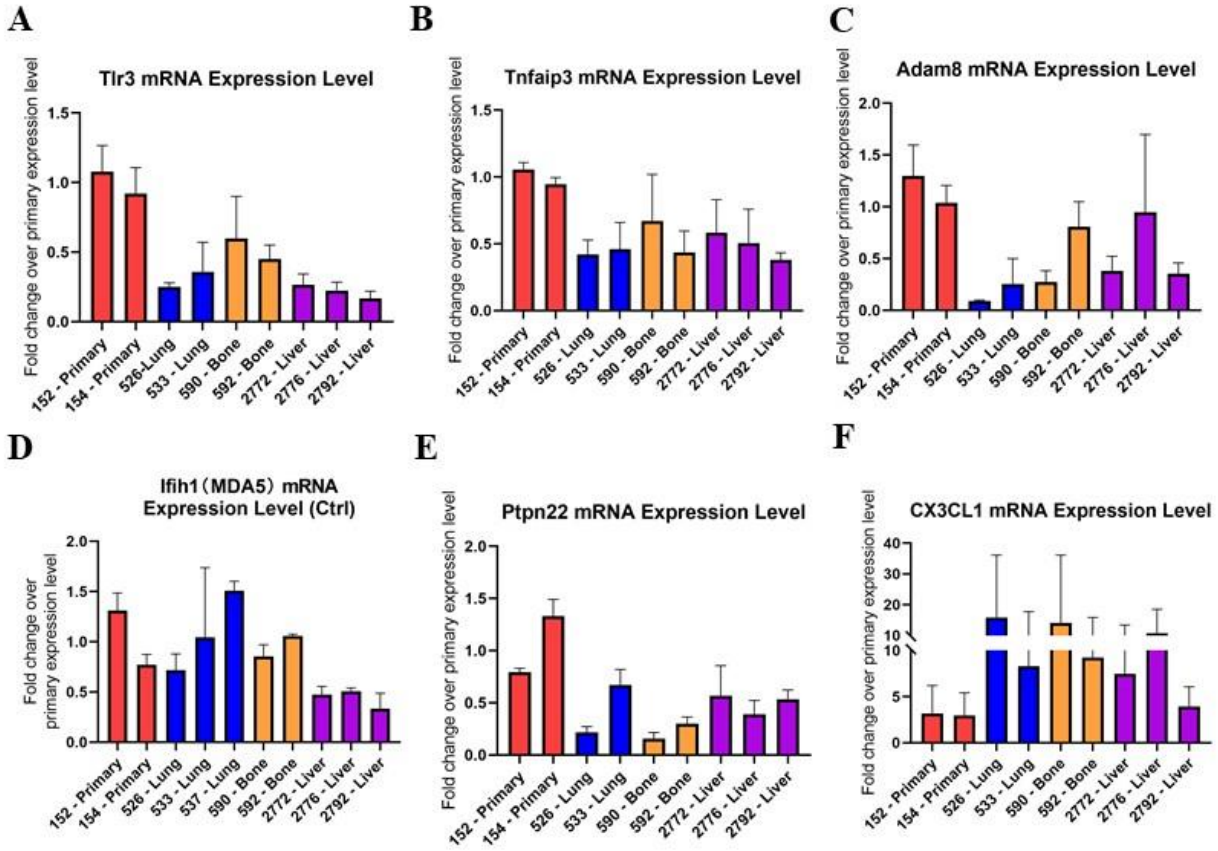

Figure 5. Panel of qPCRs performed on differentially expressed genes validates RNA-sequencing results

All data were calculated as fold change over primary tumor RNA expression level (average of expression level of primary cell lines 152 and 154)

A-E. Tlr3, Tnfaip3, Adam8, Ifih1 and Ptpn22 are involved in inflammatory processes and are expressed at lower level in the metastatic cell lines, especially in the liver-metastatic group. **F.** CX3CL1 is upregulated in metastatic cell lines. qPCR in A, B and F were performed with biological triplicates, and qPCR in C-E were performed with duplicates. All graphs were normalized using the expression level of common housekeeping genes: beta-actin and 18S.

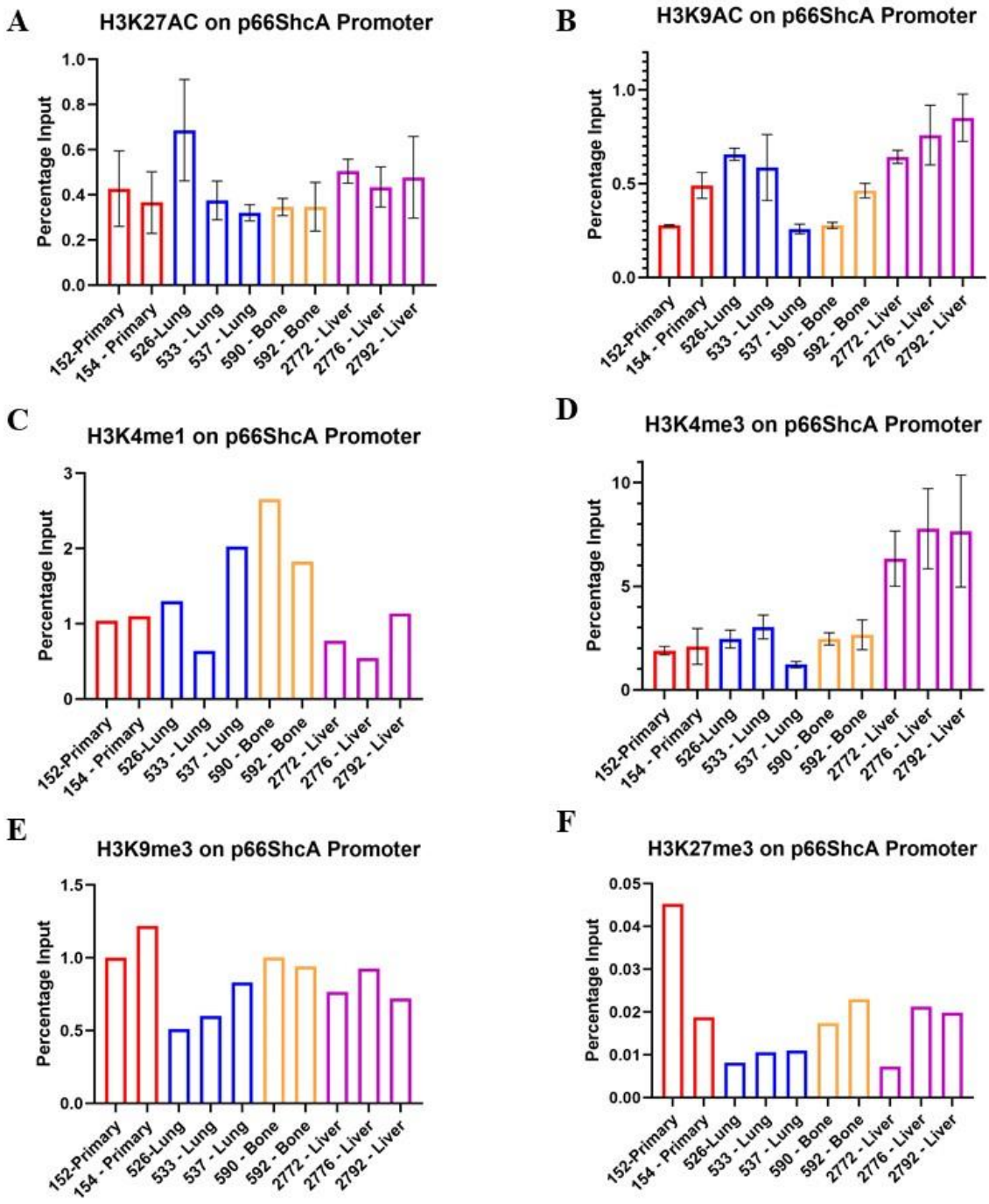

Figure 6. ChIP-qPCR on p66ShcA promoter site reveals regulatory histone modifications

A-D. H3K27ac, H3K9ac, H3K4me1 and H3K4me3 are generally involved with activating gene expression. **E-F.** H3K9me3 and H3K27me3 are involved with condensing chromatin structure and repressing gene transcription. ChIP qPCR in A, B and D were performed in biological triplicates. C, E and F were performed only once and were dropped for future study. Histone modification enrichment level was calculated as a percentage of the total input.

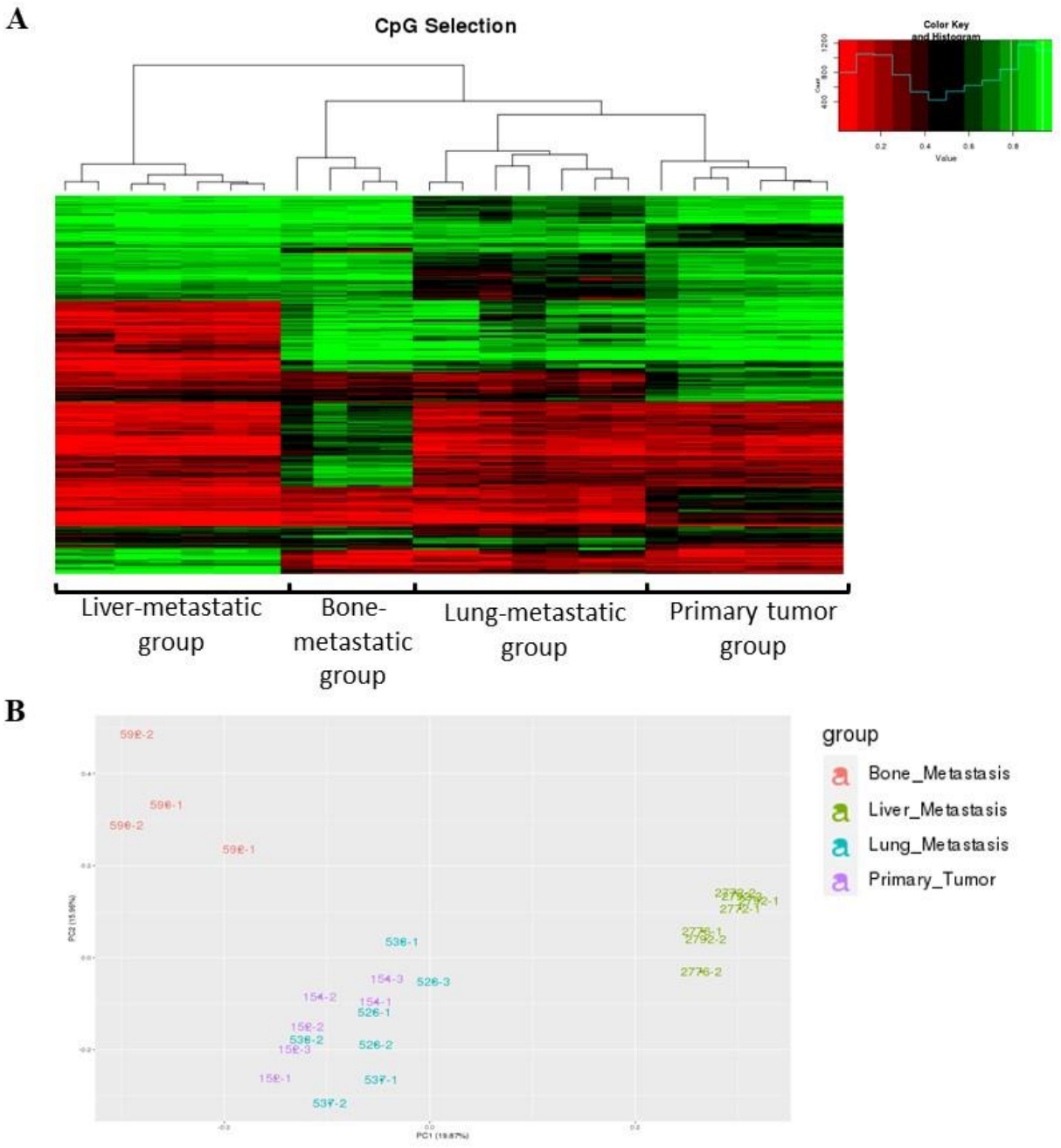

Figure 7. DNA-methylation microarray shows that DNA-methylation profile is conserved within each cell group

Heatmap of the top 400 variably methylated CpG islands reveals differential DNA-methylation profile across each cell group. **B.** PCA plot shows similar clustering of cell groups as heatmap in **(A).**

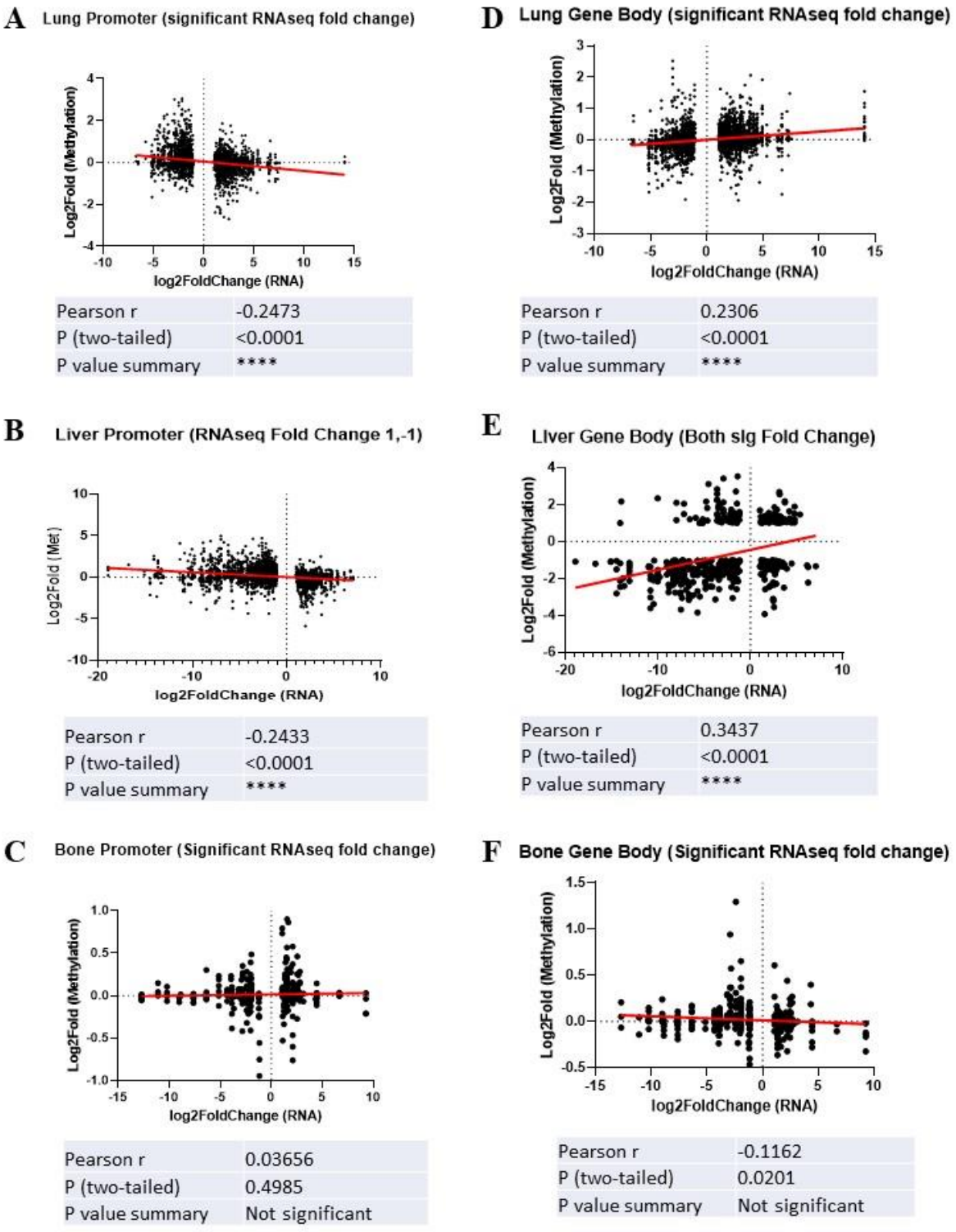

Figure 8. Correlation between DNA methylation and gene expression shows regulatory roles of DNA methylation

The Y-axis of the graphs is DNA methylation level and the x-axis is RNA-sequencing gene expression level. Pearson correlation value and p-value were calculated from the best fitted line using GraphPad. Negative Pearson r value means a negative correlation is observed and a positive value means a position correlation. Correlation value greater than 0.5 means a strong association between the two components, correlation value between 0.3 and 0.5 means a medium association, whereas correction value between 0.1 and 0.3 means a small association. **A-C.** Correlation between DNA-methylation on promoter site and gene expression is examined in lung-metastatic, liver-metastatic and bone-metastatic cell lines**. D-F**. Correlation between DNA-methylation occurring on the gene body region versus gene expression is examined.

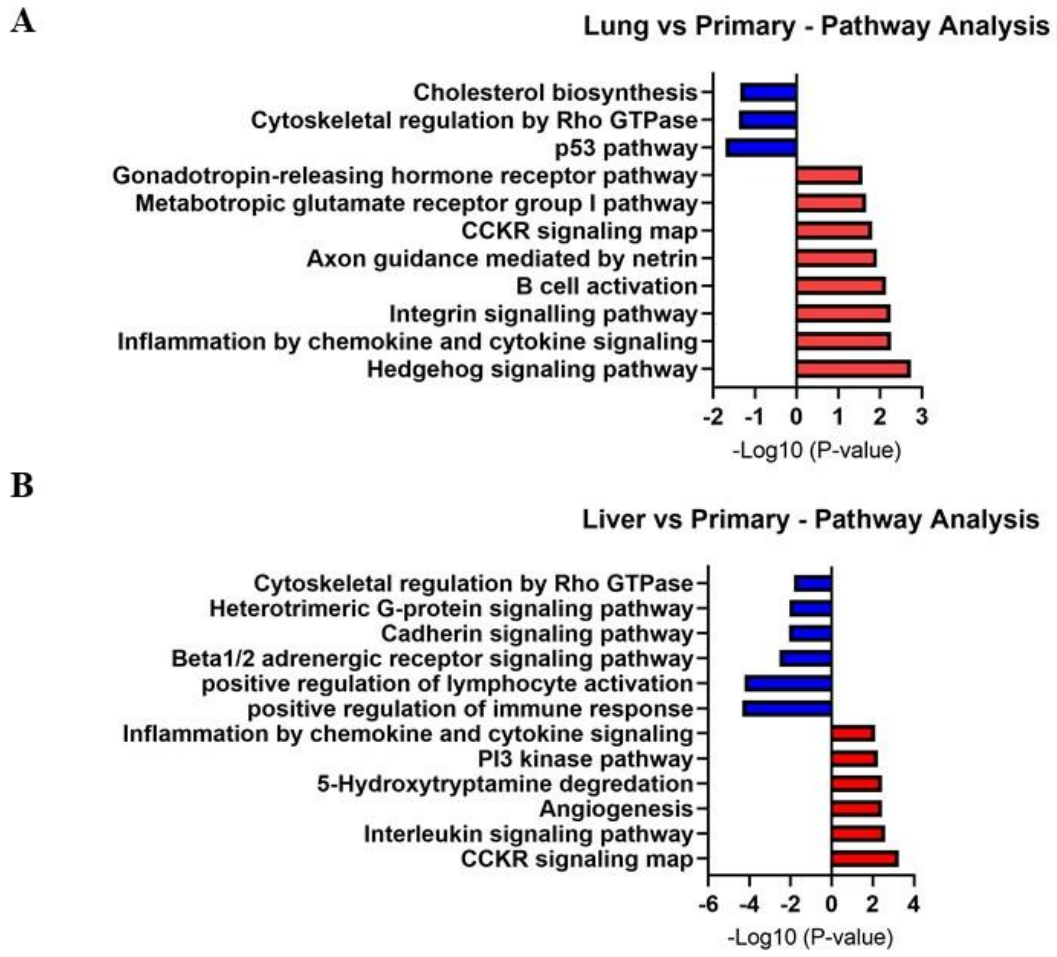

Figure 9. Pathway analysis using genes with differential DNA-methylation enrichment on the gene promoter site reveals overlapping pathways showed in the RNA-sequence analysis A-B. Pathway analysis is carried out by Gene Ontology. Pathways involved in immune signaling may be under DNA-methylation regulation.

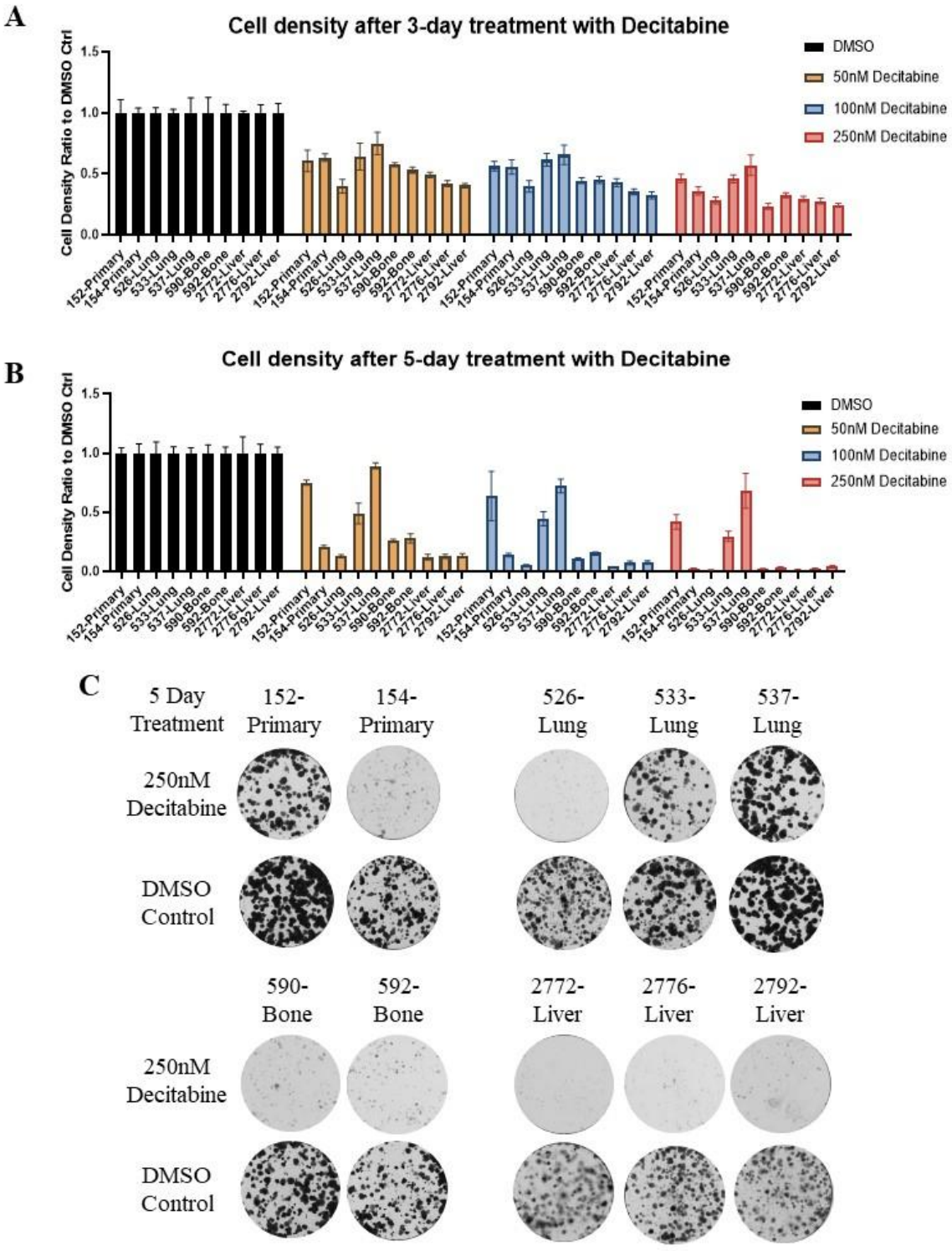

Figure 10. Decitabine treatment reveals sensitivity towards DNMT inhibitor in certain cell lines.

Growth analysis was carried out following protocol listed in **Materials and methods**. **A.** Cell density was measured using crystal violet staining 3 days after decitabine treatment at various dosage. **B.** Cell density was measured 5 days after decitabine treatment. Sensitivity towards decitabine treatment can be observed in all liver-metastatic cell lines, all bone-metastatic cell lines, one lung-metastatic cell line and one primary tumor explanted cell line. **C.** Cell plates were scanned after 5 days of treatment and after staining with crystal violet.

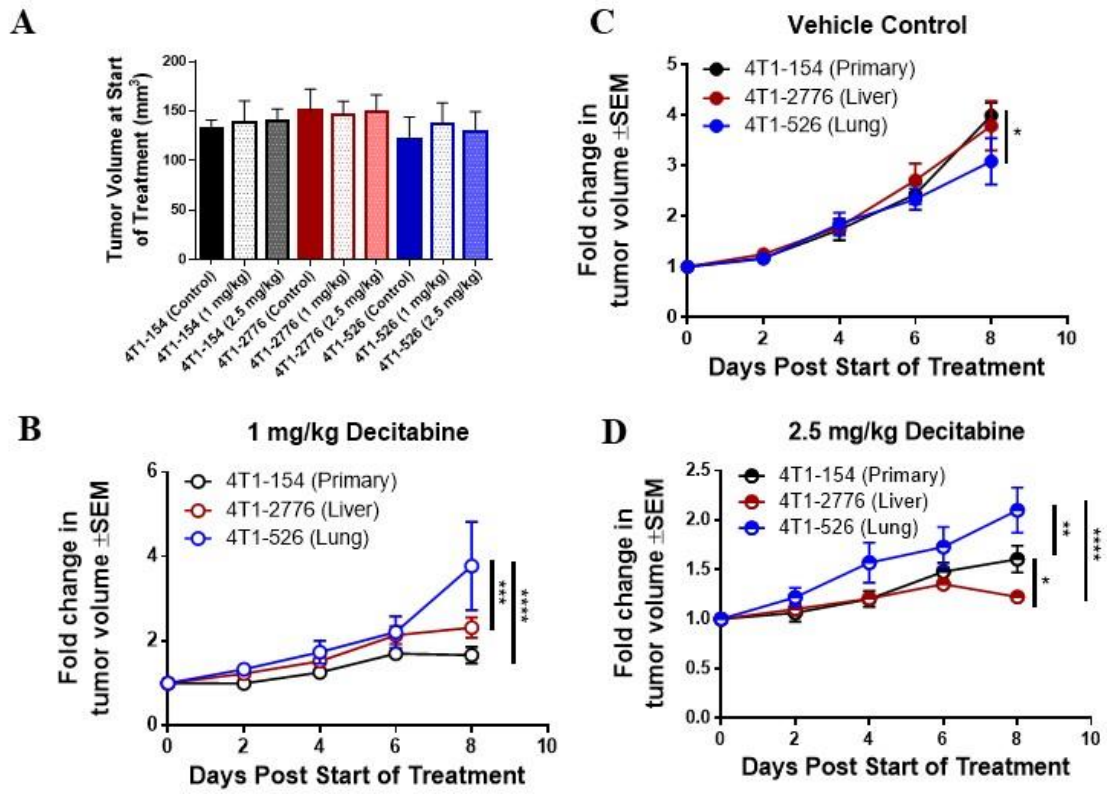

Figure 11. In-vivo decitabine treatment reveals surprising resistance in cell line that was previously sensitive towards decitabine in-vitro

Mice in-vivo experiments were kindly conducted and analyzed by Young Im. Mice were injected with cell lines that were sensitive towards decitabine in vitro (**Figure 10**). Decitabine was administered at two dosages: 1mg/kg or 2.5mg/kg. The tumor volume was then measured to track the effect of decitabine on primary tumor progression. **A.** At the start of treatment, mice in different treatment conditions had similar tumor volume. **B.** Tumor volume of the vehicle control group was measured, 526-Lung cell line showed slightly less tumor growth comparing to the other cell lines without decitabine treatment. **C-D**. Change in tumor volume was tracked for

different cell lines under the two decitabine treatment dosage, surprisingly, 526-lung cell line seems to be resistant towards decitabine in-vivo.

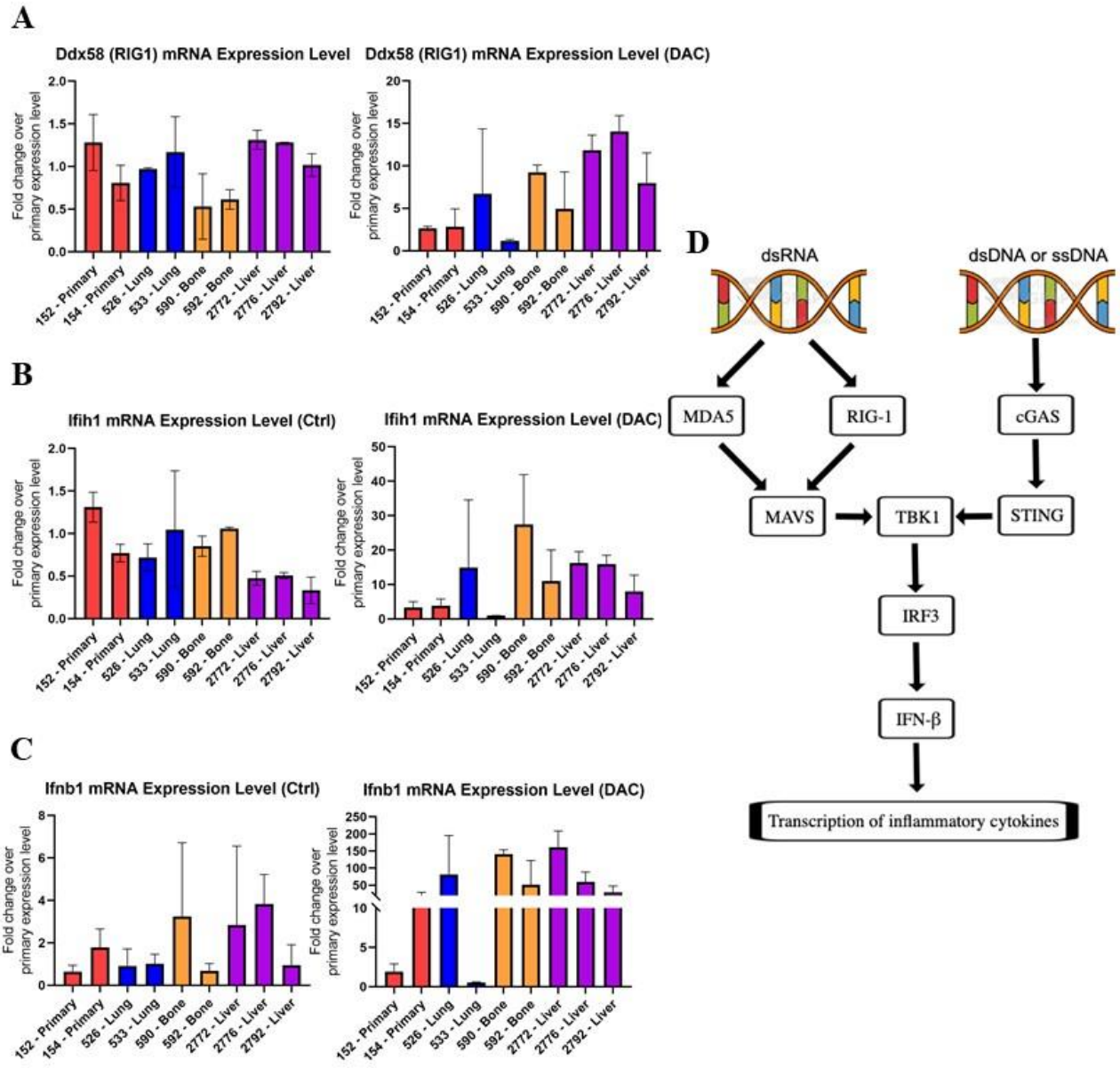

Figure 12. Immune-related gene expression are upregulated following decitabine treatment

A-C. Cell lines were treated with 50nM of decitabine for 3 days. Following treatment, RNA was extracted from cell line and qPCR was performed using specific primers. Several immune-related genes showed elevated expression level within the cell lines that are sensitive towards

decitabine treatment in the growth analysis **(Figure. 10**). **D.** Interferon-beta signaling pathway (Goubau et al., 2014; Miner & Diamond, 2014)

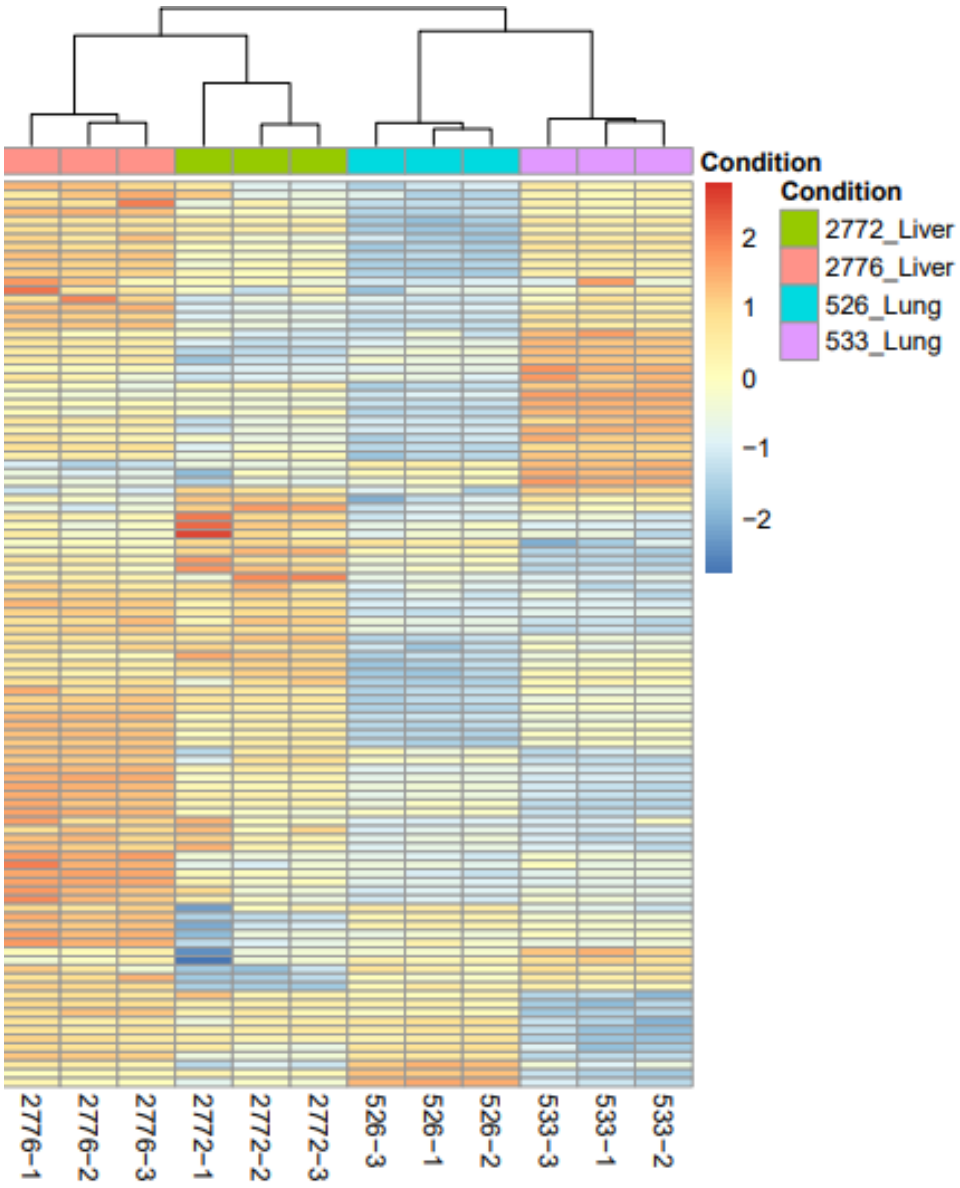

Figure 13. Heatmap comparing expression level of genes involved in cytokine production between liver-metastatic cell lines and lung-metastatic cell lines

Heatmap is generated using R package ggplot2. The lung-metastatic cell lines show a differential expression profile of cytokine-related genes comparing to the liver-metastatic cell lines. Moreover, the two lung-metastatic cell lines show variation in gene expression profile as well.

6. DISCUSSION

6.1 Characteristics of cell lines preferentially metastasize towards different organs

Organotropic metastasis is a topic that has been studied by many researchers and many theories have been proposed. Based on the RNA-sequencing heatmap and PCA plot (**Fig 2**), cell lines metastasizing towards different organs have distinct genetic profiles. This finding supports the "seed and soil" theory, where tumor cells need to cooperate with the metastatic microenvironment to facilitate site-specific metastasis (Paget, 1989). In order to complete the metastatic cascade, tumor cells need to alter their gene expression profile to be best fitted for colonization at the specific organ.

Moreover, we can also collect some information regarding the specific metastatic site and potential mechanisms from studying the individual cell line clustering on the PCA plot (**Fig 2. B**). The three liver-metastatic cell lines show a similar gene expression pattern, which is very different comparing to the primary tumor explanted cell lines that do not display organotropic metastasis. This finding suggests that the TNBC liver-metastatic cell lines may utilize a conserved mechanism to direct their invasion into the liver and require many genetic alterations to adapt to the liver microenvironment.

On the other hand, lung-metastatic cell lines show a more variable gene expression pattern among the three cell lines, suggesting that multiple mechanisms or pathways are available to facilitate lung metastasis.

Finally, based on the heatmap comparing the top 50 differentially expressed genes from RNA-sequencing and the PCA plot (**Fig 2**), the bone-metastatic cell lines have very similar gene expression profile comparing to the primary tumor explanted cell lines. This suggests that not many alterations in gene expression may be required for the 4T1 breast cancer cells to gain preference of metastasizing towards the bone. It is also possible that the organotropism towards bone is facilitated by other factor that do not require alteration in gene expression or is not regulated at the transcription level. Previous articles have shown that bone resident cells such as osteoclasts and osteocytes can express factors promoting tumor migration and attracting breast cancer cell colonization (Hiraga et al., 2001; Sottnik et al., 2015). Bone colonization can also be

initiated by interaction between N-cadherins on osteocytes and E-cadherins on tumor cells (Sottnik et al., 2015).

6.2 Identification of epigenetic modifications involved in promoting organotropism metastasis

In this study, p66ShcA was used as a model to investigate and identify epigenetic modifications involved in promoting organotropism metastasis in TNBC. Based on previous studies, p66ShcA was shown to be crucial for TNBC metastasis towards the lung (Lewis et al., 2020) and its expression was reported to be under histone acetylation and DNA methylation regulation (Ventura et al., 2002b). Using western blot and qPCR, we have established that p66ShcA expression level varies across metastatic cell groups. It is expressed at a higher level in the liver-metastatic cell lines and in two of the lung-metastatic cell lines (not in 537).

We next examined what epigenetic mechanisms are regulating p66ShcA expression within the cell lines. Using ChIp-qPCR on the p66ShcA promoter site, we showed that H3K9ac is enriched in liver- and lung-metastatic cell lines (**Fig 6. B**) and H3K4me3 is highly enriched in the liver-metastatic cell lines (**Fig 6. D**), similar to the p66ShcA expression pattern detected by western blot. In a previous study conducted by Lihua Wang et al., H3K9ac was shown to be significantly enriched in liver metastases of colorectal cancer cells comparing to in the primary tumor cells. The article proposed that this alteration in epigenetic regulation is induced by the liver microenvironment, which supports tumor cell growth in the new condition (Lihua Wang et al., 2021). Enrichment of H3K4me3 was also linked with cancer metastasis in previous studies. H3K4me3 was shown to upregulate expression of MMPs, integrins and EMT-related genes to promote tumor metastasis (Lyu et al., 2020; Salz et al., 2015; Wang et al., 2014).

To identify what epigenetic mechanisms are repressing gene transcription within the cell lines, DNA-methylation microarray and ChIP-qPCR with H3K9me3 and H3K27me3 were performed. DNA-methylation enrichment was graphed against RNA-expression level, showing a medium correlation strength between the two factors, suggesting DNA-methylation and other additional mechanisms regulate RNA-expression within the cell lines.

Previous study in promoter hypermethylation of distant breast cancer metastases have identified a few genes that are significantly more methylated in brain, lung and skin metastases compared to liver metastases, even after correction for effect of cancer subtype (Schrijver et al., 2015). Consistent with previous finding, our DNA-methylation microarray analysis reveals distinct DNA-methylation enrichment pattern on CpG islands in each cell group. Moreover, treating the cell lines with DNMT inhibitor decitabine demonstrated that the cell lines have variable sensitivity towards downregulation of DNA-methylation (**Fig 10**). Conversely, treating the cell lines with A485, an inhibitor of H3K27ac, did not show variable sensitivity across the cell lines (**Supplementary Fig. S2**), confirming the effect of DNA-methylation is not present with other treatments. The above findings suggest that the cell lines are dependent of DNA-methylation to different degrees and removing DNA-methylation activates different mechanisms within the cell lines.

The liver-metastatic cell lines cluster far away from the primary-explanted cell lines on the PCA plot (**Fig 7. B**) and the similar pattern is observed for the liver-metastatic cell lines on the PCA plot of RNA-expression level (**Fig 2. B**). Moreover, when treated with DNMT inhibitor, all liver-metastatic cell lines show a conserved sensitivity towards the treatment (**Fig 10**). As a result, we predict that DNA-methylation plays a critical role in regulating gene expression within the liver-metastatic cell lines, therefore, disruption of the DNA-methylation pattern within those cell lines is detrimental to cell growth or metastasis.

On the other hand, the lung-metastatic cell lines cluster closely with the primary explanted cell lines (**Fig 7. B**), suggesting that DNA-methylation is not the main cause of differentially expressed genes within those cell lines. Moreover, comparing the lung-metastatic cell lines with each other show variable DNA-methylation enrichment. This finding is supported by the variable sensitivity demonstrated by the lung-metastatic cell lines after decitabine treatment (**Fig 10**), supporting our previous thought that the lung-metastatic cell lines are heterogenous and may facilitate metastasis through different mechanisms.

Overall, we have discovered distinct H3K9ac, H3K4me3 and DNA-methylation enrichment across the metastatic cell lines, suggesting these epigenetic modifications may affect organotropism metastasis within TNBC.

6.3 Regulation of immune-related pathways promotes triple-negative breast cancer metastasis

Pathway analysis performed using RNA-sequencing expression showed distinct pathway information within the liver- and lung-metastatic groups. Other than pathways traditionally associated with metastasis, cell migration and EMT transition, many immune-related pathways were observed.

The immune system plays a complicated role in tumor progression and tumor metastasis. Some immune factors are pro-tumorigenic, such as tumor-associated macrophages activating angiogenesis (Loboda et al., 2012) and production of cytokines activating EMT and inhibiting cytotoxic processes (Schmieder et al., 2012). Other immune factors can inhibit tumor development and initiate tumor killing (Mittendorf et al., 2012).

A cooperation of pro-tumorigenic and anti-tumorigenic immune pathways can be observed in the cell lines. Both upregulation and downregulation of inflammation by chemokine and cytokine signaling is detected within the liver- and lung-metastatic cell lines (**Fig 3. DE**). This finding is consistent with GSEA enrichment plot, while the majority of genes related with innate immune response and cytokine signaling are downregulated, a small portion are upregulated within the metastatic cell lines (**Fig 4. CD**). Furthermore, qPCR performed on a few immune-related genes support this finding. Tlr3, Tnfaip3, Adam8 and Ptpn22 are expressed at a lower level in the metastatic cell groups comparing to the primary tumor explanted group (**Fig 5 A-E**). These genes regulate downstream cytokine production and inflammation response, which may lead to tumor cell apoptosis (Coussens & Werb, 2002). On the other hand, the expression level of CX3CL1 is upregulated in the metastatic cell lines (**Fig 5. F**), suggesting that metastasis is not promoted through a global dampening of immune response, but a more selective upregulation or downregulation of certain pathways.

Conversely, Ifih1 is only expressed at a lower level within the liver-metastatic cell lines (**Fig 5. D**). Ifih1 and Tlr3 have similar functions of detecting double stranded RNA and induce type I interferon signaling (Alexopoulou et al., 2001; Rice et al., 2014). However, previous study has shown that sensing the same dsRNA by Ifih1-encoded protein MDA5 and Tlr3 lead to

induction of unique signatures of interferon-α and interferon-β, leading to distinct immune response. Reduction of MDA5 signaling was linked with induction of protective interferon I and regulatory T cell signaling, which was not observed for Tlr3 signaling (Lincez et al., 2021). The differential role and qPCR expression pattern of Ifih1 and Tlr3 suggests that some immune-related pathways may be uniquely regulated within certain metastatic-group, promoting organ-specific TNBC metastasis.

6.4 Cell line-specific sensitivity towards DNMT inhibitor treatment

Decitabine, a DNMT inhibitor, was used to downregulate DNA-methylation within the cell lines. The cell lines show variable sensitivity towards decitabine: the liver-metastatic cell lines, bone-metastatic cell lines, one lung-metastatic cell line (526) and one primary explanted cell line (154) are sensitive and grow in low cell density after treatment (**Fig 10**).

Because previous analysis identified immune-related pathway as important regulatory pathways in the metastatic cell lines, qPCR was performed on a few immune-related genes in the cell lines with or without decitabine treatment. The three genes shown in **Fig. 12** are Ddx58 (encoding RIG-1 protein), Ifih1 (encoding MDA5 protein) and Ifnb1 (encoding interferon-beta protein). RIG-1 and MDA5 can sense distinct features of viral double strand RNA (Kato et al., 2006). Detection of viral particle leads to signaling through the MAVS adaptor protein in mitochondria and activates downstream transcription of INF-β (Rice et al., 2014; Seth et al., 2005). Production of INF-β can lead to phosphorylation of STAT1 and STAT2, which bind with IRF9 to form phosphorylated IFN-stimulated gene factor 3 (P-ISGF3), an important transcription factor activating transcription of hundreds of IFN-stimulated genes (ISG) (reviewed in (Schneider et al., 2014). IFN-β signaling is anti-tumorigenic and was shown to increase chemotherapy response and induce apoptosis within breast tumor (Doherty et al., 2019; Yoshida et al., 2004). All of the cell lines sensitive towards decitabine show significantly upregulated expression level of IFN-β, suggesting that IFN-β signaling may be the anti-tumorigenic pathway that causes growth defect within those cell lines.

IFN-β production can be induced by different pathways. As shown in **Fig 12. D**, sustained activation of either MDA5/RIG-1/MAVS pathway or cGAS/STING pathway can

recruit TANK-binding kinase-1 (TBK1) and induce downstream signaling cascade (Fang et al., 2017; Tanaka & Chen, 2012). qPCR or MDA5 and RIG-5 showed increased expression level in all of the decitabine-sensitive cell lines, except in 154-primary tumor explanted cell line. This finding proposes that the upregulated expression level of IFN-β in these decitabine-sensitive cell lines (except in 154) may be caused by increased level of MDA5/RIG-1 signaling cascade. Since MDA5/RIG-1 are not upregulated in 154-primary explanted cell line, it is possible that the cGAS/STING pathway is activated instead.

Another finding supporting the involvement of immune-related pathways in promoting sensitivity towards decitabine is that the lung-metastatic cell line 526 only gained resistance against decitabine in-vivo. It is possible that there exist additional immune pathways within 526 that can downregulate the effect of IFN-β signaling, hence protecting the cell from its anti-tumorigenic actions. However, these pathways can only be activated in-vivo, when a tumor microenvironment is present to support interaction with immune-cells or with cytokine and chemokines. The reason that the other two lung-metastatic cell lines maintain resistance against decitabine in-vitro is that different mechanisms are utilized by the cell lines to gain resistance. This theory is supported by the heatmap generated using a few cytokines and chemokines expressed within the tumor cells (**Fig 13**). The gene expression pattern is very different comparing the liver- and lung-metastatic cell lines, which makes sense since they have very different response towards decitabine. However, the gene expression pattern between the two lung-metastatic cell lines is also highly distinct, suggesting the existence of unique pathways or mechanisms that may lead to decitabine resistance.

6.5 Future direction

The first question worth further investigation is to examine the importance of histone modifications in tumor proliferation and metastasis within these cell lines. The first step would be to confirm the critical role of H3K9ac and H3K4me3 in the cell lines. ChIP-sequencing should be carried out on the two histone modification markers to study their global effect on gene transcription. Pathway analysis using the ChIP-seq results may reveal targetable pathways that can provide valuable information regarding treatment development of TNBC. Heatmap and PCA plot can be used to check if the two histone modification markers promote organotropism

metastasis. This knowledge can be used to develop prognostic markers of TNBC metastasis towards specific organ, which would be useful for determining preventative measures in a clinical setting before metastasis onset. Epigenetic drug treatments targeting the two histone modification markers can also be carried out.

Another direction is to further study and understand resistance against decitabine. In order for decitabine to be incorporated into DNA and inhibit DNMT1 function, it needs to be first activated by phosphorylation (Momparler, 2005). Deoxycytidine kinase (DCK) is a key kinase involved in decitabine activation and downregulation or mutation of DCK can cause decitabine resistance (Qin et al., 2011; Raynal et al., 2011). Resistance to decitabine can also be caused by up-regulated CDA activity, which can reduce the amount of functional decitabine through deamination (Qin et al., 2009). Previous study has shown that patients with decitabine resistance have 3-fold higher CDA/DCK ratio comparing to patients that are responsive towards decitabine treatment (Qin et al., 2009). By examining CDA/DCK RNA expression ratio in the 4T1 cell lines used for this study, we can determine whether the differential resistance across 4T1 cell lines is caused by variable level of decitabine incorporation or by other mechanisms.

Three of the cell lines sensitive towards decitabine in-vitro were also injected into mice mammary fat pads and treated with decitabine in-vivo. Primary cell line 154 and liver-metastatic cell line 2776 displayed sensitivity towards decitabine in-vivo, whereas lung-metastatic cell line 526 showed resistance against decitabine treatment (**Fig 11**). It was surprising to see the liver-metastatic cell line 2776 remained to be sensitive towards decitabine in-vivo, since liver has previously been reported to express high level of CDA, which can metabolize cytidine analogues such as decitabine into non-functional uridine (Ebrahem et al., 2012). It is possible that the liver-metastatic cell lines carry upregulated DCK or other kinases that can facilitate phosphorylation of decitabine, which balances the effect of augmented CDA level. Moreover, our in-vivo experiments measured primary tumor growth in breast. The liver-metastatic cell lines may behave differently towards decitabine at the primary tumor site comparing to the metastatic liver site. The effect of the liver microenvironment and upregulated CDA level should also be studied.

We have also discovered that signaling through IFN-β activated by decitabine treatment is an effective method of decreasing tumor growth. Moreover, decitabine is a treatment approved

by the FDA, so safety data and appropriate dosage of decitabine treatment has already been established. However, before decitabine can be suggested as a treatment option for TNBC, we need a method to determine tumor cells that may be resistance against decitabine treatment and come up with a solution to overcome this resistance. DNA-methylation analysis can be carried out pre- and post- decitabine treatment on the cell lines to gain information on cell line specific resistance towards DNMTi treatment. Furthermore, activating IFN-β signaling through the cGAS/STING pathway can be examined as a potential way to overcome this resistance. As shown in the qPCR of immune-related genes (**Fig 12, A-C**), IFN-β level in 154-primary explanted cell line may be activated through the cGAS/STING pathway instead and no resistance against decitabine treatment was detected in-vitro and in-vivo (**Fig 10, 11**). The effect of activating cGAS/STING pathway can be examined by combining a STING agonist with decitabine to treat the resistant cell lines.

Finally, most of this study focused on primary tumor growth and proliferation. Since the 4T1 cell lines show distinct metastasis profile, it would be interesting to see the effect of decitabine on general tumor metastasis and on organotropism metastasis. Because metastatic tumor cells can be highly heterogeneous and differentially affected by the tumor microenvironment, single-cell RNA-sequencing can be a useful tool to further study the metastatic potential of the 4T1 cell lines.

7. CONCLUSION

In conclusion, we have showed that H3K9ac, H3K4me3 and DNA-methylation may be important epigenetic modifications that promote TNBC organotropism metastasis. We have demonstrated that decitabine, a DNMT inhibitor, is effective in damaging tumor progression in certain cell lines. Through pathway and gene expression analysis, we propose that the effect of decitabine is exerted through activating the INF-β signaling pathway. Furthermore, we predict that by upregulating INF-β production through the STING/cGAS pathway, resistance against decitabine may be overcame.

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