Meox1 Regulates Proliferation and Metastasis of Drug Resistant P53 and PTEN Deficient Triple Negative Breast Cancer Offering a Specific Therapeutic Target

by

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Abstract

Relative to other breast cancer subtypes, triple negative breast cancer (TNBC) exhibits more aggressive clinical behavior and poorer patient outcome. Lacking specified targets to improve long-term patient outcome of overall survival, chemotherapy is the mainstay of treatment for patients with TNBC. Unfortunately, only 30% of TNBC patients achieve pathological complete response (pCR) following neoadjuvant chemotherapy, the remaining majority exhibit drug resistant residual disease. Given the aggressive characteristics of TNBC, drug resistant patients inevitably experience disease recurrence, distant metastasis, and mortality within 5 years following diagnosis. Genetic aberrations in tumor suppressor genes of p53 and PTEN are frequent critical 'driver mutations' for tumorigenesis and drug resistance in TNBC. While the significance of p53 and PTEN are well recognized, the molecular biology of their combined loss of function is not well known in TNBC; moreover, no actionable specified targets exist for TNBC patients displaying genetic aberrations in both tumor suppressor genes. To offer insight, the functional and mechanistic role of mesenchyme homeobox 1 (Meox1) is examined in the context of p53 and PTEN deficient TNBC, offering a specific therapeutic option. RNA expression analysis shows Meox1 is upregulated in TNBC. Additionally, Meox1 expression is negatively regulated

by p53 and PTEN; *in vitro* experiments show small interfering RNA (siRNA) knockdown of both tumor suppressor genes increases Meox1 expression. Furthermore, *in vitro* cell proliferation assays show siRNA knockdown of Meox1 significantly decreases cell growth of p53 and PTEN deficient TNBCs in both claudin-low and basal-like intrinsic subtypes. Interestingly, this decrease in cell growth is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like cells. *In vivo* tumor xenograft mouse models corroborate *in vitro* data, where knocking down Meox1 using doxycycline inducible short hairpin RNA (shRNA) significantly decreases tumor growth in an adjuvant and neoadjuvant setting. Meox1 knockdown in both intrinsic subtypes also significantly decreases migration and invasion, ascribing to its functional role in regulating metastasis. RNA sequencing and integrative pathway analyses show knocking down Meox1 in claudin-low and basal-like p53 and PTEN deficient TNBC inactivates important canonical pathways involved in growth and survival as well as migration and invasion. Important canonical pathways inactivated include Stat3 and Jak/Stat signaling. Western blot analyses of the Jak/Stat pathway show Meox1 knockdown decreases Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low, but only decreases Tyk2 and Stat6 protein levels in basal-like cells. These results demonstrate Meox1 function of proliferation and metastasis may be elicited via the Jak/Stat mechanistic pathway. However, it is evident that Meox1 regulates Jak/Stat signaling differently between the two different intrinsic subtypes. Furthermore, Meox1 knockdown also regulates distinct mechanistic pathways of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC. As such, data indicates Meox1 may have different transcriptional regulation in different TNBC subtypes. While

functional and mechanistic analysis of Meox1 offers insight into the complex molecular biology of p53 and PTEN deficient TNBC, results indicate that targeting Meox1 may have a critical role in ameliorating the aggressive proliferative and metastatic potential of these drug resistant tumors. Utilizing peptide inhibitors or breakthrough therapies such as siRNA delivery against Meox1 may translate to effective treatments in the clinic for chemo-resistant p53 and PTEN deficient TNBC patients with poor prognoses.

Chapter I

Introduction

Breast Cancer Heterogeneity and Classification

Breast cancer is not a single disease, but a heterogeneous disease that manifests various clinical, histological, and biological characteristics. Respectively, clinical, histopathological, and biomolecular approaches are utilized to provide meaningful prognostic and predictive patient information [1]. While prognostic factors help determine overall patient tumor outcome regardless of therapy and help determine which patients may require therapy, predictive factors determine which specific therapy is appropriate and provide meaningful knowledge regarding patient tumor response to specific therapies [1, 2].

Clinical parameters of tumor classification allow the assessment of tumor heterogeneity through nodal status, tumor grade, vascular invasion, proliferation markers, age, and tumor size [3]. Independent of treatment, such prognostic markers offer significant clinical knowledge regarding tumor growth, invasion, and metastasis [2].

Histopathology is utilized to evaluate specific morphological and cytological patterns associated with patient tumor growth and outcome [4, 5]. Identifying these

patterns allows breast cancer division into different histopathological types, where each type shows differences in clinical presentation and behavior. The World Health Organization (WHO) has classified at least twenty different types of invasive breast carcinomas, the majority of which originate from the epithelium of terminal ductal lobular units (TDLU) [4-6]. Depending on the morphological and cytological characteristics warranting classification, different histopathological types are classified as being of special or of no special type [7-9]. The most common is invasive ductal carcinoma not otherwise specified (NOS) or invasive carcinoma of no special type (NST) representing approximately 50-75% of the histopathological population [4, 5]. Invasive lobular carcinoma is the second most common, which is classified as a special type representing approximately 5-15% [5, 7-9]. The remaining majority of invasive breast carcinomas are a mixture of special types, and as a whole they encompass a much smaller percentage of the total histopathological population, making the majority of invasive breast carcinoma special types rather rare [5, 7-9].

While clinical and histopathological methods provide meaningful prognostic and predictive value for patient tumor outcome and therapeutic response, it is really the advent of gene expression profiling for subtyping of breast cancer that has proven to be most informative. Seminal gene expression profile studies conducted by Perou and Sorlie, with subsequent contributions thereafter, have shown that breast cancer can be divided into what are called the intrinsic subtypes of luminal A, luminal B, HER2-enriched, basal-like, claudin-low, and a separate normal breast-like group [10-16]. Researchers utilized different patient tumor samples from invasive breast carcinomas of special or no special type [10-14]. Intrinsic subtype classification was based on a

selective subset of genes that showed great variation of expression between different samples from different patient tumors but showed no variation of expression between different samples from the same patient tumor [10-14].

By using high throughput methods, researchers were quick to show how breast cancer phenotypic heterogeneity was attributed to differences in genetic expression [11, 12, 17]. In analyzing thousands of genes for intrinsic subtyping, more precise molecular profiles underlying biological heterogeneities of breast cancer tumors were characterized [11, 12, 17, 18]. Differences in the genetic make-up of intrinsic subtypes will affect differences in biological cell function and mechanism, as a result different intrinsic subtypes should be diagnosed and treated as distinct diseases with more specific therapeutic strategies [11, 12, 16-18]. Accordingly, researchers set out to explore the prognostic and predictive value of breast cancer intrinsic subtypes, since biological diversity can be an important determinant of breast cancer incidence, survival, and therapeutic response [11-14, 18-20]. Indeed, current data shows how intrinsic subtypes are proving to be more accurate prognostic and predictive indicators, even more so than standard clinical and histopathological methods; this undoubtable improves diagnosis of overall patient tumor outcome and helps improve selection of potential therapeutic benefits [21, 22]. Clinical and histopathological markers have not always been sufficient in providing enough information regarding the biological complexity of the tumor [18]. In addition, breast cancer patients with similar clinical and histopathological characteristics have shown differences in overall tumor outcome and therapeutic response [13, 21, 22]. Nonetheless, current knowledge dictates it is really the combination of using clinical-histopathological methods with gene expression

profiling that is proving to be most effective for patient prognosis and prediction of breast cancer [11, 18, 20].

Breast Cancer Intrinsic Subtypes: Biology, Prognosis, and Prediction (Luminal A, Luminal B, HER2-Enriched, and Normal Breast-Like)

Luminal A

Each intrinsic subtype of breast cancer shows edifying distinctions in incidence, biology, survival, and response to treatment (Figure 1.1).

Luminal A breast cancer is the most common comprising approximately 40-60% of the total intrinsic subtype population [24-27]. These tumors display a gene expression profile similar to the luminal epithelium of the normal mammary duct, hence their name [10-12, 17]. Along with having high expression of luminal epithelial cytokeratins 8 and 18, luminal A tumors are also commonly characterized by their high expression of estrogen receptor (ER) and progesterone receptor (PR) [10, 13, 28, 29]. Hormone regulation plays a critical role in luminal A tumor maintenance, particularly through ER-associated genes of GATA3, FOXA1, XBP1, LIV1, and TFF1/3 [10-14]. Signaling through ER-alpha especially has a critical role for breast cancer growth and development. Along with having an immunohistochemistry (IHC) profile of high ER and PR expression, analyses of luminal A tumors also display negative expression for human epidermal growth factor receptor 2 (HER2) and low expression for proliferative genes that include KI67, signifying minimal mitotic activity [11, 12, 14, 28, 29]. Luminal

A tumors are of low histological grade displaying well differentiated cells with low nuclear pleomorphism [28, 29]. Amongst all the intrinsic subtypes of breast cancer, luminal A breast cancer patients display the best prognosis [11, 12, 14, 17, 29]. Patients have lower rates of distant relapse 15 years after diagnosis relative to other intrinsic subtypes, with rates as low as 27.8% [30]. In addition, their survival time after first distant metastasis is significantly longer with median survival of 2.2 years [30]. Luminal A site-specific metastasis is commonly seen in the bone with a 15-year cumulative incidence rate of 18.7% [30]. Less than 10% of site-specific metastatic incidence rates are seen in the brain, liver, and lung [30]. Indeed, when looking at frequency of site-specific metastasis, luminal A patients exhibit significantly higher proportions of bone metastasis at 66.6%, while displaying lower proportions of brain metastasis at 7.6% [30, 31]. The mainstay of treatment against luminal A tumors is endocrine therapy, which includes aromatase inhibitors (AI) and selective estrogen receptor modulators (SERM) [32]. Chemotherapy is administered depending on the extent of the disease and if there is an estimated risk of relapse or metastasis [32].

Luminal B

Although similar in many ways, luminal A and B intrinsic subtypes display differences in incidence, survival, and response to treatment; in doing so they provide different prognostic and predictive value. Luminal B breast cancers comprise approximately 10-20% of the total intrinsic subtype population [24-27]. Like the luminal A, luminal B tumors also display a gene expression profile similar to the luminal epithelium of the mammary duct [10-12, 17]. They similarly express luminal epithelial

cytokeratins 8 and 18, and can commonly be characterized by their high expression of ER and PR [10, 13, 28, 29]. Hormone regulation likewise plays a critical role in luminal B tumor maintenance through ER-associated genes of GATA3, FOXA1, XBP1, LIV1, and TFF1/3 [10-14]. While it is not always simple to discern the difference between the two intrinsic subtypes, distinctions between luminal A and B tumors can be seen by their varying expression levels of ER, PR, HER2, and KI67 biomarkers [29]. Though mostly positive for ER, luminal B tumors tend to have a relatively lower expression of ER and ER-associated genes; they also may or may not express PR and may or may not express HER2 [11, 28, 29]. In addition, they are likely to be more proliferative with higher expression of proliferative genes that include KI67, signifying greater mitotic activity [14, 28, 29]. Expression of cyclin B1, cyclin D1, MYBL2, and FGFR1 are quite common, high expressions of which are associated with increased cell growth, metastasis, and negative prognosis [29, 33, 34]. Luminal B tumors are of intermediate histological grade displaying moderately differentiated cells [28, 29]. Compared to luminal A, luminal B patients have a relatively poorer prognosis, with higher rates of distant relapse 15 years after diagnosis at approximately 42.9-47.9% [11, 12, 14, 17, 29, 30]. Their survival time after first distant metastasis is also significantly lower with median survival of 1.3-1.6 years [30]. Site-specific metastasis is again commonly seen in the bone, but its 15-year cumulative incidence rate is higher at approximately 30% [30]. While less than 10% of site-specific metastatic incidence rates are seen in the brain, luminal B patients display higher metastatic rates in the liver and lung compared to luminal A with 13.8-21.3% and 13.4-17.7%, respectively [30]. However, like the luminal A, luminal B patients exhibit a significantly higher frequency of site-specific

metastasis to the bone at 71.4%, while displaying lower proportions of brain metastasis at 10.8-15.4% [30, 31]. Patients with luminal B tumors can benefit from endocrine therapy (AI or SERM), anti-HER2 therapy, and/or chemotherapy [32]. Individual or combined therapeutic administration of each treatment depends on the level and combination of hormone receptor expression, HER2 expression, and potential risk of relapse or metastasis [32]. While luminal A patients will likely respond better to endocrine therapy due to their relatively higher levels of hormone expression and low recurrence scores, luminal B patients will likely respond better to chemotherapy due their relatively higher rates of proliferation and high recurrence scores [35-37]. Indeed, following neoadjuvant chemotherapeutic treatment, luminal B patients achieve higher pathological complete response (pCR) rates of 16% compared to only 6% seen in luminal A [38].

HER2-Enriched

HER2-enriched breast cancers comprise approximately 10-25% of the total intrinsic subtype population [24-27]. Highly proliferative, these tumors are mostly characterized by the gene amplification of HER2 [39]. Amplification of HER2 increases tumor cell growth, invasion, and angiogenesis, mainly through prolonged activation of the PI3K-AKT and MAPK signaling pathways [40, 41]. HER2-enriched breast cancers display low expression for both basal and luminal epithelial genes, showing no expression for hormone biomarkers ER and PR [25, 26, 28]. These tumors are commonly characterized by increased expression for proliferative gene clusters and increased expression for GRB7, which contributes to cellular growth and migration [11,

14, 20, 25]. HER2-enriched tumors are of high histological grade displaying poorly differentiated cells with high nuclear pleomorphism [28]. Without the advent or use of HER2 targeted therapy, HER2-enriched breast cancers display a poorer prognosis relative to the luminal A and B intrinsic subtypes [11, 12]. Patients can have high rates of distant relapse 15 years after diagnosis with rates as high as 51.4% [30]. Their survival time after first distant metastasis can be significantly lower with median survival of 0.7 years [30]. While site-specific metastasis is seen in the bone, higher 15-year cumulative incidence rates are more commonly seen in the brain, liver, and lung compared to luminal A and B patients with 14.3%, 23.3%, and 24.1%, respectively [30]. Furthermore, when looking at frequency of site-specific metastasis, HER2-enriched patients exhibit greater proportions of brain metastasis at 28.7%, which are significantly higher compared to luminal patients [30]. Patients with HER2-enriched breast cancer can benefit from chemotherapy and/or anti-HER2 targeted therapy [32]. Relative to luminal A and B, HER2-enriched patients respond better to neoadjuvant chemotherapy achieving higher pCR rates of 37% [19, 38]. Anti-HER2 therapy has considerably improved patient tumor outcome both in early and advanced metastatic disease stages [42-44]. While anti-HER2 or chemotherapy alone can help improve patient outcome, combination therapy with both treatment options is proving to be the largely effective. In patients with HER2-enriched metastatic breast cancer, combination of docetaxel chemotherapy and dual anti-HER2 pertuzumab with trastuzumab targeting can increase median overall survival to 56.5 months [45].

Normal Breast-Like

The normal breast-like group comprises approximately 5-10% of the total intrinsic subtype population [26, 27]. Amongst all the intrinsic subtypes of breast cancer, it is the least characterized and the least clear, which can partly be attributed to its low levels of emergence [24-26]. The gene expression profile of the normal breast-like group displays features of fibroadenoma and adipose tissue [10, 11]. Owing to its strong genetic correlation with normal breast tissue, researchers question its validity, arguing it may be remnants of normal breast sample contamination obtained during tumor biopsy [14, 19, 20]. Though there is limited knowledge about the normal breast-like group, researchers have been able to show it displays intermediate prognosis and is not responsive to neoadjuvant chemotherapy [19, 20].

Triple Negative Breast Cancer: Biology, Prognosis, and Prediction (Basal-Like, Claudin-Low, and Lehmann/Burstein Classifications)

Biology

Brenton and colleagues were the first to publish a paper using triple negative breast cancer (TNBC) as a term in order to describe a subset of patients whose tumors lacked overexpression for the three biomarkers of ER, PR, and HER2 [46]. TNBC comprises approximately 10-20% of the total subtype population [47]. Due to its vast heterogeneity, TNBC can be considered as a separate disease, which on its own manifests into several diverse biological subtypes. Original gene expression profiling studies conducted by Perou and Sorlie identified two intrinsic subtypes of TNBC [10-16].

However, several years later the Lehmann and Burstein groups contended that the extensive heterogeneity of TNBC is more accurately portrayed in four different subtype classifications, each displaying its own unique biology [48-50]. While significant advances have been accomplished to understand TNBC biology by several groups, additional research is required to delineate a more homogeneous definition for a heterogeneous disease.

Seminal gene expression profile studies conducted by Perou and Sorlie identified the first TNBC as the basal-like intrinsic subtype [10-14]. Basal-like breast cancers comprise approximately 10-15% of the total intrinsic subtype population, but comprise nearly 50-75% majority of the TNBC population [51]. Basal-like tumors display a gene expression profile similar to the basal epithelium/myoepithelial of the normal mammary gland, hence their name [10-12]. Common cluster of basal genes utilized to identify this intrinsic subtype include cytokeratins 5, 6, 14, and 17; human epidermal growth factor receptor (EGFR); caveolins 1 and 2; c-Kit; and P-cadherin [10-12, 14, 25]. Characterized as highly proliferative, basal-like tumors demonstrate high expression for proliferative gene clusters and KI67, but low expression for luminal and HER2 gene clusters [10-12, 14, 25]. According to Nielsen and colleagues, basal-like tumors can be precisely categorized by staining for the biomarkers of ER, EGFR, HER2, and cytokeratins 5, 6; tumors should stain positive for EGFR and cytokeratins 5, 6 while staining negative for ER and HER2 [52]. Precise categorization of basal-like tumors by staining for these five biomarkers provides more significant prognostic and predictive value; more so than staining for ER, PR, and HER2 alone, which are not considered definitive classifications [53]. Comprehensive research has linked the risk of developing

basal-like breast cancer to loss of function mutations or epigenetic silencing of the breast cancer gene one (BRCA1) [12, 54]. More than 75% of BRCA1 mutated cancers display basal-like tumors [55, 56]. The BRCA1 gene is crucial for mechanisms of DNA repair by homologous recombination, loss of function prevents repair of DNA double strand breaks causing genomic instability that leads to uncontrolled tumor cell growth [57].

Initially, basal-like and TNBC were used as two synonymous terms. It wasn't until several years later that researchers were able to further grasp the vast heterogeneity of TNBC and show it was not solely embodied by the basal-like intrinsic subtype. Additional studies conducted by Herschkowitz and colleagues identified a new intrinsic subtype called claudin-low, which lacked overexpression for ER, PR, and HER2, thus accordingly classified as TNBC [15]. Claudin-low breast cancers comprise approximately 5-10% of the total intrinsic subtype population, but comprise nearly 25-50% of the TNBC population [16, 51]. Claudin-low tumors display a low gene expression profile for epithelial cell-cell adhesions and tight junctions, low expression of such genes include claudins 3, 4, and 7; occludin; and E-cadherin [15, 16, 18, 25]. Critical signaling pathways of immune response, cell communication, extracellular matrix interactions, cell differentiation, cell migration, and angiogenesis are common characteristics of this intrinsic subtype [16, 18]. Although proliferative, claudin-low tumors are identified as comparatively low mitotic with lower expression of proliferative genes and lower expression of KI67 relative to basal-like tumors; however, they are still considered to be more proliferative compared to luminal A and the normal breast-like group [16, 18]. While characterized by downregulation of luminal and HER2 gene

expressions, these tumors also exhibit a high epithelial to mesenchymal gene signature of increased Vimentin, Snail1, Slug, and Twist1 [15, 16, 18, 25]. Extensive research on claudin-low shows this intrinsic subtype displays enrichment for stem like properties, resembling the mammary stem cell profile and displaying features of tumor initiating cells or cancer stem cells [16, 18]. Interestingly, epithelial to mesenchymal transitions and stem cell properties have shown an important functional and mechanistic interplay in breast cancer maintenance, of which may be resembled in the cluadin-low intrinsic subtype [16, 18].

Beyond intrinsic subtype classification of basal-like and claudin-low, the extensive heterogeneity of TNBC was further demonstrated by the Lehmann group who declared that TNBC should be divided into six different subtypes [48]. Using methods of differential gene expression and gene ontology, six TNBC subtypes were identified as basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) [48]. However, several years later Lehmann and colleagues published a correction for their work, arguing two of the original six subtypes did not represent true TNBC gene signatures, specifically referring to the IM and MSL, but rather resembled tumor infiltrating lymphocytes and tumor associated stromal mesenchymal cells, respectively [49]. The authors affirmed that the IM and MSL should not be considered as distinct TNBC subtypes, but as separate informative states of the tumor immune and environmental biology [49]. Thus, the original six were further refined to four subtypes of BL1, BL2, M, and LAR [49].

Each of the four TNBC subtypes identified by Lehmann and colleagues display unique differences in incidence and gene ontology [48, 49]. The BL1, BL2, M, and LAR subtypes comprise 35%, 22%, 25%, and 16% of the total TNBC population, respectively [49]. In the BL1 subtype, gene ontology analysis shows upregulation in cell division, cell cycle, and DNA damage response signaling [48]. Pathways include G1 to S cell cycle, G2 cell cycle, DNA replication reactome, and ATR/BRCA [48]. Aberrations in these signaling pathways are affiliated with uncontrolled proliferation, a characteristic that is reinforced by increased levels of KI67 seen in BL1 tumors [48]. Like the BL1, the BL2 subtype also shares features of uncontrolled proliferation through, but not limited to, increased levels of KI67 [48]. BL2 demonstrates distinct signaling of growth factors such as EGF, NGF, MET, IGF1R, and WNT/beta-catenin pathways [48]. Gluconeogenesis and glycolysis also seem to play critical roles in BL2 tumor biology [48]. The M subtype shows increase in actin reformation by RHO signaling, signifying increase in cell motility [48]. These tumors display upregulation of cell growth and differentiation through WNT/beta-catenin, ALK, and TGF-beta signaling [48]. Extracellular matrix receptor interactions and epithelial to mesenchymal transition genes of increased Slug, Twist1, Zeb1, and Zeb2 with downregulation of E-cadherin are distinct features of the M subtype [48]. The LAR subtype represents hormone mediated androgen receptor (AR) tumors [48]. These tumors display an increase in the hormone mediated signaling of steroid biosynthesis as well as porphyrin, androgen, and estrogen metabolism [48]. Differential in their pattern of gene expression, LAR subtypes also seem to manifest luminal characteristics seen with increase in expression of cytokeratin 18, FOXA1, and XBP1 [48].

The categorization of four TNBC subtypes was further confirmed by Burstein and colleagues who also used gene expression profiling to show indeed TNBC should be divided into four subtypes of basal-like immune-activated (BLIA), basal-like immunosuppressed (BLIS), mesenchymal (MES), and luminal androgen receptor (LAR) [50]. Although similar in several ways, the Burstein classification of TNBC heterogeneity differs from the Lehmann [50]. The BLIA subtype exhibits an increase in immune mediated signaling of natural killer cells, B cells, and T cells [50]. BLIA tumors can be characterized by upregulation of STAT transcription, signaling of which is associated with a wide array of immune, apoptotic, and proliferative functions [50]. In contrast to BLIA, the BLIS subtype is characterized by decreased expression of immune mediated signaling [50]. BLIS tumors exhibit upregulation of SOX transcription, commonly associated with cell fate and differentiation [50]. The MES subtype displays signaling pathways associated with DNA damage, mismatch repair, and cell cycle [50]. Increased growth factor signaling through IGF1 seems to be an important component in MES tumors [50]. Resembling the Lehmann classification, the Burstein LAR subtype is also characterized by increased hormone mediated AR signaling [50]. Likewise, these tumors display gene expressions similar to the luminal phenotype, as seen with increased expression of ER-alpha and ER-associated genes of GATA3, FOXA1, XBP1, and PR [50].

While gene expression profiling has significantly helped advance TNBC understanding, it is evident that not all TNBC gene expression profile classifications are precisely and consistently reproducible [47]. Therefore, further research is required for more accurate and stable classifications of TNBC subtypes. Continued extensive and

concerted efforts will help improve the knowledge underlying TNBC biological heterogeneity. As previously mentioned, biological diversity can be an important determinant of breast cancer incidence, survival, and therapeutic response [11-14, 18-20]. Given that TNBC is a vastly heterogeneous disease, identifying precise TNBC subtypes will considerably help improve prognosis and prediction. Furthermore, classification of TNBC into different subtypes has very significant therapeutic applications, as characterization of accurate molecular profiles underlying TNBC functional and mechanistic heterogeneity will help identify specific therapeutic targets. Since one size does not always fit all, different TNBC subtypes may need to be treated as separate diseases.

Prognosis

Patients diagnosed with TNBC exhibit a more aggressive rate of clinical behavior and outcome [28, 58]. TNBC patients display a significantly younger onset of the disease, where women of African American and Hispanic descent exhibit higher incidences relative to Caucasians, with 32%, 34%, and 19%, respectively [28, 59]. TNBC tumors are characterized as highly proliferative with increased mitotic index and KI67 expression [28]. Along with displaying a larger mean tumor size and higher nuclear pleomorphism, patients with TNBC also exhibit higher grade tumors upon diagnosis, with 66% displaying grade III tumors compared to only 28% seen in non-TNBC [28, 58].

Disease recurrence also seems to manifest a more aggressive role, as TNBC patients not only achieve higher proportions of distant recurrence, but they also achieve distant recurrence faster. Patients with TNBC are more likely to experience distant

recurrence within 5 years following diagnosis relative to non-TNBC (hazard ratio 2.6; 95% CI 2.0-3.5; $p<0.0001$) [58]. The peak interval of recurrence in TNBC patients increases very rapidly within 1 to 3 years of diagnosis, however it decreases just as rapidly after 3 to 5 years [58]. If recurrence does not occur within the peak interval, little to no recurrence is exhibited in TNBC patients thereafter. Indeed, following 8 years of diagnosis, TNBC patients exhibit little to no distant recurrence [58]. In contrast, patients displaying non-TNBC subtypes show a continual constant risk of recurrence after diagnosis, demonstrating no association between time of diagnosis and peak time of preferred recurrence. Distant recurrence in non-TNBC patients can manifest at consistent rates for up to 17 years following diagnosis [58].

TNBC patients display site-specific metastasis to the brain, liver, lung, bone, and node with 15-year cumulative incidence rates of 7.2-10.9%, 9.3-10.7%, 12.5-18.5%, 15.1-16.6%, and 12.3-17.2%, respectively [30]. However, when looking at frequency of site-specific metastasis, patients with TNBC exhibit significantly higher proportions of brain metastasis at 22.0-25.2%, which are analogous to that seen in the HER2-enriched but significantly higher relative to luminal A and B intrinsic subtypes [30]. In addition, research also shows patients with TNBC exhibit the lowest proportions of bone metastasis at 39.0-43.1%, much lower relative to the 59.6-71.4% seen in non-TNBC [30]. Indeed, other studies have confirmed that the brain is the preferential site of relapse for patients with TNBC, while bone relapse is least expected [31]. Furthermore, frequency of site-specific metastasis to the distant node is higher in TNBC patients with proportions as high as 35.8-39.6% [30]. Studies show that nodal status varies greatly between TNBC and non-TNBC patients. While patients with non-TNBC only display

high lymph node positivity when tumor sizes are large, TNBC display high lymph node positivity regardless of tumor size. Non-TNBC patients with tumors sizes <1cm display 19% lymph node positivity, this is significantly lower compared to the 55% positivity seen in TNBC patients with tumor sizes <1cm [58]. High lymph node positivity in non-TNBC patients is only seen when tumor sizes are >5cm [58].

The majority of cancer related deaths in patients with TNBC occur within 10 years of diagnosis, non-TNBC have a longer outcome of 18 years [58]. The first few years are the most critical, as TNBC patients have a higher chance of death within 5 years of diagnosis (hazard ratio 3.2; 95% CI 2.3-4.5; $p<0.0001$) [58]. While mortality proportions for patients with TNBC are 70% within 5 years of diagnosis, lower proportions of 44% are seen in non-TNBC [58]. Interestingly, this rapid and high mortality seen in TNBC patients abates and ceases after 5 years of diagnosis [58]. In contrast, patients with non-TNBC exhibit a more continual constant risk of death, demonstrating no association between time of diagnosis and peak time of death. Disease recurrence also seems to have a critical interplay with patient mortality rates. Patients with TNBC who exhibit distant recurrence will have a median survival time of 9 months, significantly shorter compared to the longer median survival time of 20 months seen in non-TNBC [58]. Furthermore, TNBC patients also have a higher chance of death following distant recurrence (hazard ratio 1.6; 95% CI 1.2-2.1; $p=0.003$) [58]. Thus, patients with TNBC not only experience a shorter survival time after disease recurrence, but they also display an increased likelihood of death if recurrence does occur.

Prediction

Amongst all the subtypes of breast cancer, TNBC is the only subtype that lacks targeted therapy to improve long-term patient outcome of overall survival. Lacking overexpression for the three biomarkers of ER, PR, and HER2, TNBC is unresponsive to endocrine and HER2-targeted therapies. While promising therapeutic developments are in progress to target genomic instability in basal-like TNBC patients harboring BRCA1 mutations, applications are still in their infancy and more time is needed to determine benefits for overall patient survival, for which no data exists. As such, research is still currently ongoing to establish new strategies for targeted therapy against TNBC. Given the extensive heterogeneity of TNBC subtypes, more than one targeted treatment option may be a necessity. Since the lack of precise targets prevents specific treatment of individual subtypes, chemotherapy is still the mainstay of treatment for all subtypes of TNBC. Fortunately, patients with TNBC are responsive to chemotherapy, more so than non-TNBC, a predictive value that has significant impact in the neoadjuvant setting.

Liedtke and colleagues conducted one of the largest patient studies known to determine long-term outcomes of neoadjuvant chemotherapy in TNBC versus non-TNBC patients [59]. Interestingly, results show patients with TNBC who receive neoadjuvant chemotherapy are able to achieve pCR rates higher than non-TNBC, with 22% and 11%, respectively [59]. Indeed, other studies also demonstrate significantly higher pCR rates exhibited in TNBC patients, confirming their high sensitivity to chemotherapeutic treatment [49]. While there may be several potential reasons why TNBC patients display an increased response to chemotherapy, researchers argue that

it can be attributed to higher rates of proliferation, which is associated with increased expression of proliferative gene clusters seen in TNBC tumors [11, 25, 60-62].

Studies demonstrates a strong correlation between achieving pCR following neoadjuvant chemotherapy with long-term patient outcome. Patients who achieve pCR without displaying residual disease after neoadjuvant chemotherapy have improved disease-free survival and overall survival; this prognostic association has significant impact for patients with TNBC [59, 61-63]. Liedtke and colleagues were able to show that TNBC and non-TNBC patients who exhibit pCR without displaying residual disease have a similar increase in 3-year overall survival of 94% and 98%, respectively [59]. Studies further corroborate that patients who do exhibit pCR are able to display improved long-term outcome irrespective of breast cancer subtype [59, 61]. However, not all patients are able to achieve pCR with neoadjuvant chemotherapy. Patients who do not achieve pCR and display residual disease following neoadjuvant chemotherapy show a significant decrease in long-term outcome [59, 61-63]. This is especially detrimental for TNBC patients, as patients with TNBC who do not achieve pCR and display residual disease have a considerably decreased 3-year overall survival of 68% compared to 88% seen in non-TNBC [59]. Several additional studies looking at the survival benefit of achieving pCR following neoadjuvant chemotherapy have also been conducted, confirming TNBC patients who are not able to achieve pCR exhibiting residual disease have a significantly worse long-term outcome [61-63]. Researchers argue this can be associated with at least two potential reasons, one being that patients with TNBC display more severe and aggressive prognostic characteristics relative to non-TNBC [59]. In addition, while non-TNBC patients have the advantage of

combination therapy with chemotherapeutic agents and specific endocrine or HER2 targeting, TNBC patients have only chemotherapy as their one treatment option [59].

While displaying unique differences in incidence and biological behavior, each of the TNBC intrinsic and Lehmann classified subtypes also display differences in neoadjuvant chemotherapeutic response. Following neoadjuvant chemotherapy, the claudin-low intrinsic subtype demonstrates a pCR rate lower than that seen in the basal-like intrinsic subtype, with 38.9% versus 73.3%, respectively [16]. In the Lehmann classification of TNBC, several studies using chemotherapy in a neoadjuvant setting confirm the BL1 to consistently demonstrate a pCR rate higher than any of the BL2, M, and LAR subtypes [38, 48, 49, 64]. Furthermore, while the study did not display details about treatment information, Burstein and colleagues were also able to show the BLIA subtype demonstrates a relatively good prognosis for disease free survival and disease specific survival compared to the BLIS, MES, and LAR subtypes following clinical treatment [50]. However, regardless of dataset, all patient cohorts of TNBC were able to display higher pCR rates when compared to non-TNBC following neoadjuvant chemotherapy [49].

Even though patients with TNBC are more responsive to chemotherapy and exhibit higher pCR rates in a neoadjuvant setting compared to non-TNBC, patients with TNBC still display a relatively worse prognosis. This is known as the triple negative paradox [61]. Since there still remains a large proportion of TNBC patients who do not achieve pCR and who display residual disease following neoadjuvant chemotherapy, susceptibility of disease relapse is inevitable [61]. Due to the aggressive nature of the disease, TNBC patients are more likely to experience relapse within 5 years of

diagnosis, thus increasing the likelihood of death within those 5 years [58]. Rightfully so, alternative treatment options other than chemotherapy must be taken into serious consideration to improve patient outcome.

There is continued progressive research to develop new strategies for targeted therapy against TNBC. Promising therapeutic applications to target genomic instability in basal-like TNBC patients with BRCA1 mutations are making headway. Studies have shown that inhibiting alternate DNA repair mechanisms in cancer cells can be utilized for effective therapy, such as targeted inhibition of poly(adenosine diphosphate-ribose) polymerase (PARP) responsible for repairing DNA single strand breaks [65]. With BRCA1 loss of function and PARP inhibition, DNA repair mechanisms in breast cancer cells are significantly impaired, causing an overwhelming accumulation of genomic instability that leads to tumor cell death [65]. Very recently in January of 2018, FDA approved PARP inhibitor olaparib as the first targeted form of therapy for patients with HER2 negative metastatic breast cancer harboring BRCA germline mutations. In phase III clinical trials conducted by Robson and colleagues, olaparib as a monotherapy PARP inhibitor was able to afford longer median progression-free survival of 2.8 months and a 42% decrease in disease progression or death relative to patients receiving standard chemotherapeutic treatment [66]. Although encouraging, the clinical trial was unable to show any benefit for overall patient survival with olaparib monotherapy [66]. Furthermore, the study was not exclusive for patients with TNBC, results shown not only include HER2 negative metastatic breast cancer patients with BRCA germline mutations, but they also include ER/PR hormone receptor positive and negative patients [66]. While a promising start, application of olaparib is still in its early stages and further

research is a necessity to ascertain benefits for TNBC patients alone as well as determine benefits for long-term patient outcome for overall survival.

Besides PARP inhibition, several alternate approaches are also underway for targeted therapy against TNBC. This includes inhibitors for microtubule dynamic stabilization, EGFR, angiogenesis, AR, histone deacetylase (HDAC), PI3K-AKT-mTOR pathway, mitogen-activated protein kinase (MAPK) signaling, checkpoint kinase 1 (Chk1), stem cell pathways (NOTCH, Hedgehog, WNT), and immune modulation [67, 68]. With multiple clinical trials ongoing, more time will be needed to properly ascertain their benefits in the long-term.

Since TNBC exhibits vast biological heterogeneity, different TNBC subtypes may require distinct therapeutic approaches. Indeed, preliminary results from Lehmann and colleagues show the potential therapeutic applications associated with dividing TNBC into individual subtypes [48]. Based on the biological heterogeneity governed by differential gene expression, TNBC subtypes of BL1, BL2, M, and LAR not only display distinct differences in incidence, gene ontology, and pCR following neoadjuvant chemotherapy, but they also display differences in therapeutic response to specific treatments. The BL1 and BL2 subtypes respond well to the cisplatin platinum agent, the M subtype exhibits sensitivity to PI3K/mTOR inhibition, and the LAR subtype demonstrates susceptibility to therapies targeted against androgen activity [48]. While it is a significant achievement, additional research is required to fully understand the therapeutic implications associated with distinct TNBC subtypes.

Although new targeting strategies are progressive developments to help improve TNBC treatment, there currently is no effective targeted therapy against TNBC known to

display improved long-term patient outcome of overall survival. As a result, there is a great need to further explore the biological heterogeneity of TNBC and identify effective therapeutic targets. With the advent of gene expression profiling, researchers have made significant progress to classify multiple subtypes of TNBC in order to better grasp the biology that underlies tumor heterogeneity. While this provides valuable knowledge in understanding functional and mechanistic pathways, further efforts using similar methods will be necessary to help precisely and continually define and redefine novel therapeutic strategies to advance TNBC targeting in its evolving biology. Ultimately, this will help improve and shift TNBC treatment from a general chemotherapeutic standard of care to a more specific therapeutic regimen, which will provide hope to ameliorate the disease in its distinct subtypes.

Drug Resistant Triple Negative Breast Cancer

(TP53, PTEN, and Meox1)

Drug Resistance

Approximately 30% of TNBC patients achieve pCR following neoadjuvant chemotherapy [69]. The remaining majority of TNBC patients who do not achieve pCR and display residual disease after neoadjuvant treatment are not responsive to chemotherapy, as such they are considered to be drug resistant. Since chemotherapy is the mainstay of treatment for TNBC patients, limited or no alternative therapeutic options exist. Thus, there is not only an important need to develop effective targeted

therapies against TNBC and its specific subtypes, but there is also an important need for applying such new therapeutic strategies to patients who display resistance to the mainstay of TNBC treatment. Intrinsic and acquired drug resistance is governed by the heterogeneity of tumor residual disease. Deciphering the biology underlying tumor residual disease may offer insight to molecular pathways associated with drug resistant. However, the vast complexity of tumor heterogeneity and its evolving biology presents a challenge for ascertaining new therapeutic alternatives. The fundamental objective is to understand the combination of genetic changes that lead to the functional and mechanistic interplay responsible for tumor maintenance, as well as drug resistance.

Sources of tumor heterogeneity can be described in the context of clonal evolution, extrinsic environmental signals, and cancer stem cells [70]. Neither of these sources are mutually exclusive and each can contribute to tumor heterogeneity enabling the six biological hallmarks of cancer [70, 71]. The most established source of clonal evolution describes the stochastic accumulation of genetic and epigenetic changes that confer a selective advantage for tumorigenesis [70]. Using high throughput platforms to analyze DNA copy numbers, RNA expression profiles, and proteomic landscapes allow identification of ‘driver mutations’ responsible for tumor functional and mechanistic biology. Extensive and concerted efforts have repeatedly been able to determine several common gene alterations implicated in breast cancer and its specific subtypes [72, 73]. Due to its vast heterogeneity, comprehensive analysis of TNBC especially has acquired much attention. By using multiple platforms, The Cancer Genome Atlas Network has been able to identify TP53, PIK3CA, RB1, BRCA1, BRCA2, PTEN, INPP4B, MYC, ATM, CCNE1, CDKN2A, MDM2, and AKT3 as frequent genetic

aberrations that occur in TNBC [74]. Gene amplifications such as KRAS, BRAF, and EGFR involved in the RAS-RAF-MEK pathway, as well as amplifications of growth factor and tyrosine kinase receptors of FGFR1, FGFR2, IGFR12, KIT, MET, and PDGFRA are also commonly seen in TNBC tumors [74]. Evaluating these genetic alterations with integrated pathway analyses has confirmed functions of cell survival, cell proliferation, cell cycle arrest, and apoptosis to be significantly perturbed in TNBC [74]. Shah and colleagues have also been able to demonstrate high somatic mutations in TP53, PIK3CA, and PTEN as drivers responsible for dominating TNBC tumor clonal frequency [75]. Using mass spectrometry, Lawrence and colleagues have likewise been able to corroborate these commonly found genetic alterations of TP53, PIK3CA, BRCA2, ATM, NF1, CDKN2A, RB1, CCND1, CCNE1, and EGFR by characterizing the TNBC proteomic landscape and analyzing potential avenues for drug targeting [76].

Not only are driver genetic alterations identified to understand the heterogeneity of TNBC tumor biology as a whole, but they are also reported in the context of residual disease and drug resistance. A research study conducted by Balko and colleagues published genetic and molecular pathways commonly altered in drug resistant residual tumors obtained from TNBC patients who had received neoadjuvant chemotherapy but did not achieve pCR [69]. TNBC residual disease tumor samples that did not respond to chemotherapeutic treatment had frequent genetic aberrations in TP53, MCL1, MYC, PTEN, PIK3CA, RB1, BRCA1, JAK2, CDKN2A, NF1, KRAS, AKT3, CCND1, CCND2, CCND3, IGF1R, CDK6, CCNE1, and EGFR [69]. The authors were able to further organize these commonly altered genes into five functional and targetable pathways, of which include PI3K/mTOR, DNA repair, RAS/MAPK, cell cycle, and growth factor

receptor alterations [69]. Their study was able to show that over 90% of drug resistant residual disease TNBC tumor samples had aberrations in at least one of the five molecular pathways governed by frequent genetic alterations in aforementioned genes [69].

TP53 and PTEN

Although there are several diverse genetic aberrations that are important for tumorigenesis and drug resistance in TNBC, genetic alterations that repeatedly manifest as key drivers are TP53 and PTEN.

TP53, also known as tumor protein 53 or p53, is the most frequently mutated gene in cancer and rightfully named as the guardian of the genome [77, 78]. Germline mutations in p53 are commonly found in families with Li-Fraumeni syndrome, a hereditary disorder predisposing patients to breast cancer and other spectra of neoplasms [79]. A tumor suppressor gene predominantly functioning as a transcription factor, p53 mediates target genes responsible for cell cycle arrest, DNA repair, senescence, and apoptosis [80]. To prevent genomic instability and malignant transformation, p53 activity is induced by multiple cellular stress signals of hypoxia, DNA damage, heat/cold shock, oncogene expression, and nutrient deprivation, just to name a few [81]. Loss of p53 wild-type function can occur through loss of protein expression caused by nonsense or frameshift mutations [82]. However, more prominent are single amino acid missense substitutions, which are frequently seen in the p53 DNA binding domain and predisposed to six ‘hotspot’ amino acid residues: G245, R175, R248, R249, R273, and R282 [83]. Missense substitutions cause high expression of

stable mutant p53 proteins that are unable to bind to DNA and activate normal p53 tumor suppressive canonical genes [80, 82]. Research suggests that such amino acid substitutions not only incur loss of p53 tumor suppressor wild-type function, but also enable distinct novel gain of function oncogenic traits for most mutations [84]. These oncogenic mutant forms of p53 are linked to tumor promoting properties of avoiding cell death, proliferation, invasion, migration, and angiogenesis [82]. This has important therapeutic implications, as restoring wild-type p53 tumor suppressor function may not be sufficient and oncogenic p53 mutant activity might need to be simultaneously suppressed.

PTEN, also known as phosphatase and tensin homolog, is one of the most frequently mutated genes in brain, breast, and prostate cancers [85, 86]. Germline mutations in PTEN are commonly found in hamartoma syndromes, which result in benign hyperplastic growths [87]. One of the most well-known hamartoma syndromes is Cowden syndrome, a hereditary disorder predisposing patients to breast and thyroid cancers [87]. As a tumor suppressor gene, PTEN dephosphorylates PIP_3 at the plasma membrane to negatively regulate PI3K activation of AKT, which subsequently drives downstream signals of cell proliferation, survival, senescence, angiogenesis, energy metabolism, and migration [81, 88]. While a plethora of signaling molecules are activated downstream of AKT, PTEN mediated tumor suppressive effects are mostly conducted through inhibition of the PI3K-AKT-mTOR pathway, as the pathway plays a prominent role in tumorigenesis [88]. Independent of its phosphatase activity in the cytoplasm, PTEN also seems to have tumor suppressive functions in the nucleus by controlling genomic stability and cell cycle progression [89, 90]. Nuclear PTEN is known

to increase expression of RAD51, a DNA repair protein for double-strand breaks; furthermore, nuclear PTEN promotes association of the APC/C-CDH1 complex increasing cell cycle regulation [89, 90]. Both genetic and epigenetic aberrations of PTEN can promote malignant transformation. While frequent PTEN somatic mutations, insertions, and deletions manifest, it is PTEN allelic or complete deletions that are more commonly associated with brain, breast, and prostate cancers [88]. Loss of PTEN expression can also be attributed to epigenetic hypermethylation of the PTEN promoter [91, 92]. Several studies demonstrate an association between promoter hypermethylation of PTEN and increased risk of breast carcinoma [91, 92].

Both p53 and PTEN have dependent and independent mechanistic interplay as tumor suppressor genes. PTEN transcription can be induced by p53 direct binding to its promoter [93]. Furthermore, independent of PTEN, p53 can downregulate the transcription of the PI3K subunit p110 [94]. In both situations, similar to PTEN, p53 functions as negative regulator of PI3K activity. Not only can p53 promote PTEN activity, but PTEN likewise can increase p53 function. PTEN is known to directly interact with p53 in order to increase and promote its DNA binding activity [95]. Furthermore, loss of PTEN activity can result in activated AKT and its subsequent phosphorylation of MDM2; phosphorylated MDM2 activates ubiquitination and proteolysis of p53 [95]. With increased activity of PTEN, AKT-mediated MDM2 phosphorylation is inhibited, preventing degradation of p53 and promoting its tumor suppressor function.

Comprehensive sequencing analyses identify increased genetic aberrations of both p53 and PTEN in TNBC [74]. The Cancer Genome Atlas Network reports TNBC tumors to display the highest frequency of p53 and PTEN genetic alterations relative to

all other breast cancer subtypes, with 84% and 35%, respectively [74]. Indeed, seminal gene expression profile studies conducted by Perou and Sorlie identified a significant difference in p53 genetic aberrations amongst the intrinsic subtypes, showing TNBCs harbor the highest frequency of p53 mutations [11]. Furthermore, studies demonstrate TNBC BRCA1/2 mutants to frequently exhibit p53 mutations [96]. While p53 genetic alterations manifest a higher frequency compared to PTEN, epigenetic alterations by hypermethylation are relatively more common with PTEN in breast cancer, silencing protein expression [91, 92]. Although frequencies can vary greatly, one study shows breast cancer patients can display a 31.3% frequency of PTEN promoter hypermethylation and 64.3% of those patients can exhibit a decrease in protein expression [91]. The frequency of combined aberrations in both tumor suppressor genes of p53 and PTEN manifests in approximately 20-30% of TNBC tumors [97, 98].

Research shows the combined loss of wild-type function in p53 and PTEN tumor suppressor genes induces the formation of TNBC [97-99]. Mammary specific epithelial deletion of both p53 and PTEN in mice causes TNBC-like tumors [97]. These tumors exhibit a gene signature profile resembling the claudin-low intrinsic subtype, expressing epithelial to mesenchymal and cancer stem cell characteristics [97]. Furthermore, these tumors also display a gene set enrichment profile for increased proliferation and migration while displaying a decrease for genes associated with immune response and cell death [97]. Concomitant deletion of both p53 and PTEN was able to manifest faster and more aggressive TNBC hallmarks with decreased tumor free survival compared to deletion of either p53 or PTEN alone [97]. Indeed, analyzing human TNBC tumors shows patients with low p53 and PTEN expressions display poorer prognosis with

worse metastatic free survival relative to patients with normal p53 and PTEN levels [97]. In a separate yet similar study, mammary specific epithelial deletion of PTEN with mutant p53 expressing mouse models induced formation of TNBC tumors with both basal-like and claudin-low intrinsic subtype gene signatures [98]. Results were able to show tumors harboring concurrent deletion of PTEN with mutant p53 expression share similar aggressive TNBC characteristics with decreased tumor free survival as tumors with combined deletion of both p53 and PTEN [98]. However, tumors harboring deletion of PTEN with mutant p53 expression display a comparatively higher metastatic potential than p53 and PTEN deleted tumors [98]. Last but not least, *in vitro* stable knockdown of p53 and PTEN in normal immortalized MCF-10A breast cancer transforms cells to resemble basal-like and claudin-low TNBCs [99]. These cells are not only able exhibit more aggressive metastatic tumors in NOD/SCID mice after orthotropic injections into mammary fat pads, but they are also able to manifest increased epithelial to mesenchymal and cancer stem cell characteristics [99].

Mesenchyme Homeobox 1 (Meox1)

Although the importance of tumor suppressor genes p53 and PTEN are well established in TNBC, the molecular biology involved in their concomitant loss of function is not well understood. Incomplete knowledge of the molecular mechanisms governing the aggressive nature of TNBC presents a challenge for developing effective therapeutic strategies. Establishing effective therapeutic targets will require analyzing the interplay of signaling pathways transformed by multiple genetic alterations, which ultimately govern functions of tumor maintenance as well as drug resistance. In this

dissertation, insight is offered into the complex molecular biology of p53 and PTEN deficient TNBC by studying the functional and mechanistic role of mesenchyme homeobox 1 (Meox1).

Meox1 belongs to the sub-family of Meox homeobox transcription factors. It is well known for its role in the development of the vertebrate embryo, where its expression and function is observed in the epithelial somites of the paraxial mesoderm [100-102]. In concerted action with sub-family member Meox2, Meox1 is responsible for mesodermal regional specification and normal differentiation of cells originating from the somites [100-102]. Expressions of both Meox1 and Meox2 are observed in the early phase of somite differentiation, prior to terminal differentiation of cells [100-102]. The somites are comprised of sclerotome, myotome, and dermamyotome compartments, where normal function of Meox transcription factors in each compartment is responsible for proper development of the axial skeleton, skeletal muscles, and dermis, respectively [100-102]. Accordingly, mice with null mutations in Meox transcription factors display improper somitogenesis, patterning, and differentiation, causing defects in axial skeleton and skeletal muscle formations [103, 104]. Humans with truncating mutations in Meox1 develop Klippel-Feil Syndrome (KFS), which is associated with improper development of the cervical spine, resulting in short neck, reduced movement, and low posterior hairline [105, 106].

Although mainly studied in the context of development, recent research demonstrates a link between Meox1 and cancer. Thiaville and colleagues show evidence involving Meox1 in ovarian cancer, describing Meox1 as an important Pre-B-cell leukemia homeobox-1 (PBX1) cofactor and target gene for tumor cell growth [107].

Furthermore, Sun and colleagues correlate nuclear expression of Meox1 with poor overall breast cancer patient survival, as well as increased lymph node metastasis and high tumor stage [108]. Authors also link the function of Meox1 to increased breast cancer cell proliferation and epithelial to mesenchymal stem cell regulation [108].

In this dissertation, the functional and mechanistic role of Meox1 is further studied in association with cancer, specifically in the context of p53 and PTEN deficient TNBC. In Chapter II, RNA analysis of Meox1 expression demonstrates the transcription is specifically upregulated in TNBC and negatively regulated by both tumor suppressor genes of p53 and PTEN. *In vitro* functional assays of cell proliferation show knockdown of the transcription factor in two different p53 and PTEN deficient TNBC intrinsic subtypes of claudin-low and basal-like significantly decreases cell proliferation. *In vivo* tumor xenograft studies corroborate *in vitro* results, where decrease in Meox1 expression decreases *in vivo* tumor growth of basal-like p53 and PTEN deficient TNBC. Interestingly, *in vitro* cell based assays demonstrate that decrease in cell proliferation is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like intrinsic subtypes. Aside from cell proliferation, Meox1 is also an effective regulator of metastasis. *In vitro* results demonstrate knockdown of the transcription factor decreases cell migration and invasion in both claudin-low and basal-like p53 and PTEN deficient TNBC. In Chapter III, the mechanistic role of Meox1 is explored using RNA sequencing and integrative pathway analyses in both claudin-low and basal-like p53 and PTEN deficient TNBC. Results show knockdown of the transcription factor inactivates important canonical pathways associated with growth and survival as well as migration and invasion. Important canonical pathways inactivated include Stat3 and Jak/Stat

signaling. Continued investigation of the Jak/Stat pathway shows knockdown of Meox1 has a significant impact on decreasing Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low as well as decreasing Tyk2 and Stat6 protein levels in basal-like intrinsic subtypes. As such, results indicate Meox1 regulation of proliferation and metastasis in p53 and PTEN deficient TNBC may be conducted through the Jak/Stat mechanistic pathway. In Chapter IV, the distinct mechanistic and transcriptional roles of Meox1 are explored in the different claudin-low and basal-like p53 and PTEN deficient TNBCs. As just discussed, Meox1 knockdown largely elicits apoptosis in claudin-low but cell cycle arrest in basal-like intrinsic subtypes. Furthermore, regulation of Jak/Stat signaling upon knockdown of the transcription factor is effected differently between the two TNBC intrinsic subtypes. As such, these preliminary results suggest Meox1 has ability to manifest distinct mechanistic and transcriptional regulation in the different intrinsic subtypes of claudin-low and basal-like p53 and PTEN deficient TNBC, where mechanistic regulation can be elicited by direct or indirect transcriptional control.

Conclusive results indicate targeting homeobox transcription factor Meox1 may have a critical impact on decreasing the aggressive proliferative and metastatic properties of p53 and PTEN deficient TNBC. Since frequent genetic aberrations in tumor suppressor genes of p53 and PTEN are drivers of tumorigenesis and drug resistance in TNBC, targeting Meox1 has the ability to help ameliorate patients' resistance to the mainstay of chemotherapeutic treatment.

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Intrinsic Subtype	Incidence	Biomarkers	Proliferative Gene Cluster	Tumor Grade	Prognostic Outcome	Prediction Therapy	TP53 Genetic Aberrations	PTEN Genetic Aberrations
Luminal A	40-60%	ER+, PR+, HER2-	Low	I (Good)	Good	Hormone Targeted, Chemotherapy	Low	Low
Luminal B	10-20%	ER-/+, PR-/+, HER2-/+	High	II (Intermediate)	Intermediate	Hormone Targeted, Chemotherapy, HER2 Targeted	Intermediate	Intermediate/High
HER2-Enriched	10-25%	ER-, PR-, HER2+	High	III (Poor)	Intermediate/Poor	HER2 Targeted, Chemotherapy	Intermediate/High	Intermediate/High
Basal-Like	10-15%	ER-, PR-, HER2-	High	III (Poor)	Poor	Chemotherapy, PARP Inhibitors	High	High
Claudin-Low	5-10%	ER-, PR-, HER2-	High	III (Poor)	Poor	Chemotherapy	High	High
Normal Breast-Like	5-10%	ER-/+, PR-/+, HER2-	Low	II (Intermediate)	Intermediate	Hormone Targeted, Chemotherapy	Low	Low

Figure 1.1 - Summary of incidence, biomarker expression, proliferative gene cluster expression, tumor grade, prognostic outcome, predictive therapy, as well as p53 and PTEN genetic aberration frequencies based on intrinsic subtype classification of breast cancer.

Chapter II

Meox1 Regulates Proliferation and Metastasis of Drug Resistant P53 and PTEN Deficient Triple Negative Breast Cancer

Abstract

Triple negative breast cancer (TNBC) lacks targeted therapy known to improve long-term patient outcome of overall survival. While chemotherapy is the mainstay of treatment, a great majority of TNBC patients do not respond to chemotherapeutic treatment and display drug resistance. A clinically aggressive disease, drug resistant patients inevitably experience disease recurrence, distant metastasis, and death within 5 years of diagnosis. As such, specific and alternative therapeutic strategies are in dire need. Tumor suppressor genes of p53 and PTEN are frequent genetic aberrations governing tumorigenesis and drug resistance in TNBC. However, their combined loss of function in TNBC is poorly understood. Mesenchyme homeobox 1 (Meox1) presents insight into the complex molecular biology of p53 and PTEN deficient TNBC tumors. RNA expression analyses show Meox1 is upregulated in TNBC and negatively regulated by tumor suppressor genes of p53 and PTEN. *In vitro* Meox1 knockdown experiments demonstrate the transcription factor significantly decreases cellular

proliferation of p53 and PTEN deficient TNBC. *In vivo* tumor xenograft experiments corroborate *in vitro* data, where decrease in Meox1 expression significantly decreases *in vivo* tumor growth. Decrease in cellular proliferation upon Meox1 knockdown is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like p53 and PTEN deficient TNBC intrinsic subtypes. Additionally, knocking down Meox1 decreases metastatic properties of cell migration and invasion in these cells. These results reveal Meox1 regulates proliferation and metastasis of p53 and PTEN deficient TNBC; as such, Meox1 presents a specific therapeutic target for aggressive tumorigenic and drug resistant p53 and PTEN deficient TNBC cells.

Introduction

Relative to other breast cancer subtypes, triple negative breast cancer (TNBC) manifests more aggressive clinical behavior and poorer patient outcome [1, 2]. TNBC tumors are characterized as highly proliferative with increased mitotic index [1]. Compared to non-TNBC patients, patients with TNBC exhibit a larger mean tumor size, increased nuclear pleomorphism, and higher grade tumors upon diagnosis [1, 2]. Additionally, TNBC patients exhibit more rapid and increased frequencies of disease recurrence, distant metastasis, and death, especially within 5 years following diagnosis [2].

Lacking overexpression for the three biomarkers of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2),

TNBC is the only subtype that lacks specified targeted therapy known to improve long-term patient outcome of overall survival. As such, chemotherapy is the mainstay of treatment for TNBC patients. Fortunately, patients with TNBC are more responsive to chemotherapeutic treatment compared to non-TNBC [3, 4]. Following neoadjuvant chemotherapy patients with TNBC exhibit higher pathological complete response (pCR) rates, significantly improving disease-free survival and overall survival [3, 4]. However, only 30% of TNBC patients exhibit pCR after neoadjuvant chemotherapy [5]. TNBC patients who do not achieve pCR and display residual disease following neoadjuvant chemotherapy are resistant to chemotherapeutic treatment displaying significantly poorer long-term outcome [3, 6-8]. As such, alternative therapeutic strategies other than chemotherapy are in dire need.

It is crucial to explore the biological heterogeneity of TNBC to not only identify specific therapeutic targets, but to also apply such new therapeutic strategies towards patients displaying resistance to the mainstay of chemotherapeutic treatment. Tumor heterogeneity can be described in the context of clonal evolution, where ‘driver mutations’ conferring a selective advantage for tumorigenesis and drug resistance define TNBC functional and mechanistic biology [9]. Genetic aberrations that repeatedly manifest as critical drivers for tumorigenesis and drug resistance in TNBC are tumor suppressor genes of p53 and PTEN [5, 10-12]. Amongst all the subtypes of breast cancer, TNBC exhibits the highest frequencies of p53 and PTEN genetic alterations, with 84% and 35%, respectively [10]. Concurrent alterations in both tumor suppressor genes of p53 and PTEN are exhibited in approximately 20-30% of TNBC tumors [13, 14]. While genetic alterations in p53 are higher in frequency relative to PTEN,

epigenetic hypermethylation causing protein silencing is more common with PTEN in breast cancer [15, 16]. Research shows mammary specific epithelial loss of wild-type p53 and PTEN function induces TNBC-like tumors in mice [13, 14]. Compared to loss of either p53 or PTEN alone, combined loss of p53 and PTEN wild-type function manifests faster and more aggressive TNBC tumor hallmarks, displaying increased proliferation and migration gene expression profiles [13, 14]. Mice with loss of p53 and PTEN wild-type function manifest decreased tumor free survival relative to loss of p53 or PTEN alone [13, 14]. Furthermore, human TNBC tumors with low expression of p53 and PTEN exhibit poorer prognosis with worse metastatic free survival relative to patients with normal p53 and PTEN levels [13].

While the significance of p53 and PTEN are essential in TNBC tumor function, the molecular biology involved with combined loss of function for both tumor suppressor genes is not well known. To offer further insight, this chapter explores the function of mesenchyme homeobox 1 (Meox1) in the context of p53 and PTEN deficient TNBC, focusing on intrinsic subtypes of claudin-low and basal-like. Meox1 is a homeobox transcription factor well known for its role of somite development in the vertebrate embryo [17-19]. The transcription factor has been previously linked to increased tumor cell growth in ovarian and breast cancers [20, 21]. Nuclear expression of Meox1 is associated with poor overall breast cancer patient survival, along with increased lymph node metastasis and high tumor stage [21]. In this chapter, data shows Meox1 expression is upregulated in TNBC. Additionally, expression of Meox1 is negatively regulated by both tumor suppressor genes of p53 and PTEN. Both *in vitro* and *in vivo* studies demonstrate knockdown of Meox1 decreases aggressive cellular proliferation of

p53 and PTEN deficient TNBC. Interestingly, this decrease in cellular proliferation is largely attributed to apoptosis in claudin-low, but cell cycle arrest in basal-like TNBC subtypes. Furthermore, knockdown of Meox1 in these cell lines decreases cell migration and invasion, ascribing to its functional role of regulating metastasis. As such, Meox1 may serve as a specific therapeutic target to help ameliorate the aggressive proliferative and metastatic properties of p53 and PTEN deficient TNBC, particularly in a drug resistant setting.

Materials and Methods

In Vitro Cell Culture Growth Conditions

In vitro breast cancer cell lines were grown according to ATCC guidelines. While ATCC formulated media could not be purchased, base media from Gibco Life Technologies was used. Supplements were added to Gibco Life Technologies base media to match ATCC media formulations accordingly. For certain cell lines, ATCC suggested the use of Leibovitz's L-15 media in an atmospheric gas exchange environment without CO_2 (suggested for MDA-MB-231, MDA-MB-361, MDA-MB-453, and MDA-MB-468 breast cancer cell lines). However, these conditions could not be accommodated in our lab and the next best alternative of media formulation was utilized. Two breast cell lines were grown according to Asterand guidelines, which are SUM149 and SUM159.

MCF-10A cells were grown in DMEM/F12 base media supplemented with antibiotic-antimycotic, 5% horse serum, 20ng/mL epidermal growth factor (EGF), 100ng/mL cholera toxin, 500ng/mL hydrocortisone, and 10µg/mL insulin. MCF-7 cells were grown in EMEM base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 10µg/mL insulin, and 1mM sodium pyruvate. T-47D cells were grown in RPMI base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 5µg/mL insulin, 1mM sodium pyruvate, and 10mM HEPES. ZR-75-1, BT-474, and ZR-75-30 cells were grown in RPMI base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 1mM sodium pyruvate, and 10mM HEPES. MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells were grown in DMEM base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, and 1mM sodium pyruvate. MDA-MB-361 cells were grown in DMEM base media supplemented with antibiotic-antimycotic, 20% fetal bovine serum, and 1mM sodium pyruvate. BT-549 cells were grown in RPMI base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 0.85µg/mL insulin, 1mM sodium pyruvate, and 10mM HEPES. SUM149 and SUM159 cells were grown in Ham's F12 base media supplemented with antibiotic-antimycotic, 5% fetal bovine serum, 1µg/mL hydrocortisone, 5µg/mL insulin, and 10mM HEPES. All cells were grown in a humidified 37^{o}C incubator with 5% CO_2.

In Vitro Cell Culture Transient Transfection for Small Interfering RNA (siRNA) Knockdown

Different subtypes of *in vitro* breast cancer cell lines show distinct differences in cell morphology, growth rate, confluency, and transfection efficiency. As such, experimental siRNA protocols required optimization for each cell line.

For siRNA knockdown experiments, cell lines were grown with media specified above. However, antibiotic-antimycotic was omitted from the media in order to increase transfection efficiency and avoid unnecessary toxicity to cells during transfection. Breast cancer cell lines were plated onto 6-well plates at approximately 50% confluency per well. For each well, $2x10^5$ cells of BT-549, MDA-MB-468, SUM159, MCF-10A; $6x10^5$ cells of ZR-75-1; and $9x10^5$ cells of BT-474 were required to achieve 50% confluency. After 24 hours of plating, cells were transfected with siRNA using Invitrogen Lipofectamine RNAiMAX Reagent (#13778-150); transfection was conducted according to manufacturer's instructions. For Meox1 knockdown experiments, 50nM of Negative Control siRNA and 50nM of Meox1 siRNA for each treatment group was utilized. For HER2, p53, and PTEN knockdown experiments, 30nM of Negative Control siRNA and 30nM of HER2, p53, and PTEN siRNA was utilized. Cells were incubated with transfection reagent and siRNA for only 24 hours to avoid toxicity, after which media was removed and fresh media without antibiotic-antimycotic was added. Following 48-72 hours from start of transfection, cells were trypsinized or harvested for RNA, protein, or for specified experimental analyses.

All siRNAs used to conduct experiments were purchased from Qiagen. Negative Control siRNA (#1027281) contained target sequence 5'-CAGGGTATCGACGATTACAAA-3', sense strand 5'-GGGUAUCGACGAUUACAAAUU-3', and antisense strand 5'-UUUGUAAUCGUCGAUACCCUG-3'. Three different Meox1

siRNA treatments were used to validate results. Meox1 siRNA 1 (#SI00630266) contained target sequence 5'-CAGGCTTGACTGGGTGGACAA-3', sense strand 5'-GGCUUGACUGGGUGGACAATT-3', and antisense strand 5'-UUGUCCACCCAGUCAAGCCTG-3'. Meox1 siRNA 2 (#SI00630280) contained target sequence 5'-AAGCTAATTGTGCGAGCTCAA-3', sense strand 5'-GCUAAUUGUGCGAGCUCAATT-3', and antisense strand 5'-UUGAGCUCGCACAAUUAGCTT-3'. Meox1 siRNA Mixture is a pool of four different Meox1 siRNAs mixed together for a final concentration of 50nM, matching the concentration of Meox1 siRNA 1 and Meox1 siRNA 2. This is a technique commonly carried out by using four different siRNAs each at low concentrations to ensure there are no off target affects that may be caused when using one siRNA alone. The Meox1 siRNA Mixture pools together 12.5nM of each Meox1 siRNA 1, Meox1 siRNA 2, Meox1 siRNA 3 (#SI03145205) with target sequence 5'-AGCTGGCGACTCGGAAAGTAA-3', sense strand 5'-CUGGCGACUCGGAAAGUAATT-3', and antisense strand 5'-UUACUUUCCGAGUCGCCAGCT-3', as well as Meox1 siRNA 4 (#SI04293310) with target sequence 5'-TCCACGATTTCTGGATTGAAA-3', sense strand 5'-CACGAUUUCUGGAUUGAAATT-3', and antisense strand 5'-UUUCAAUCCAGAAAUCGUGGA-3'.

To knockdown HER2, Qiagen FlexiTube GeneSolution (#GS2064) was tested. Functionally verified HER2 siRNA (#SI02223571) validated by Qiagen to knockdown HER2 contained target sequence 5'-AACAAAGAAATCTTAGACGAA-3', sense strand 5'-CAAAGAAAUCUUAGACGAATT-3', and antisense strand 5'-UUCGUCUAAGAUUUCUUUGTT-3'. To knockdown p53, Qiagen FlexiTube

GeneSolution (#GS7157) was tested. Functionally verified p53 siRNA (#SI02655170) validated by Qiagen to knockdown p53 contained target sequence 5'-AAGGAAATTTGCGTGTGGAGT-3', sense strand 5'-GGAAAUUUGCGUGUGGAGUTT-3', and antisense strand 5'-ACUCCACACGCAAAUUUCCTT-3'. To knockdown PTEN, Qiagen FlexiTube GeneSolution (#GS5728) was tested. Functionally verified PTEN siRNA (#SI00301504) validated by Qiagen to knockdown PTEN contained target sequence 5'-AAGGCGTATACAGGAACAATA-3', sense strand 5'-GGCGUAUACAGGAACAAUATT-3', and anti-sense strand 5'-UAUUGUUCCUGUAUACGCCTT-3'.

In Vitro Cell Culture Stable Transduction for Lentiviral Overexpression

Normal immortalized MCF-10A cells were used to overexpress Meox1 with lentiviral vectors. MCF-10A cells were virally transduced with Abmgood pLenti-CMV-RFP-2A-Puro-Blank Control (#LV591) and Abmgood pLenti-GIII-CMV-hMeox1-RFP-2A-Puro (#LV217710) lentiviral vectors. Viral particles were generated at the University of Michigan Vector Core. Cells were infected with lentiviral particles for 24 hours using 8ug/mL of polybrene. Following 72 hours from start of infection, selection for infected cells was conducted using fluorescence-activated cell sorting (FACS) of RFP positive MCF-10A pLenti-CMV-RFP-2A-Puro-Blank Control and RFP positive MCF-10A pLenti-GIII-CMV-hMeox1-RFP-2A-Puro transduced cells. MCF-10A cells with pLenti-CMV-RFP-2A-Puro-Blank Control and pLenti-GIII-CMV-hMeox1-RFP-2A-Puro are here on after labeled as pLenti Control and pLenti Meox1 Overexpression, respectively.

Two-Step Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was isolated from cells using either Life Technologies TRIzol Reagent (#15596018) or Qiagen RNeasy Kit (#74104). RNA isolation was conducted according to manufacturer's instructions. Invitrogen SuperScript III First-Strand Synthesis SuperMix (#11752-050) was used to make cDNA from isolated RNA. The maximum amount of RNA allotted by the kit was utilized, 1ug, to make cDNA. Afterwards, cDNA was diluted no more than five-fold to perform qPCR. Applied Biosystems SYBR Green PCR Master Mix (#4309155) was used to perform qPCR. All primers were obtained from Integrated DNA Technologies (IDT) and used at a final concentration of 500nM. The ACTB primer (#Hs.PT.39a.22214847) was used with forward sequence of 5'-CCTTGCACATGCCGGAG-3' and reverse sequence of 5'-ACAGAGCCTCGCCTTTG-3'. Three different Meox1 primers were used to validate results: Meox1 primer 1 (#Hs.PT.58.26021003.g) with forward sequence of 5'-CTCAGTGAAGATGTGCTCCTC-3' and reverse sequence of 5'-CAGACTTCCTGGCGACA-3'; Meox1 primer 2 (#Hs.PT.58.2405084) with forward sequence of 5'-GCTTCCCTCTGTTCTCCTG-3' and reverse sequence of 5'-GAGCACTGCCAATGAGACA-3'; and finally, Meox1 primer 3 (#Hs.PT.58.38645298) with forward sequence of 5'-CACGCTTCCACTTCATCCTT-3' and reverse sequence of 5'-GGCTCCGCAGATATGAGATTG-3'. Interestingly, Meox1 primer 1 worked well to detect endogenous and overexpressed Meox1 RNA levels of *in vitro* breast cancer cell lines, while Meox1 primer 3 worked well to detect siRNA and shRNA knockdown of Meox1 RNA levels.

It is important to note that when performing RT-qPCR for Meox1, extra care must be taken to handle the RNA samples. Meox1 expression in breast cancer cell lines is

extremely low. As such, when isolating RNA, proper technique must be utilized to obtain good quality RNA. When making cDNA, regardless of preferred kit, maximum amount of RNA allotted by the kit must be used to make ample cDNA. Then finally, cDNA should not be diluted more than five-fold when performing qPCR. Ensuring these techniques will help obtain Ct values that will be reliable for Meox1 detection. Furthermore, results will be more reliable if more than one Meox1 qPCR primer is used for analysis, different primers can give vastly different results. Unfortunately, Meox1 was only detected at RNA levels using *in vitro* breast cancer cell lines; while many attempts were made, no successful antibody was found to detect Meox1 expression at a protein level.

Cell Proliferation Assay

As mentioned above, different subtypes of *in vitro* breast cancer cell lines show distinct differences in cell morphology, growth rate, confluency, and transfection efficiency. As such, cell proliferation assays also required optimization for each cell line. Following 48 hours with transfection of 50nM Negative Control siRNA and 50nM Meox1 siRNA treatments, cells were trypsinized and counted three times so as to ensure accurate plating of cell number. Using 96-well plates, each cell line required plating a different number of cells per well to achieve normal exponential growth within five days of analysis. As such, for each well, $2x10^3$ cells of BT-549, MDA-MB-468, MCF-10A; $6x10^3$ cells of ZR-75-1; and $9x10^3$ cells of BT-474 were plated. For each siRNA treatment, cells were plated into six different wells for statistical analysis. Invitrogen CyQUANT Cell Proliferation Assay (#C35011) was conducted for a period of five days.

Apoptosis Assay

Following 72 hours with transfection of 50nM Negative Control siRNA and 50nM Meox1 siRNA treatments, media containing loose floating cells was collected and combined with trypsinized adherent cells. Cells were counted three times, washed with 1XPBS, and resuspended to a final concentration of $1x10^6$ cells/mL using BD Biosciences Annexin V Binding Buffer (#556454). For analysis, 100uL of this cell suspension ($1x10^5$ cells) was pipetted into separate tubes for staining. To each 100uL of $1x10^5$ cells, DAPI was added to a final concentration of 1ug/mL and BD Biosciences PE Annexin V (#556422) was added at 5uL as instructed by manufacturer. Cells were then incubated for 15 minutes at room temperature in the dark. Following incubation, 400uL of BD Biosciences Annexin V Binding Buffer (#556454) was added to each tube and analyzed by flow cytometry within one hour.

Cell Cycle Assay

Similar to the apoptosis assay, following 72 hours with transfection of 50nM Negative Control siRNA and 50nM Meox1 siRNA treatments, media containing loose floating cells was collected and combined with trypsinized adherent cells, counted three times and washed with 1XPBS. Ice cold 66% ethanol was gently added to the cells to a final concentration of $1x10^6$ cells/mL and fixed by storing at 4^oC for at least 2 hours or up to 4 weeks. Cells were removed from 4^oC and equilibrated to room temperature then washed with 1XPBS. Given previously counted cell numbers, $1x10^5$ cells in 200uL was used for staining, to which Propidium Iodide was added a final concentration of 0.05mg/mL and RNase was added to final units of 550U/mL. Cells were incubated for

20-30 minutes at 37°C in the dark, then analyzed by flow cytometry. Assay can also be performed by using Abcam Propidium Iodide Flow Cytometry Kit for Cell Cycle Analysis (#ab139418).

Mammosphere Formation Assay

Following 48 hours with transfection of 50nM Negative Control siRNA and 50nM Meox1 siRNA treatments, cells were trypsinized and counted three times. Cells were resuspended to a final concentration of $1x10^6$ cells/mL and a syringe was utilized with a 23G needle to gently aspirate and dissociate the cells three times in order to achieve a single cell suspension. Stem Cell Technologies MammoCult Medium (#05620) was utilized and supplemented with heparin to a final concentration of 4ug/mL and hydrocortisone to a final concentration of 0.48ug/mL. Ultra-Low attachment 6-well plates were necessary for this experiment to ensure cells would not adhere to the bottom but grow suspended in media. To each ultra-low attachment 6-well plate, 3mL of complete MammoCult Medium was added per well and to each well $5x10^3$ cells were plated; each treatment group was plated into at least three wells for statistical analysis. Primary mammospheres were allowed to grow undisturbed for five days, after which mammospheres > 25um were counted under a microscope. After counting, mammospheres for each treatment group were collected and combined for secondary mammosphere experiments. Combined wells for each treatment group were trypsinized and counted three times. Cells were resuspended to a final concentration of $1x10^6$ cells/mL, and again a 23G needle was utilized to gently aspirate and dissociate the cells three times in order to achieve a single cell suspension. Using ultra-low attachment 6-

well plates, 3mL of complete MammoCult Medium was added per well and to each well $5x10^3$ cells were plated. All cells from primary mammospheres were plated for secondary mammosphere formation. Secondary mammospheres were allowed to grow undisturbed for five days, after which mammospheres > 25um were counted under a microscope. Mammosphere formation efficiency (%) was calculated as (number of mammospheres per well / number of cells plated per well) x 100 [22].

Cell Migration and Invasion Assay

Following 72 hours with transfection of 50nM Negative Control siRNA and 50nM Meox1 siRNA treatments, Corning BioCoat Control Inserts (#354578) and Corning BioCoat Matrigel Invasion Chamber (#354480) 24-well was utilized for cell migration and invasion assays. Protocol was conducted according to manufacturer's instructions with one exception, an optimized seeding density of $7.5x10^4$ cells per 24-well chamber were plated. While the protocol gives you two options to fix and stain cells, in this experiment the Diff-Quick stain option was chosen, Thermo Scientific Shandon Kwik-Diff Stain Kit (#9990701) was utilized.

Cloning for Short Hairpin RNA (shRNA) Stable Cell Lines

The Addgene Tet-pLKO-puro plasmid (#21915) was used to clone doxycycline inducible Meox1 shRNA, a gift from Dmitri Wiederschain [23]. As such, protocols to clone and establish stable doxycycline inducible Meox1 shRNA cell lines were conducted according to Wiederschain and Addgene instructions manuals. Sequences for Meox1 shRNA were obtained from the Mission shRNA Library of The RNAi

Consortium; the sequences used for Meox1 cloning were TRCN0000016108 and TRCN0000016110. TRCN0000016108 contained top sequence of 5'-CCGGGCTGTTATTGTGGGAGGAAATCTCGAGATTTCCTCCCACAATAACAGCTTTTT-3' and bottom sequence of 5'-AATTAAAAAGCTGTTATTGTGGGAGGAAATCTCGAGATTTCCTCCCACAATAACAGC-3'. TRCN0000016110 contained top sequence of 5'-CCGGCCAATGAGACAGAGAAGAAATCTCGAGATTTCTTCTCTGTCTCATTGGTTTTT-3' and bottom sequence of 5'-AATTAAAAACCAATGAGACAGAGAAGAAATCTCGAGATTTCTTCTCTGTCTCATTGG-3'. The Addgene Tet-pLKO-puro-scrambled plasmid (#47541) was used as a negative control shRNA, a gift from Charles Rudin [24]. The Tet-pLKO-puro-scrambled contained negative control shRNA top sequence of 5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTT-3' and bottom sequence of 5'-AATTAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3'. The following stable cell lines were established using the basal-like MDA-MB-468 TNBC cells: MDA-MB-468 Tet-pLKO-puro-scrambled (labeled as Scrambled Control shRNA), MDA-MB-468 Tet-pLKO-puro Meox1 shRNA TRCN0000016108 (labeled as Meox1 shRNA 1), and MDA-MB-468 Tet-pLKO-puro Meox1 shRNA TRCN0000016110 (labeled as Meox1 shRNA 2).

In Vivo Mammary Fat Pad Injections

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. Female nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice at the ages of 8-10 weeks were used for orthotopic fourth inguinal mammary fat pad injections. Xenograft tumor models were established by injecting $5x10^4$ human breast cancer cells suspended in a 2:3 ratio of 1XPBS:matrigel, respectively. Three groups of cells were injected, the MDA-MB-468 Tet-pLKO-puro-scrambled (labeled as Scrambled Control shRNA), MDA-MB-468 Tet-pLKO-puro Meox1 shRNA TRCN0000016108 (labeled as Meox1 shRNA 1), and MDA-MB-468 Tet-pLKO-puro Meox1 shRNA TRCN0000016110 (labeled as Meox1 shRNA 2). Five mice per group were injected for statistical analysis.

To induce Meox1 shRNA knockdown, Envigo doxycycline 625mg/kg pellet diet (#TD.08541) was administered to the mice once a week. In an adjuvant setting, Meox1 shRNA knockdown was induced with administration of doxycycline 3 days following surgery. In a neoadjuvant setting, Meox1 shRNA knockdown was induced with administration of doxycycline after palpable or visible tumor growth.

Statistical Analysis

GraphPad Prism 7.0 was used for calculations of all statistical analyses. An unpaired t-test was conducted when comparing two means of two unmatched groups. One-way ANOVA was conducted when measuring one variable and comparing three or more means of unmatched groups. Two-way ANOVA was conducted when determining a change in response based on two factors and comparing three or more means of matched or unmatched groups.

Results and Discussion

Meox1 is upregulated in TNBC; p53 and PTEN negatively regulate Meox1 expression.

RT-qPCR analysis using a panel of *in vitro* breast cancer cell lines shows Meox1 expression is upregulated in TNBC (Figure 2.1A). Data shows TNBC subtypes of MDA-MB-231, BT-549, SUM149, MDA-MB-468, SUM159, and MDA-MB-453 have high expression of the transcription factor when calibrated to the normal immortalized breast cancer cell line MCF-10A. No expression of Meox1 is seen in the luminal subtypes of MCF-7, T-47D, and ZR-75-1. Similarly, no expression of Meox1 is seen in the HER2-enriched subtypes of BT-474, MDA-MB-361, and ZR-75-30.

Interestingly, RT-qPCR analyses further show that the expression of Meox1 is negatively regulated by p53 and PTEN. Knockdown experiments in normal immortalized MCF-10A cells using siRNA for p53 and PTEN effect Meox1 expression. While Meox1 expression is increased with siRNA knockdown of either p53 or PTEN alone in MCF-10A, no statistically significant increase in expression is seen (Figure 2.1B). Greatest influence on increased Meox1 expression is observed with dual knockdown of both tumor suppressor genes in MCF-10A cells. Similar results are displayed when conducting the same experiment in the TNBC cell line SUM159. SUM159 cells harbor loss of wild-type function of p53 with mutant expression, however they have normal expression and function of PTEN (Figure 2.1G) [25-34]. Finding a TNBC cell line with no p53 genetic aberrations is challenging, as such SUM159 cells are used as proof of

concept. SUM159 siRNA knockdown of p53 alone has no effect on Meox1 expression, however knocking down PTEN induces a small yet significant increase (Figure 2.1C). As seen with MCF-10A, dual knockdown of both tumor suppressor genes in SUM159 has the greatest influence on increased Meox1 expression.

Besides p53 and PTEN, HER2 also negatively regulates Meox1 expression. In luminal MCF-7, stable knockdown of PTEN alone increases the expression of the transcription factor (Figure 2.1D), however stable overexpression of HER2 downregulates its expression. Furthermore, increase of HER2 prohibits Meox1 expression when PTEN is decreased, as evident with concurrent overexpression of HER2 and knockdown of PTEN. Similar results are observed in TNBC SUM159 cells. Stable knockdown of PTEN alone in SUM159 increases the expression of Meox1, but stable overexpression of HER2 downregulates its expression (Figure 2.1E). As seen with MCF-7, overexpression of HER2 prohibits Meox1 expression when PTEN is concurrently knocked down in SUM159. This point is further established when performing HER2, p53, and PTEN siRNA experiments in the HER2-enriched breast cancer cell line BT-474. Knockdown of p53 or PTEN alone has no effect on Meox1 expression in BT-474, and neither does dual knockdown of both p53 and PTEN (Figure 2.1F). Only during concurrent knockdown of p53 with HER2 or concurrent knockdown of PTEN with HER2 does expression of Meox1 start to increase. However, most significant increase of Meox1 expression is seen when all three genes of HER2, p53, and PTEN are downregulated.

While further research is required to offer more mechanistic insight as to how HER2, p53, and PTEN regulate Meox1 expression upstream, it is nonetheless clear that

the expression of the transcription factor is specific to TNBC and negatively regulated by both tumor suppressor genes of p53 and PTEN.

Knockdown of Meox1 decreases *in vitro* cell proliferation of p53 and PTEN deficient TNBC.

To assess changes in cell proliferation upon knockdown of Meox1 in p53 and PTEN deficient TNBC, *in vitro* cell lines of claudin-low BT-549 and basal-like MDA-MB-468 are utilized; both of these TNBC cell lines harbor innate genetic aberrations in p53 and PTEN conferring loss of wild-type function for both tumor suppressor genes (Figure 2.1G) [25-34]. TNBC intrinsic subtypes of claudin-low BT-549 and basal-like MDA-MB-468 show significant decrease in cellular proliferation when knocking down Meox1 using three different siRNA treatments (Figure 2.2A and 2.2B). Knocking down Meox1 in normal immortalized MCF-10A, luminal ZR-75-1, and HER2-enriched BT-474 shows minor decrease in cell proliferation and minimal off-target effects (Figure 2.2C, 2.2D, and 2.2E). As such, Meox1 functions to regulate cell proliferation, specifically in p53 and PTEN deficient TNBC. Thus, targeting Meox1 may decrease the aggressive proliferative behavior of p53 and PTEN deficient TNBC tumors with low potential of off-target effects.

Meox1 knockdown largely causes apoptosis in claudin-low but cell cycle arrest in basal-like p53 and PTEN deficient TNBC.

In vitro assays of apoptosis with Annexin V plus DAPI as well as cell cycle arrest with Propidium Iodide (PI) are utilized to assess Meox1 effects on cell proliferation in p53 and PTEN deficient TNBC.

Whether looking at Annexin V only or total Annexin V positive cells, knockdown of Meox1 significantly increases apoptosis in claudin-low BT-549 using all three siRNA treatments (Figure 2.3A and 2.3B). Highest levels of apoptosis are exhibited with a 20% increase in Annexin V and 30% increase in total Annexin V positive cells following 72 hours of Meox1 siRNA 1 knockdown in claudin-low BT549. However, a less profound effect of apoptosis is seen in basal-like MDA-MB-468 cells (Figure 2.3C and 2.3D). A relatively lower level of 10% increase in Annexin V and 15% increase in total Annexin V positive cells are evident in basal-like MDA-MB-468 after 72 hours of Meox1 siRNA 1 knockdown.

Interestingly, reverse roles are displayed between the two intrinsic subtypes of TNBC when performing cell cycle arrest assays. While apoptosis significantly dominates in claudin-low BT-549, cell cycle arrest in the G2/M phase significantly dominates in basal-like MDA-MB-468 after Meox1 siRNA treatments (Figure 2.4C and 2.4D). Highest levels of cell cycle arrest are exhibited with a 30% increase in the G2/M phase following 72 hours of Meox1 siRNA 1 knockdown in basal-like MDA-MB-468. A relatively lower level of 15% increase in the G2/M phase is evident in claudin-low BT-549 after 72 hours of Meox1 siRNA 1 knockdown (Figure 2.4A and 2.4B). It is important to note that unfortunately cell cycle arrest in the G2/M phase is not observed with the Meox1 siRNA 2 treatment in either cell line; one plausible reason could be more time is required for its effects to manifest. Interestingly, Meox1 siRNA 2 treatment does seem to slightly increase the G0/G1 phase following 72 hours of knockdown; one potential reason could be that the cells were not synchronized prior to conducting the assay.

Nonetheless, data shows decrease in cell proliferation upon Meox1 knockdown is largely attributed to apoptosis in claudin-low BT549 but cell cycle arrest in basal-like MDA-MB-468 p53 and PTEN deficient TNBCs.

Knockdown of Meox1 decreases self-renewal of p53 and PTEN deficient TNBC.

Not only does Meox1 regulate cell proliferation in p53 and PTEN deficient TNBC, but it also regulates cell self-renewal. Ability to affect mammosphere formation efficiency for consecutive passages *in vitro* is a measure of regulating cell self-renewal function [22]. Using three different siRNA treatments, mammosphere formation assays show knocking down Meox1 in p53 and PTEN deficient TNBC of claudin-low BT-549 and basal-like MDA-MB-468 significantly decreases both primary and secondary consecutive mammosphere formations (Figure 2.5A, 2.5B, 2.5C, and 2.5D). As such, Meox1 knockdown decreases self-renewal function of p53 and PTEN deficient TNBC. Appropriately, stable overexpression of Meox1 in normal immortalized MCF-10A increases mammosphere formation, regulating increase in cell self-renewal (Figure 2.5E).

It is important to note the ability to regulate cell self-renewal is a property attributed to stem or progenitor cell function [22]. While Meox1 will not be discussed in the context of stem or progenitor cells in this dissertation, the transcription factor's ability to regulate cell self-renewal offers insight that it may harbor stem or progenitor cell properties.

Meox1 knockdown decreases cell migration and invasion of p53 and PTEN deficient TNBC.

Chemotaxis of cell directional migration and invasion through an extracellular matrix are important functional properties of metastasis [35-37]. *In vitro* cell migration and invasion assays were conducted to ascertain the role of Meox1 as a metastatic regulator of p53 and PTEN deficient TNBC. Knocking down Meox1 in both claudin-low BT-549 and basal-like MDA-MB-468 significantly decreases both migration and invasion of TNBC cells after 72 hours (Figure 2.6A and 2.6C). Furthermore, the invasion index decreases by approximately 50% in claudin-low BT-549 and approximately 30% in basal-like MDA-MB-468 cells (Figure 2.6B and 2.6D). These results indicate Meox1 regulates metastasis and targeting the transcription factor may have a crucial role in decreasing the aggressive metastatic potential of p53 and PTEN deficient TNBC.

Knockdown of Meox1 effects cellular morphology in p53 and PTEN deficient TNBC.

Claudin-low cell lines such as BT-549 display a more mesenchymal cellular structure with flattened bodies and spindle like protrusions. Basal-like cell lines such as MDA-MB-468 exhibit rounder pebble-like morphologies. Knocking down Meox1 using three different siRNA treatments effects p53 and PTEN deficient TNBC cellular morphology, seen after 96 hours post-transfection (Figure 2.7). Following 96 hours of Meox1 knockdown mesenchymal claudin-low BT-549 cells display longer and sharper cellular protrusions, while basal-like MDA-MB-468 cells lose their pebble-like circularity displaying more flattened cell bodies with spindle protrusions.

Changes in cellular morphology are attributed to rearrangement of the cytoskeletal structure, a process required for cell migration, invasion, and subsequent metastasis [35-37]. Thus, not only do cell migration and invasion assays show the metastatic capability of Meox1, but cellular morphological changes induced upon knockdown of the transcription factor may further accredit its metastatic potential.

Meox1 knockdown inhibits *in vivo* tumor growth of p53 and PTEN deficient TNBC.

Using a doxycycline inducible vector system, stable Meox1 shRNA cell lines were established with p53 and PTEN deficient TNBC of basal-like MDA-MB-468. Xenograft tumor growth effects after orthotopic mammary fat pad injections using Meox1 shRNA MDA-MB-468 cells was studied in an adjuvant and neoadjuvant setting. In an adjuvant setting, Meox1 shRNA knockdown is induced with administration of doxycycline 3 days following surgery. In a neoadjuvant setting, Meox1 shRNA knockdown is induced with administration of doxycycline after palpable or visible tumor growth. Data shows that regardless of when doxycycline is administered to induce shRNA, Meox1 knockdown significantly decreases *in vivo* tumor growth of p53 and PTEN deficient TNBC (Figure 2.8A and 2.8B). However, knockdown of Meox1 seems to have a greater impact on decreasing tumor growth in an adjuvant setting. As such, targeting Meox1 particularly in an adjuvant setting may serve a benefit to decreasing the rapid proliferative property of p53 and PTEN deficient TNBC tumors.

Conclusion

Compared to other breast cancer subtypes, patients with TNBC exhibit more aggressive clinical behavior and poorer outcome [1, 2]. TNBC tumors are characterized as highly proliferative, manifesting larger mean tumor size and higher tumor grade upon diagnosis [1, 2]. Relative to non-TNBC, TNBC patients display increased frequencies of disease recurrence, distant metastasis, and mortality within 5 years of diagnosis [2]. Lacking targeted therapy known to improve long-term patient outcome of overall survival, chemotherapy is the mainstay of treatment for TNBC. While TNBC patients are sensitive to chemotherapy and exhibit high pCR rates following neoadjuvant chemotherapy, a great majority are unable to achieve pCR displaying residual disease and drug resistance [3-5]. It is thus critical to further investigate the biological heterogeneity underlying TNBC and drug resistance in hopes of identifying specific therapeutic targets. Genetic aberrations in tumor suppressor genes of p53 and PTEN repeatedly manifest as frequent crucial drivers for tumorigenesis and drug resistance in TNBC [5, 10-12]. However, the molecular biology involved with combined loss of function for both tumor suppressor genes is poorly understood.

Homeobox transcription factor Meox1 offers insight into the complex molecular biology of p53 and PTEN deficient TNBC. RT-qPCR analysis of *in vitro* breast cancer cell lines show Meox1 expression is upregulated in TNBC, no expression is seen in luminal or HER2-enriched subtypes. Expression of Meox1 is further regulated by p53 and PTEN, where loss of both tumor suppressor genes significantly increases levels of the transcription factor. High expression of Meox1 in p53 and PTEN deficient TNBC has a crucial role for regulating cell proliferation *in vitro* and *in vivo*. Knocking down Meox1

significantly decreases cellular proliferation, largely causing apoptosis in claudin-low but cell cycle arrest in basal-like *in vitro* cell lines. *In vivo* knockdown of Meox1 corroborates *in vitro* data, where knocking down the transcription factor significantly decreases p53 and PTEN deficient TNBC tumor proliferation in an adjuvant and neoadjuvant setting. However, knockdown of Meox1 seems to have a greater impact in an adjuvant setting for decreasing tumor growth. Not only is Meox1 a potent regulator of cell proliferation, but it also regulates migration and invasion of p53 and PTEN deficient TNBC. Knockdown of the transcription factor decreases migration and invasion of both claudin-low and basal-like cells, attributing to its functional role in regulating metastasis.

These results reveal Meox1 regulates proliferation and metastasis of p53 and PTEN deficient TNBC. While studying Meox1 offers insight into the molecular biology of p53 and PTEN deficient TNBC cells, it also offers potential avenues for specified targeting. Current data indicates targeting Meox1 may have a crucial role in decreasing the aggressive proliferative and metastatic potential of p53 and PTEN deficient TNBC tumors. Since genetic alterations in tumor suppressor genes of p53 and PTEN are common drivers for tumorigenesis as well drug resistance in TNBC, targeting Meox1 may further help ameliorate patients' resistance to the mainstay of chemotherapeutic treatment.

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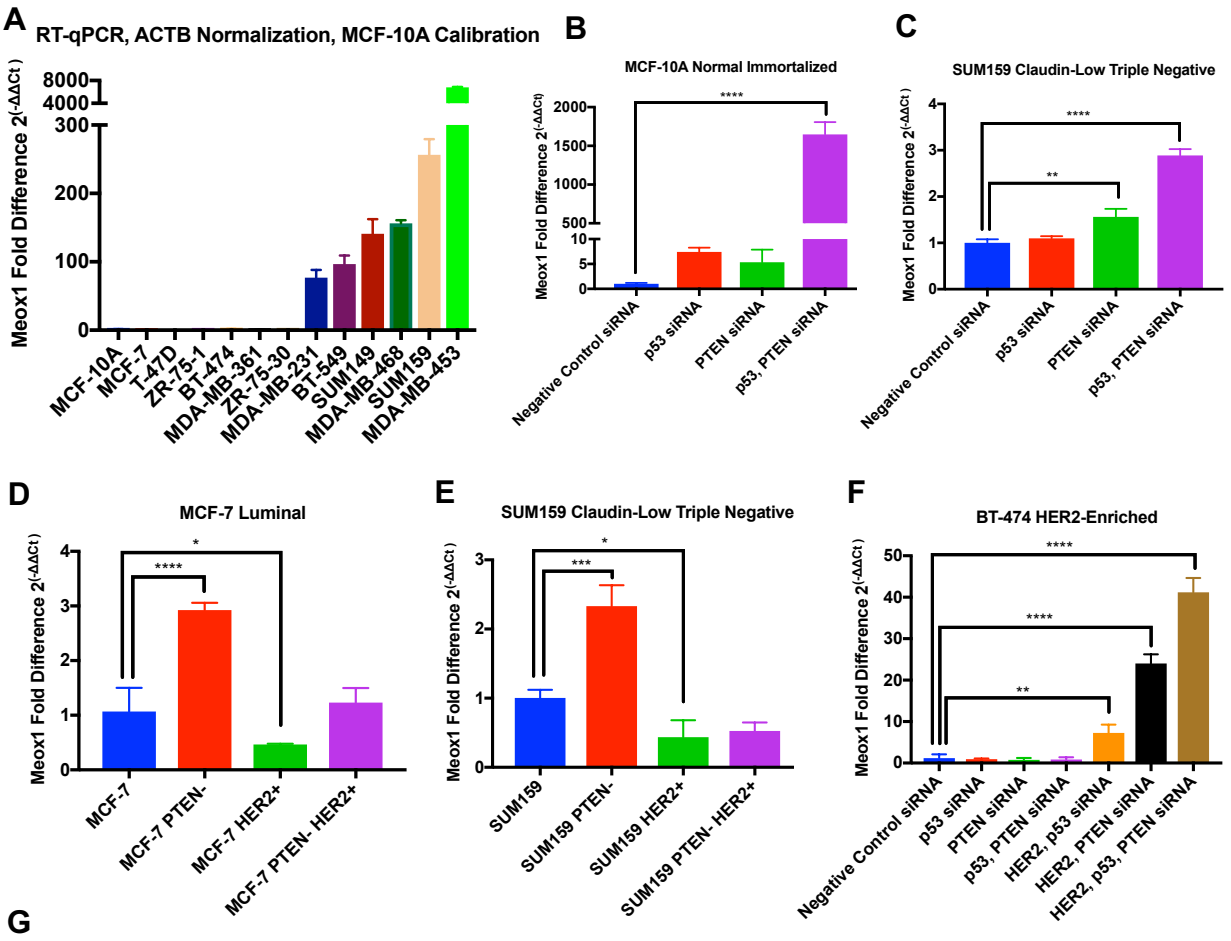

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Breast Cancer Subtype	Cell Line	TP53 Mutation Status	TP53 Expression	PTEN Mutation Status	PTEN Expression
Normal Immortalized	MCF-10A	wild-type	wild-type expression	wild-type	wild-type expression
Luminal	MCF-7	wild-type	wild-type expression	wild-type	wild-type expression
	T-47D	homozygous c.580C>T, p.L194F	mutant expression	wild-type	wild-type expression
	ZR-75-1	wild-type	wild-type expression	homozygous c.323T>G, p.L108R	mutant expression
HER2-Enriched	BT-474	homozygous c.853G>A, p.E285K	mutant expression	wild-type	wild-type expression
	MDA-MB-361	wild-type / homozygous c.166G>T, p.E56X ?	wild-type / mutant expression ?	wild-type	wild-type expression
	ZR-75-30	wild-type	wild-type expression	wild-type	wild-type expression
Triple Negative	MDA-MB-231	homozygous c.839G>A, p.R280K	mutant expression	wild-type	wild-type expression
	BT-549	homozygous c.747G>C, p.R249S	mutant expression	homozygous c.823delG, p.V275fsX1	null
	SUM149	homozygous c.711G>A, p.M237I	mutant expression	wild-type / mutation ?	wild-type / null ?
	MDA-MB-468	homozygous c.818G>A, p.R273H	mutant expression	homozygous c.253+1G>T, p.A72fsX5	null
	SUM159	homozygous c.472_473ins3, p.S158insS	mutant expression	wild-type	wild-type expression
	MDA-MB-453	homozygous c.991_1182del192, p.0	null	heterozygous c.919G>A, p.E307K	wild-type and mutant expression

Figure 2.1 - Meox1 is upregulated in TNBC; p53 and PTEN negatively regulate Meox1 expression. (A) Meox1 expression is upregulated in TNBC cell lines of MDA-MB-231, BT-549, SUM149, MDA-MB-468, SUM159, and MDA-MB-453. No Meox1 expression is seen in luminal cell lines of MCF-7, T-47D, and ZR-75-1. No Meox1 expression is also seen HER2-enriched cell lines of BT-474, MDA-MB-361, and ZR-75-30. (B and C) siRNA knockdown experiments of p53 and PTEN in normal immortalized MCF-10A and TNBC SUM159 cells show p53 and PTEN negatively regulate Meox1 expression. Most

significant increase in Meox1 expression is seen with dual knockdown of both tumor suppressor genes, compared to individual knockdown of either tumor suppressor gene alone. (D and E) Besides p53 and PTEN, HER2 also seems to negatively regulate Meox1 expression. While stable knockdown of PTEN significantly increases Meox1 expression in luminal MCF-7 and TNBC SUM159, stable overexpression of HER2 downregulates its expression. Furthermore, overexpression of HER2 prohibits Meox1 expression when PTEN is concurrently knocked down. (F) siRNA knockdown of p53 and/or PTEN in HER2-enriched BT-474 has no effect on Meox1 expression. Only when knocking down HER2 concurrently with p53 or PTEN does Meox1 expression start to increase. Most significant increase in Meox1 expression is seen when all three genes of HER2, p53, and PTEN are simultaneously knocked down. (G) Summary of p53 and PTEN genetic aberrations seen in different subtypes of *in vitro* breast cancer cell lines. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$)

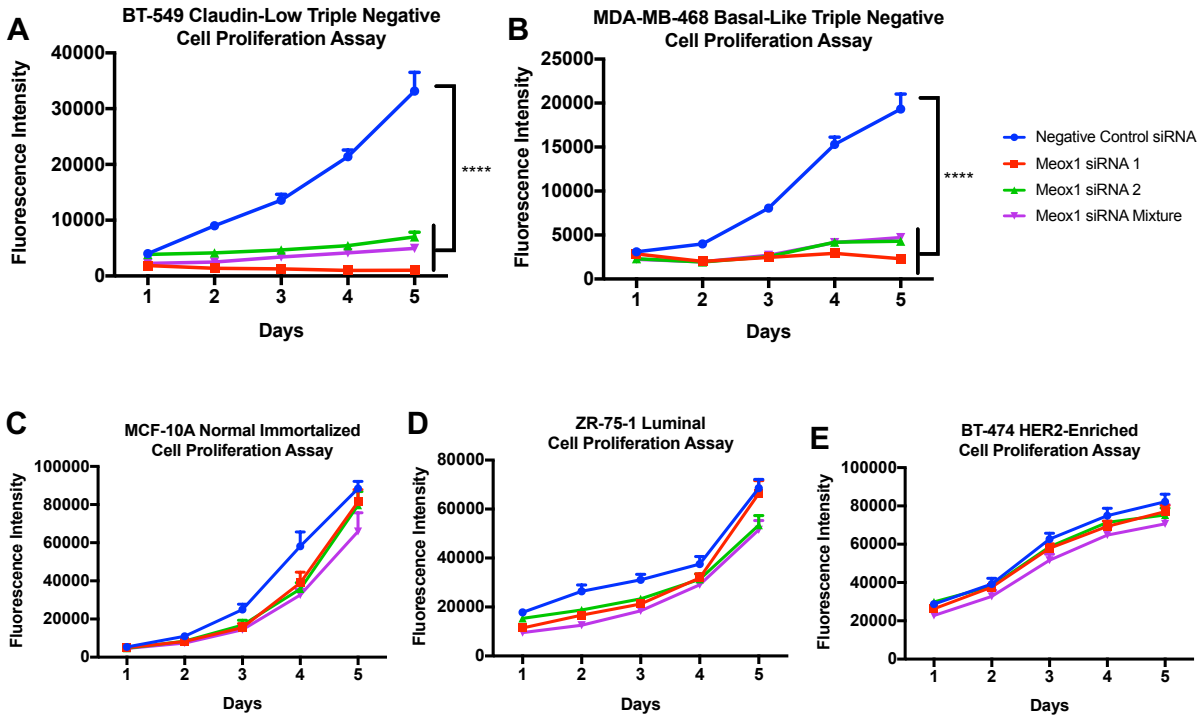


Figure 2.2 - Knockdown of Meox1 decreases *in vitro* cell proliferation of p53 and PTEN deficient TNBC. (A and B) Using three different siRNA treatments, Meox1 knockdown in claudin-low BT-549 and basal-like MDA-MB-468 significantly decreases cell proliferation of these p53 and PTEN deficient TNBCs. (C, D, and E) Using three different siRNA treatments, Meox1 knockdown in normal immortalized MCF-10A, luminal ZR-75-1, and HER2-enriched BT-474 shows minor decrease in cell proliferation and low potential of off-target effects. (****$p \leq 0.0001$)

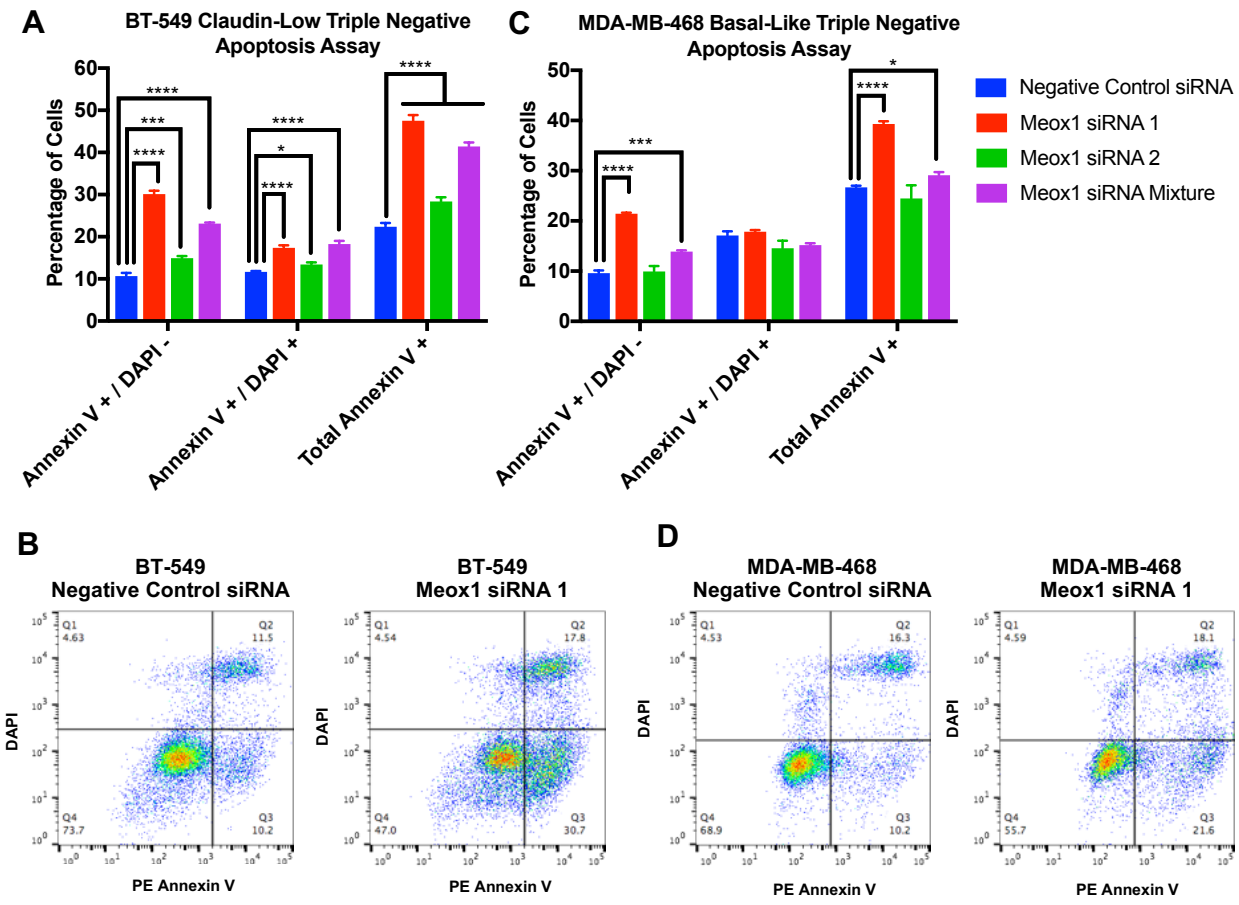


Figure 2.3 - Meox1 knockdown largely causes apoptosis in claudin-low p53 and PTEN TNBC. (A and B) Following 72 hours of Meox1 knockdown in claudin-low BT-549, three different siRNA treatments show significant increase in both Annexin V + / DAPI – and total Annexin V + positive cells. High levels of 20% increase in Annexin V + / DAPI – and 30% increase in total Annexin V + positive cells are observed following 72 hours of Meox1 siRNA 1 knockdown. (C and D) A less profound effect of apoptosis is seen in basal-like MDA-MB-468 cells. A relatively lower level of 10% increase in Annexin V + / DAPI – and 15% increase in total Annexin V + positive cells are evident after 72 hours of Meox1 siRNA 1 knockdown. (*p≤0.05, ***p≤0.001, ****p≤0.0001)

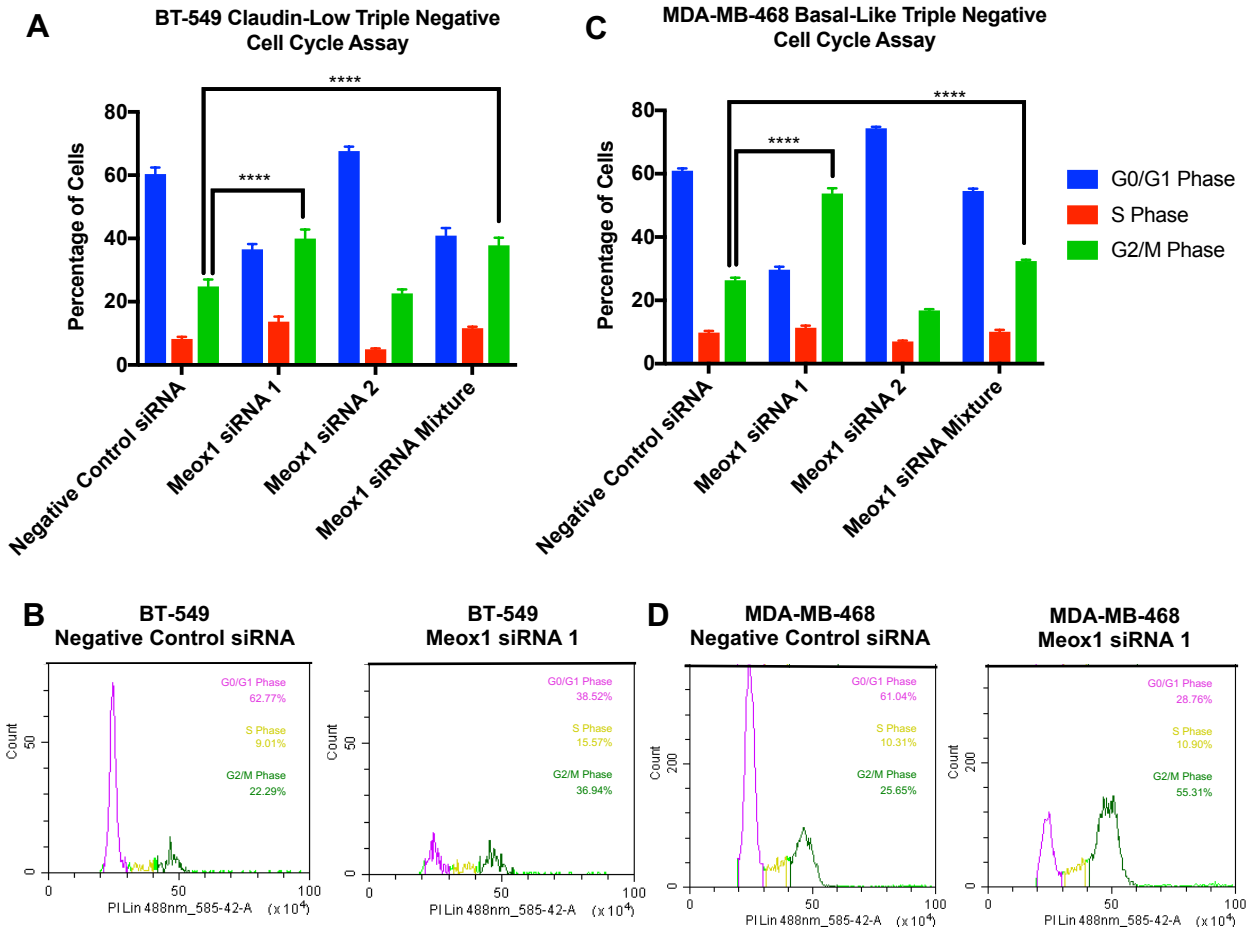


Figure 2.4 - Meox1 knockdown largely causes cell cycle arrest in basal-like p53 and PTEN deficient TNBC. (A and B) Compared to basal-like MDA-MB-468 cells, a less profound effect of cell cycle arrest is seen in claudin-low BT-549. A relatively lower level of 15% increase in G2/M cell cycle arrest is observed after 72 hours of Meox1 siRNA 1 knockdown. Looking at the flow cytometric PI histogram, it is evident that BT-549 cells are very unhealthy with Meox1 knockdown; this likely can be attributed to high levels of apoptosis exhibited within this cell line. (C and D) Higher levels of cell cycle arrest are seen in basal-like MDA-MB-468 cells, where following 72 hours of Meox1 siRNA 1 knockdown a 30% increase in the G2/M phase is observed. (****$p \leq 0.0001$)

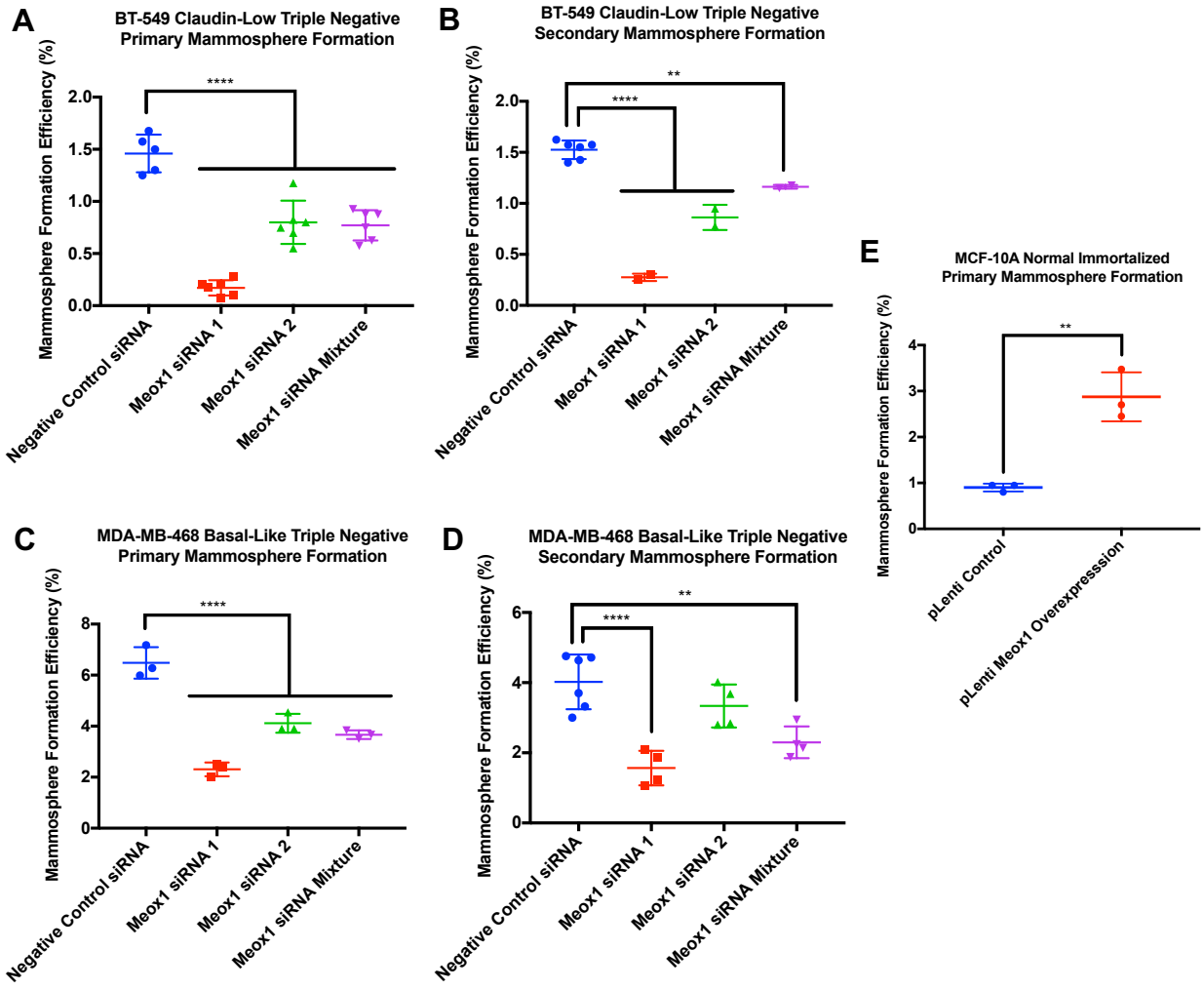
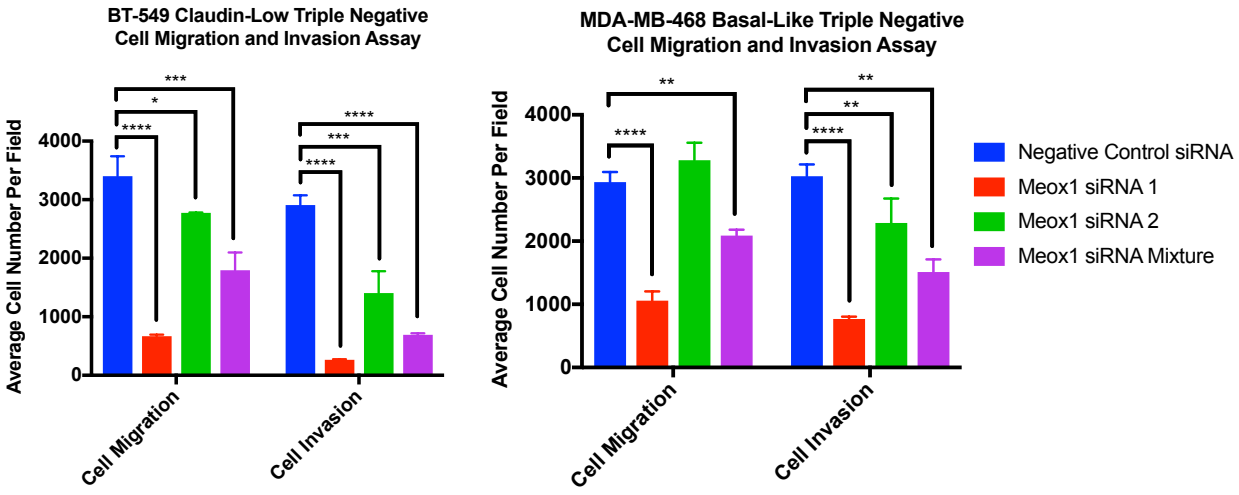


Figure 2.5 - Knockdown of Meox1 decreases self-renewal of p53 and PTEN deficient TNBC. (A, B, C, and D) Using different siRNA treatments, decrease in Meox1 expression significantly decreases primary and secondary mammosphere formations in both claudin-low BT-549 and basal-like MDA-MB-468 cells. As such, knockdown of Meox1 significantly decreases self-renewal properties of p53 and PTEN deficient TNBCs. (E) Appropriately, using normal immortalized MCF-10A cells for stable overexpression of Meox1 increases mammosphere formation, regulating increase in cell self-renewal. (**$p \leq 0.01$, ****$p \leq 0.0001$)



B

BT-549	% Invasion	Invasion Index
Negative Control siRNA	85.50	1
Meox1 siRNA 1	39.58	0.46
Meox1 siRNA 2	50.57	0.59
Meox1 siRNA Mixture	38.64	0.45

D

MDA-MB-468	% Invasion	Invasion Index
Negative Control siRNA	100	1
Meox1 siRNA 1	72.56	0.70
Meox1 siRNA 2	69.76	0.68
Meox1 siRNA Mixture	72.38	0.70

Figure 2.6 - Meox1 knockdown decreases cell migration and invasion of p53 and PTEN deficient TNBC. (A and B) Following 72 hours of Meox1 siRNA treatments, cell migration and invasion are significantly decreased in claudin-low BT-549. Invasion index decreases by approximately 50%. (C and D) Decrease in migration and invasion are also observed in basal-like MDA-MB-468 cells after 72 hours of Meox1 knockdown, although results are less profound compared to claudin-low BT-549. Invasion index decreases by approximately 30% in basal-like MDA-MB-468 cells. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$)

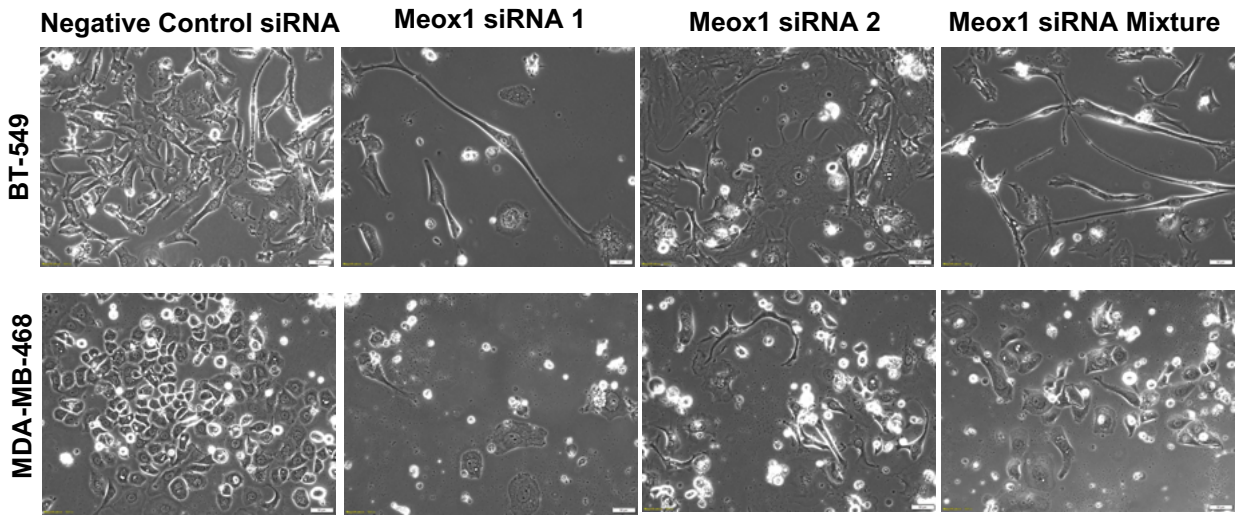


Figure 2.7 - Knockdown of Meox1 effects cellular morphology in p53 and PTEN deficient TNBC. Claudin-low BT-549 are mesenchymal cells with flattened bodies and sharp protrusions. Knocking down Meox1 using three different siRNA treatments show BT-549 cells forming longer and sharper cellular protrusions after 96 hours. Basal-like MDA-MB-468 cells exhibit more rounder pebble-like morphologies. After 96 hours of Meox1 knockdown, cells lose their pebble-like circularity displaying more flattened cell bodies and spindle-like protrusions.

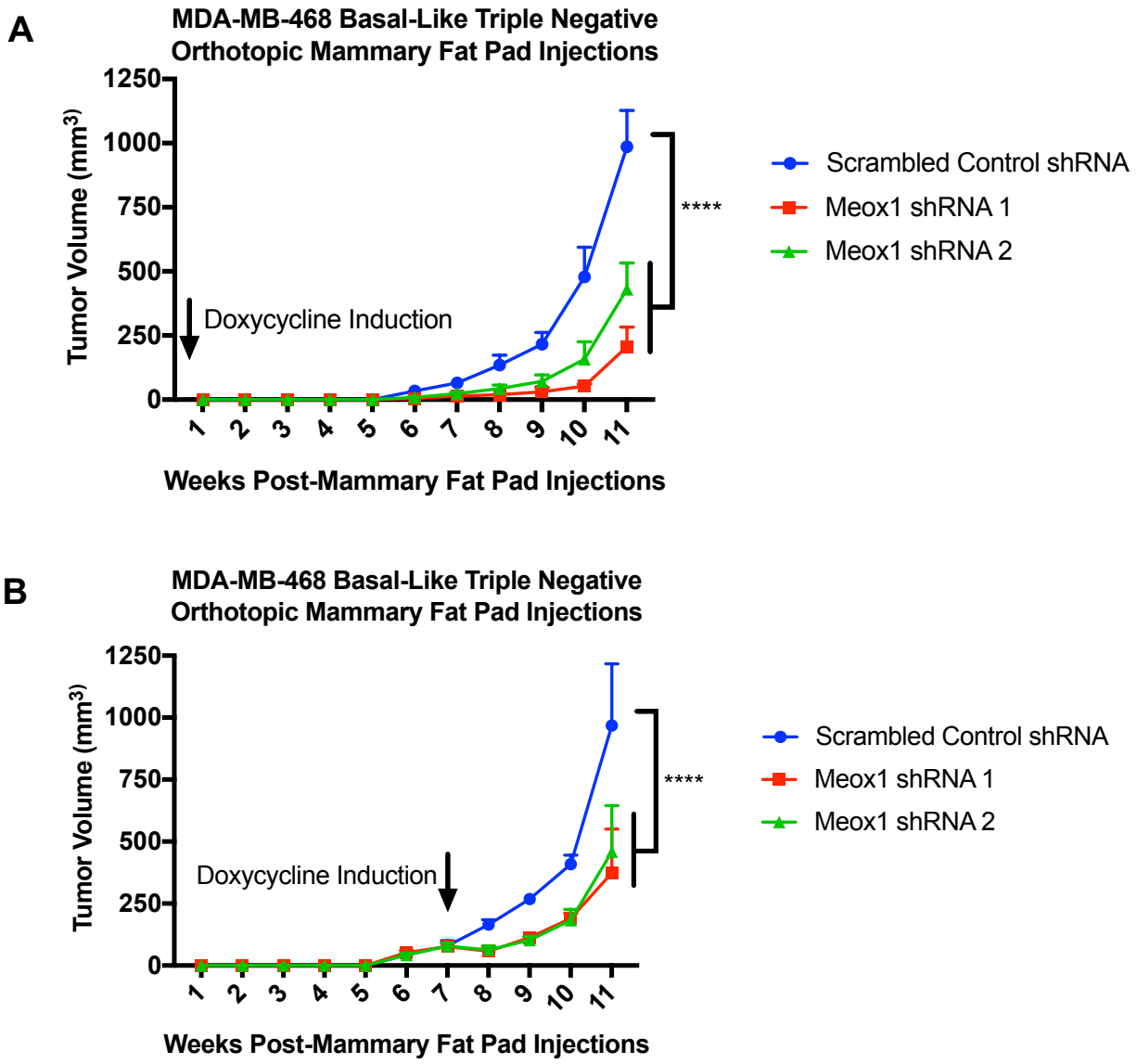


Figure 2.8 - Meox1 knockdown inhibits *in vivo* tumor growth of p53 and PTEN deficient TNBC. (A) In an adjuvant setting, Meox1 shRNA knockdown is induced with doxycycline 3 days after orthotopic mammary fat pad injections. (B) In a neoadjuvant setting, Meox1 shRNA knockdown is induced with doxycycline after palpable or visible tumor growth following orthotopic mammary fat pad injections. In both settings, knocking down Meox1 significantly decreases growth of MDA-MB-468 p53 and PTEN deficient TNBC xenograft tumors. However, a greater impact on decreasing tumor growth with Meox1 knockdown is seen in the adjuvant setting. (****$p \leq 0.0001$)

Chapter III

Meox1 Regulates Proliferation and Metastasis of Drug Resistant P53 and PTEN Deficient Triple Negative Breast Cancer Through Jak/Stat Signaling

Abstract

Mesenchyme homeobox 1 (Meox1) is specifically upregulated in triple negative breast cancer (TNBC) and negatively regulated by tumor suppressor genes of p53 and PTEN. Functional analysis of Meox1 shows it is an effective regulator of proliferation and metastasis in p53 and PTEN deficient TNBC. TNBC lacks specified targeted therapy known to improve long-term patient outcome of overall survival. As such, chemotherapy is the first line of treatment for patients with TNBC; however, a great majority are not sensitive to chemotherapeutic treatment and display drug resistance. TNBC properties of tumorigenesis and drug resistance are associated with frequent genetic aberrations in p53 and PTEN, of which combined inactivation is poorly understood. Homeobox transcription Meox1 not only offers insight into the complex molecular biology of p53 and PTEN deficient TNBC, but it also offers a specific therapeutic target for drug resistant p53 and PTEN deficient TNBC patients. A homeobox transcription factor mostly studied in the context of vertebrate development,

the mechanism of how Meox1 effectively regulates proliferation and metastasis in p53 and PTEN deficient TNBC is not well known. RNA sequencing and integrative pathway analyses offer mechanistic insight of Meox1 in p53 and PTEN deficient TNBC cells. Results show knockdown of Meox1 in claudin-low and basal-like p53 and PTEN deficient TNBC inactivates important canonical pathways involved in growth and survival as well as migration and invasion. Important canonical pathways inactivated in both cell lines include Stat3 and Jak/Stat signaling. Western blot analyses of the Jak/Stat pathway show Meox1 knockdown decreases Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low, but decreases Tyk2 and Stat6 protein levels in basal-like p53 and PTEN deficient TNBC. These results indicate Meox1 functional regulation of proliferation and metastasis in p53 and PTEN deficient TNBC may be conducted through the Jak/Stat mechanistic pathway, further elucidating the role of Jak/Stat signaling in TNBC.

Introduction

The Jak/Stat signaling pathway is comprised of Janus kinase (Jak) family of proteins Jak1, Jak2, Jak3, and tryrosine kinase 2 (Tyk2) as well as signal transducer and activator of transcription (Stat) family of proteins Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B, and Stat6 [1-4]. The Jak/Stat pathway is regulated throughout several steps in its signal transduction with suppressor of cytokine signaling (SOCS), protein inhibitor of activated Stat (PIAS), and protein tyrosine phosphatases (PTPs) [1-4]. Research shows

aberrations in Jak/Stat signaling promotes oncogenic properties of proliferation and metastasis such as increased cell growth, survival, anti-apoptosis, migration, invasion as well as angiogenesis and immune evasion [1-4].

Recent publications have investigated aberrations in Jak/Stat signaling in association with triple negative breast cancer (TNBC) [5, 6]. Balko and colleagues show TNBC patients harbor amplifications of Jak2, high frequencies of which are found in tumor samples displaying drug resistant residual disease following neoadjuvant chemotherapy [5]. Amplification of Jak2 in TNBC mediates Stat6 phosphorylation, activating stem cell characteristics [5]. Additionally, patients with TNBC and amplifications in Jak2 exhibit poorer recurrence-free and overall survival [5]. An alternative study demonstrating aberrations in Jak/Stat signaling in association with TNBC is shown by Kim and colleagues [6]. Their research demonstrates *in vitro* stable knockdown of p53 and PTEN in normal immortalized MCF-10A transforms cells to resemble basal-like and claudin-low TNBCs [6]. These cells manifest aggressive metastatic tumors in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice and exhibit increased epithelial to mesenchymal cancer stem cell characteristics via activation of the IL6-Stat3-NFkappaB signaling pathway, which is dependent on proteolytic degradation of SOCS3 [6].

While research is underway to elucidate the role of Jak/Stat signaling in TNBC, further investigation is required to gain more accurate mechanistic insight between Jak/Stat signaling in association with TNBC.

Lacking specified targets known to improve long-term patient outcome of overall survival, chemotherapy is the mainstay of treatment for patients with TNBC. Although

TNBC patients display sensitivity to chemotherapy, only 30% of TNBC exhibit pathological complete response following neoadjuvant chemotherapy [7]. The remaining majority of TNBC patients display drug resistant residual disease. Genetic aberrations that frequently manifest as key drivers of tumorigenesis and drug resistance in TNBC are tumor suppressor genes of p53 and PTEN [7-10]. The Cancer Genome Atlas Network reports TNBC patients to harbor the highest frequencies of p53 and PTEN genetic alterations, with 84% and 35%, respectively [8]. Combined alterations in both tumor suppressor genes of p53 and PTEN can be seen in approximately 20-30% of TNBC tumors [11, 12]. *In vitro* and *In vivo* studies show loss of wild-type function for p53 and PTEN induces the formation of TNBC-like tumors, exhibiting fast and aggressive properties of cancer hallmarks [6, 11, 12]. While the importance of tumor suppressor genes of p53 and PTEN are evident in TNBC, the molecular biology governing their concurrent loss of function is poorly understood. In Chapter II, the functional role of mesenchyme homeobox 1 (Meox1) offers insight into molecular biology of p53 and PTEN deficient TNBC, presenting a specific therapeutic target. Data shows Meox1 is upregulated in TNBC and negatively regulated by both tumor suppressor genes of p53 and PTEN. Knocking down Meox1 in p53 and PTEN deficient TNBC *in vitro* cell lines significantly decreases metastatic properties of cell migration and invasion. Furthermore, *in vitro* and *in vivo* knockdown experiments of Meox1 show decrease in expression of the transcription factor significantly decreases proliferation of p53 and PTEN deficient TNBC. Interestingly, this decrease in cellular proliferation is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like TNBC intrinsic subtypes.

Meox1 is a homeobox transcription factor predominantly studied for its role in somitogenesis throughout vertebrate development [13-15]. Only a few articles establish the role of Meox1 in tumor cell growth in ovarian and breast cancers [16, 17]. While in Chapter II the functional role of Meox1 is described in association with p53 and PTEN deficient TNBC, the mechanism of how Meox1 regulates p53 and PTEN deficient TNBC is not well known. In this chapter, the mechanistic role of Meox1 in p53 and PTEN deficient TNBC is investigated, divulging a molecular connection between Meox1 and Jak/Stat signaling. RNA sequencing is utilized to ascertain mechanisms involved in Meox1 function to regulate proliferation and metastasis. Integrative pathway analyses of RNA sequencing results show knockdown of Meox1 in p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468 inactivates important canonical pathways involved in growth and survival as well as migration and invasion. Interestingly, important canonical pathways inactivated include Stat3 and Jak/Stat signaling. Further investigation of the Jak/Stat pathway using western blot analyses reveal Meox1 knockdown has a significant impact on decreasing Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low BT-549, but only has a significant impact on decreasing Tyk2 and Stat6 protein levels in basal-like MDA-MB-468. These results demonstrate Meox1 function of proliferation and metastasis in p53 and PTEN deficient TNBC may be elicited through the Jak/Stat mechanistic pathway, further offering insight to the role of Jak/Stat signaling in TNBC.

Materials and Methods

In Vitro Cell Culture Growth Conditions

Recommended ATCC culture methods were followed to grow breast cancer cell lines *in vitro*. Although ATCC formulated media could not be used, Gibco Life Technologies base media was utilized. Required supplements were added to Gibco Life Technologies base media to match recommended ATCC culture methods. ATCC proposes the use of Leibovitz's L-15 media in an atmospheric gas exchange environment without CO_2 for certain cell lines, which was suggested for MDA-MB-468. Unfortunately, these conditions could not be met and other suitable alternatives were used.

BT-549 cells were grown in RPMI base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 0.85µg/mL insulin, 1mM sodium pyruvate, and 10mM HEPES. MDA-MB-468 cells were grown in DMEM base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, and 1mM sodium pyruvate. All cells were grown in a humidified 37^{o}C incubator with 5% CO_2.

In Vitro Cell Culture Transient Transfection for Small Interfering RNA (siRNA) Knockdown

Knockdown experiments using siRNA were conducted according to culture methods specified above. There was however one exception, antibiotic-antimycotic was not added to the media in order to prevent excessive toxicity to cells during transfection and to increase transfection efficiency. BT-549 and MDA-MB-468 cells were plated onto 6-well plates to achieve approximately 50% confluency per well. For both of these cell

lines this required plating $2x10^5$ cells per well. Following 24 hours of plating, cells were transfected using Invitrogen Lipofectamine RNAiMAX Reagent (#13778-150) with 50nM Negative Control siRNA and 50nM Meox1 siRNA Mixture. Transfection protocol was performed based on manufacturer's outlined methods. To prevent toxicity, cells were incubated with transfection reagent and siRNA for only 24 hours. After 24 hours of transfection, media was removed and fresh media was added without antibiotic-antimycotic. After 72 hours from start of transfection, cells were harvested for RNA or protein extraction.

Qiagen siRNA was purchased to conduct experiments. Negative Control siRNA (#1027281) contained target sequence 5'-CAGGGTATCGACGATTACAAA-3', sense strand 5'-GGGUAUCGACGAUUACAAAUU-3', and antisense strand 5'-UUUGUAAUCGUCGAUACCCUG-3'. Meox1 siRNA Mixture is four different Meox1 siRNAs pooled together for a final concentration of 50nM. This method allows the utilization of four different siRNAs each at low concentrations to ensure there are no off target affects that may be caused when using one siRNA alone. The Meox1 siRNA Mixture pools together 12.5nM of each Meox1 siRNA 1 (#SI00630266) with target sequence 5'-CAGGCTTGACTGGGTGGACAA-3', sense strand 5'-GGCUUGACUGGGUGGACAATT-3', and antisense strand 5'-UUGUCCACCCAGUCAAGCCTG-3'; Meox1 siRNA 2 (#SI00630280) with target sequence 5'-AAGCTAATTGTGCGAGCTCAA-3', sense strand 5'-GCUAAUUGUGCGAGCUCAATT-3', and antisense strand 5'-UUGAGCUCGCACAAUUAGCTT-3'; Meox1 siRNA 3 (#SI03145205) with target sequence 5'-AGCTGGCGACTCGGAAAGTAA-3', sense strand 5'-

CUGGCGACUCGGAAAGUAATT-3', and antisense strand 5'-UUACUUUCCGAGUCGCCAGCT-3'; and Meox1 siRNA 4 (#SI04293310) with target sequence 5'-TCCACGATTTCTGGATTGAAA-3', sense strand 5'-CACGAUUUCUGGAUUGAAATT-3', and antisense strand 5'-UUUCAAUCCAGAAAUCGUGGA-3'.

RNA Sequencing (RNA-Seq)

BT-549 and MDA-MB-468 cell lines were transfected with 50nM Negative Control siRNA and 50nM Meox1 siRNA Mixture. Two biological replicates were performed. Cells were harvested to extract RNA following 72 hours from start of transfection. Qiagen RNeasy Kit (#74104) was used to isolate RNA according to manufacturer's instructions. To afford better statistical analysis, both biological replicates along with the two technical replicates of each biological replicate were used for library preparation and sequencing RNA. Samples were submitted for library preparation and sequencing RNA at the University of Michigan DNA Core. Libraries were prepared for mRNA, non-strand-specific, using polyA-selection, approximately 120 nucleotide fragment lengths. RNA-seq was conducted using Illumina Sequencing with the HiSeq-4000 platform. Samples were multiplexed together and split into two lanes. Single-end sequencing was conducted with 50 nucleotide read lengths. Gene expression values were measured in reads per kilobase per million mapped reads (RPKM). Genes with average RPKM less than 1.0 across all the samples were removed from analysis. Calculations for $\log_2$(fold change), p-value, and false discovery rate (FDR) were conducted using edgeR in order

to ascertain differential gene expression between Meox1 knockdown versus negative control.

Integrative Pathway Analysis (IPA) was used to assess how differential gene expression between Meox1 knockdown versus negative control effects biological pathways. Using IPA, differential gene expressions were filtered with $\log_2$(fold change) > 1.5 and FDR < 0.05. To ensure statistically significant predicted pathways are analyzed, IPA advises the use of canonical pathway activated z-scores > 2 and canonical pathway inactivated z-scores of < -2. Further statistical significance is ensured by analyzing canonical pathways scoring p-values < 0.05.

Western Blots

BT-549 and MDA-MB-468 cell lines were transfected with 50nM Negative Control siRNA and 50nM Meox1 siRNA Mixture. Following 72 hours from start of transfection cells were washed with cold 1XPBS twice and harvested by scraping. Harvested cells were pelleted using centrifugation and Pierce RIPA Buffer (#89900) with Thermo Scientific protease inhibitor cocktail (#78410), Halt phosphatase inhibitor cocktail (#78420), and 5mM EDTA were used to lyse cells for protein extraction. Pierce BCA Assay Kit (#23227) was performed to ascertain protein concentrations. A total of 20ug of protein was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Nitrocellulose or PVDF membranes were utilized for protein transfers. Depending on the antibody, blocking was performed for one hour with either 5% milk or 5% BSA. Primary antibodies were incubated rotating overnight at 4^{o}C. Following several washes, appropriate secondary antibodies conjugated with horseradish peroxidase

(HRP) were added and incubated rotating for one hour at room temperature. After several washes, enhanced chemiluminescence (ECL) western blotting substrate was added and blots were imaged using film. Jak/Stat pathway antibodies were purchased from Cell Signaling Technology which were supplied as Stat Antibody Sampler Kit (#9939), Phospho-Stat Antibody Sampler Kit (#9914), Phospho-Jak Family Antibody Sampler Kit (#97999), and Jak/Stat Pathway Inhibitors Antibody Sampler Kit (#8343). Loading control was purchased from Santa Cruz Biotechnology beta-actin (#sc-47778). Manufacturer's instructions were followed for all antibodies in order to use specific concentrations required for optimal staining.

Results and Discussion

Meox1 regulates proliferative and metastatic pathways in p53 and PTEN deficient TNBC.

RNA-seq helps ascertain mechanisms involved in Meox1 function of regulating proliferation and metastasis in p53 and PTEN deficient TNBC. IPA is used to assess how RNA-seq differential gene expression between Meox1 knockdown versus negative control effects biological pathways in p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468 cells. Differential gene expressions are filtered with $\log_2$(fold change) > 1.5 and FDR < 0.05. To ensure significance in assessing canonical pathways activated and inactivated upon knockdown of Meox1, cutoff z-score values of > 2 and less than < -2 are considered for accurate prediction, respectively. In addition to

using z-score, the most statistically significant canonical pathways with p-values < 0.05 that are perturbed after Meox1 knockdown are further filtered.

RNA-seq comparison analysis of p53 and PTEN deficient claudin-low BT-549 and basal-like MDA-MB-468 shows important biological pathways involved in growth and survival as well as migration and invasion are inactivated in both cell lines upon knockdown of Meox1 (Figure 3.1). This includes well-known canonical pathways involving Stat3 as well as Jak/Stats, MAPK, p70S6K, CXCR4, actin cytoskeleton signaling, integrin signaling, G-alpha-q, RhoA and Rho family of GTPases. These pathways offer insight to potential mechanistic transductions involved in Meox1 regulation of proliferation and metastasis in these p53 and PTEN deficient TNBC cell lines.

Meox1 regulates Jak/Stat signaling in p53 and PTEN deficient TNBC.

Whether looking at comparison analyses or individual analyses of p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468, a canonical pathway inactivated upon Meox1 knockdown is Stat3 signaling, with significant p-values < 0.05 and significant z-scores of -2.132 and -2.309, respectively (Figure 3.1, Figure 3.2A and 3.2B). Interestingly, in the comparison analysis, signaling of the Jak/Stat pathway as a whole is also disrupted in both cell lines (Figure 3.1). While perturbation of Jak/Stat signaling in claudin-low BT-549 has an insignificant inactivation z-score of -0.2, it has a significantly high p-value of 0.00000892. The reverse holds true for basal-like MDA-MB-468, with significant inactivation z-score of -2.714, but a close to significant p-value of 0.0597. Since these are computerized calculated predictions based on

differential gene expression upon Meox1 knockdown, it is worthwhile to not only further analyze the role of Stat3 but also Jak/Stat signaling altogether in p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468.

Western blot analysis of the four Jak family of proteins reveals knockdown of Meox1 significantly decreases Jak1 and Tyk2 protein levels in claudin-low BT-549, but only significantly decreases Tyk2 protein levels in basal-like MDA-MB-468 (Figure 3.3A). Analyzing the Stat family of proteins shows a moderate increase in Stat1, complete loss of Stat5, and significant decrease in Stat6 protein levels in claudin-low BT-549 (Figure 3.3B). However, a small decrease in Stat5 and significant decrease in Stat6 protein levels are seen in basal-like MDA-MB-468 (Figure 3.3B). Further analysis of phosphorylated Stats shows a significant decrease of P-Stat3 (Tyr705) in claudin-low BT-549, however no change in phosphorylated Stats are seen in basal-like MDA-MB-468 cells (Figure 3.4A and 3.4B). In analyzing inhibitors of the Jak/Stat signaling pathway, a moderate decrease of PIAS1 and moderate increase of PIAS4 is observed in claudin-low BT-549, but only a moderate increase in PIAS4 is evident in basal-like MDA-MB-468 (Figure 3.5A and 3.5B).

In summary, western blot analyses for the Jak/Stat pathway show Meox1 knockdown has a significant impact on decreasing Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low BT-549, but only has a significant impact on decreasing Tyk2 and Stat6 protein levels in basal-like MDA-MB-468. It is important to note that the moderate increase in Stat1 in claudin-low BT-549 and small decrease in Stat5 in basal-like MDA-MB-468 should not be overlooked upon knockdown of Meox1. While these results display the ability of Meox1 to regulate Jak/Stat signaling in p53 and

PTEN deficient TNBC, further detailed investigation is necessary to ascertain how the mechanistic interplay of change in Jak/Stat signaling proteins affect functions of proliferation and metastasis.

RNA-seq results support Meox1 functional role of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC.

Previous data in Chapter II has shown Meox1 knockdown largely causes apoptosis in claudin-low BT-549 but cell cycle arrest in basal-like MDA-MB-468 p53 and PTEN deficient TNBC. Using IPA to investigate claudin-low BT-549 and basal-like MDA-MB-468 individually, more defined differences between the two cell lines upon Meox1 knockdown are evident. In claudin-low BT-549, canonical pathways associated with cell death are significantly activated, such as apoptosis signaling and well-known mediators of cell death TNFR1/TNFR2 (Figure 3.2A). In basal-like MDA-MB-468, no striking activation of pathways associated with cell death occurs, however a great majority of the pathways disrupted are significantly associated with cell cycle arrest (Figure 3.2B). Furthermore, IPA comparison assessment of RNA-seq shows apoptosis signaling is activated in both claudin-low BT-549 and basal-like MDA-MB-468 cells upon Meox1 knockdown (Figure 3.1). However, activation of apoptosis signaling is more pronounced in BT-549 with near significant z-score of 1.964 and p-value of 0.000822, compared to MDA-MB-468 with z-score of 1.633 and p-value of 0. These RNA-seq results are in direct accordance with our previously presented functional data in Chapter II. Data in Chapter II shows upon Meox1 knockdown apoptosis largely dominates in claudin-low BT-549 with low proportions of cell cycle arrest observed; however, while low

proportions of apoptosis are observed in basal-like MDA-MB-468, cell cycle arrest largely manifests.

Furthermore, it is interesting to note that IPA comparison assessment of RNA-seq shows p53 signaling is activated in both claudin-low BT-549 and basal-like MDA-MB-468 cells upon Meox1 knockdown (Figure 3.1). However, activation of p53 signaling is more significant in BT-549 with higher significant z-score of 2.117 and p-value of 0.000000491, compared to MDA-MB-468 with z-score of 1.387 and p-value of 0.0025. While this requires further detailed investigation, we hypothesize increase in activation of p53 signaling can be attributed to increase in apoptosis and cell cycle arrest pathways seen upon Meox1 knockdown, pathways of which overlap and are commonly activated by wild-type p53 function.

Interestingly, canonical pathways in IPA also show opposite directions of activation and inactivation between p53 and PTEN deficient claudin-low BT-549 and basal-like MDA-MB-468. Such well-known canonical pathways include Wnt/beta-catenin and interferon signaling (Figure 3.1). Further investigation of these oppositely activated and inactivated pathways may offer mechanistic insight for reasons as to why different regulatory roles of apoptosis dominate in claudin-low BT-549 and cell cycle arrest dominate in basal-like MDA-MB-468 upon Meox1 knockdown; a discussion continued in Chapter IV.

Conclusion

Abnormalities in the Jak/Stat signaling pathway promote cancer characteristics of proliferation and metastasis including increased cell growth, survival, anti-apoptosis, migration, invasion as well as angiogenesis and immune evasion [1-4]. Researchers have recently examined the role of Jak/Stat signaling in TNBC [5, 6]. Data shows TNBC residual disease tumor samples displaying drug resistance to neoadjuvant chemotherapy harbor increased amplifications of Jak2, which mediates signaling via phosphorylation of Stat6 [5]. Jak2 amplifications in TNBC are associated with poor recurrence-free and overall survival [5]. Additionally, aberrations in Jak/Stat signaling are associated with loss of p53 and PTEN wild-type function [6]. Research demonstrates *in vitro* stable knockdown of p53 and PTEN in normal immortalized MCF-10A not only transforms cells to resemble basal-like and claudin-low TNBCs, but it also activates the IL6-Stat3-NFkappaB signaling pathway via proteolytic degradation of SOCS3 [6]. These transformed cells exhibit aggressive metastatic tumors when injected into mammary fat pads of NOD/SCID mice [6]. While such research is edifying, more investigation is required to gain further mechanistic insight of Jak/Stat signaling in TNBC.

Frequent genetic aberrations in p53 and PTEN are known to promote tumorigenesis and drug resistance in TNBC [7-10]. Although the molecular biology underlying the combined inactivation of both tumor suppressor genes is poorly understood, data in Chapter II shows homeobox transcription factor Meox1 to play a critical functional role in regulating proliferation and metastasis of p53 and PTEN deficient TNBC. To further elucidate the mechanistic role of Meox1 RNA-seq was utilized. Interestingly, RNA-seq mechanistic assessment using IPA demonstrates

Meox1 knockdown in claudin-low and basal-like p53 and PTEN deficient TNBC inactivates canonical pathways of Stat3 and Jak/Stat signaling in both cell lines. Further investigation of the Jak/Stat pathway using western blot analyses show Meox1 knockdown decreases Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low BT-549, but only decreases Tyk2 and Stat6 protein levels in basal-like MDA-MB-468. These results offer mechanistic insight into Meox1 functional regulation of proliferation and metastasis in p53 and PTEN deficient, further elucidating the role of Jak/Stat signaling in TNBC. While promising, more research is required to assess how the mechanistic crosstalk of Jak/Stat protein levels affect functions of proliferation and metastasis in p53 and PTEN deficient TNBC via Meox1 regulation.

Previous data in Chapter II has shown knockdown of Meox1 causes apoptosis in claudin-low but cell cycle arrest in basal-like p53 and PTEN deficient TNBC. RNA-seq assessment of alterations in canonical pathways shown in this chapter are in direct accordance with functional data seen in Chapter II. RNA-seq IPA assessment of claudin-low BT-549 shows canonical pathways associated with cell death are activated upon Meox1 knockdown, such as apoptosis signaling and cell death mediators of TNFR1/TNFR2. Basal-like MDA-MB-468 shows no striking activation in cell death, however the majority of canonical pathways altered are associated with cell cycle signaling. While knockdown of Meox1 considerably decreases cell proliferation in both cell lines of p53 and PTEN deficient TNBC, it is important to further investigate the distinct mechanistic roles underlying apoptosis in claudin-low and cell cycle arrest in basal-like intrinsic subtypes; a discussion continued in Chapter IV.

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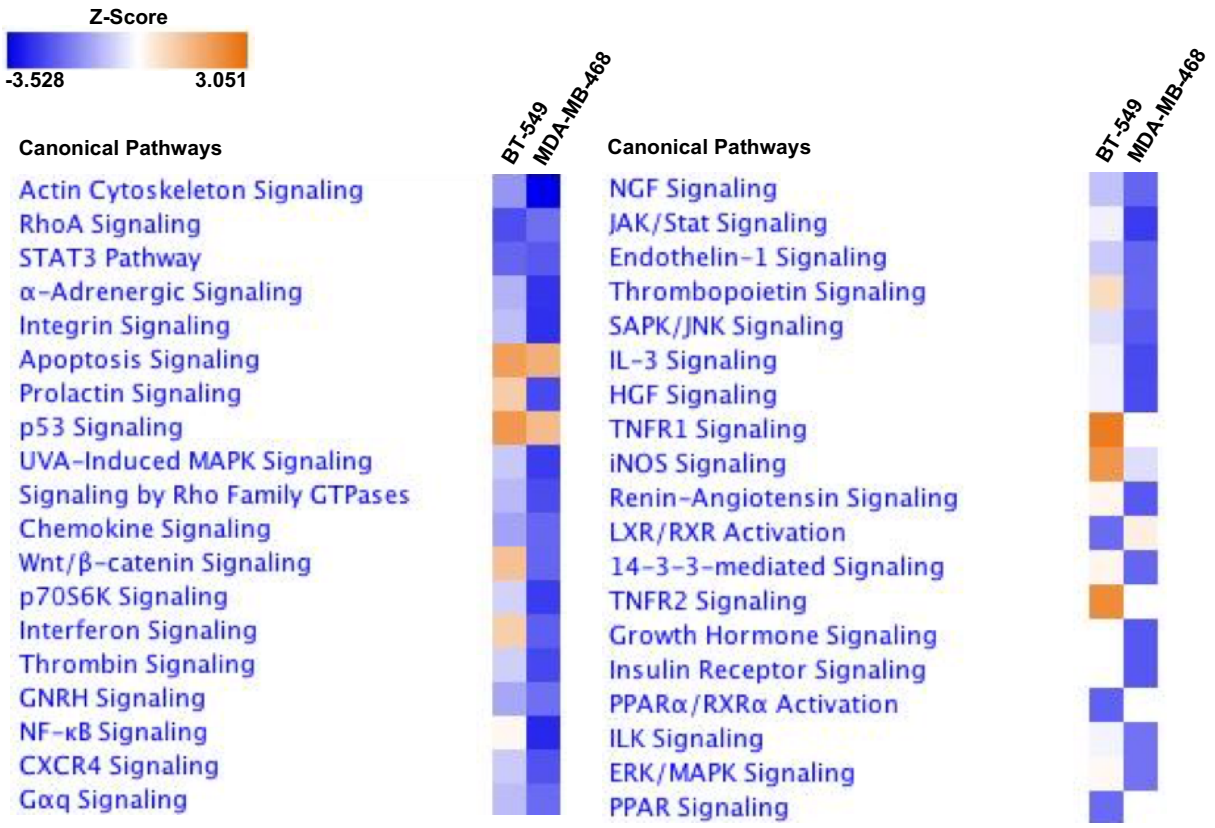

Figure 3.1 - Meox1 regulates proliferative and metastatic pathways in p53 and PTEN deficient TNBC. IPA is utilized to assess and compare biological pathways significantly altered in p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468 upon Meox1 knockdown. Alterations in biological pathways are analyzed based on differential gene expression between Meox1 knockdown versus negative control. Differential gene expressions are filtered according to $\log_2$(fold change) > 1.5 and FDR < 0.05. Predicted activated pathways (orange) with z-scores > 2 and predicted inactivated pathways (blue) with z-scores < -2 are analyzed as statistically significant. Results are further filtered to include statistically significant pathways with p-values < 0.05. Integrative pathway comparison analysis shows important canonical pathways effected upon Meox1 knockdown involve growth and survival for proliferation as well as migration and invasion for metastasis, seen in both p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468. (Although not all z-scores and p-values can be reported here, important canonical pathways mentioned have the following z-scores and p-values, respectively: BT-549 p53 signaling 2.117 and 0.000000491, MDA-MB-468 p53 signaling 1.387 and 0.0025, BT-549 apoptosis signaling 1.964 and 0.000822, MDA-MB-468 apoptosis signaling 1.633 and 0, BT-549 Stat3 signaling -2.132 and 0.0000378, MDA-MB-468 Stat3 signaling -2.309 and 0.0123, BT-549 Jak/Stat signaling -0.2 and 0.00000892, MDA-MB-468 Jak/Stat signaling -2.714 and 0.0597.)

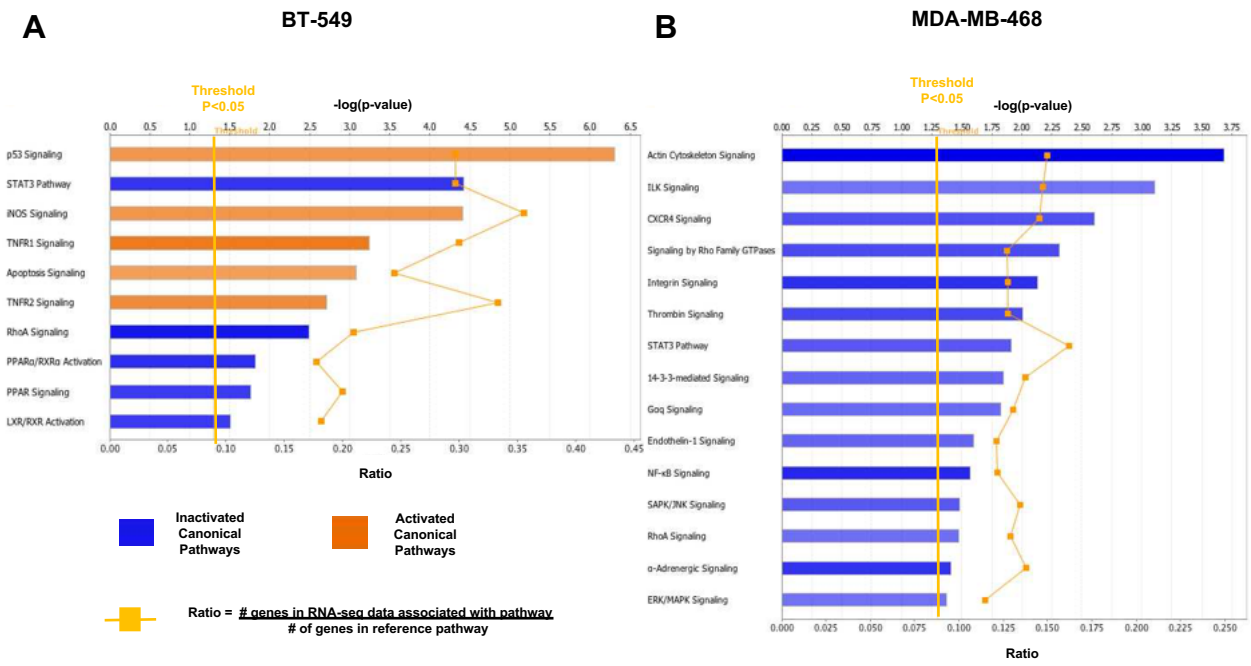

Figure 3.2 - The Stat3 pathway is significantly inactivated in both intrinsic subtypes of claudin-low and basal-like p53 and PTEN deficient TNBC. Additionally, RNA-seq results support Meox1 dominating functional roles of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC. (A) Individual IPA assessment of Meox1 knockdown in p53 and PTEN deficient claudin-low BT-549 shows profound activation of canonical pathways associated with cell death, such as apoptosis signaling and well-known mediators of cell death TNFR1/TNFR2. (B) Individual IPA assessment of Meox1 knockdown in p53 and PTEN deficient basal-like MDA-MB-468 shows no prominent activation of cell death, however a great majority of pathways disrupted are significantly associated with cell cycle arrest. (Differential gene expressions of Meox1 knockdown versus control with $\log_2$(fold change) > 1.5 and FDR < 0.05. Predicted activated pathways (orange) with z-scores > 2 and predicted inactivated pathways (blue) with z-scores < -2 are analyzed as statistically significant.)

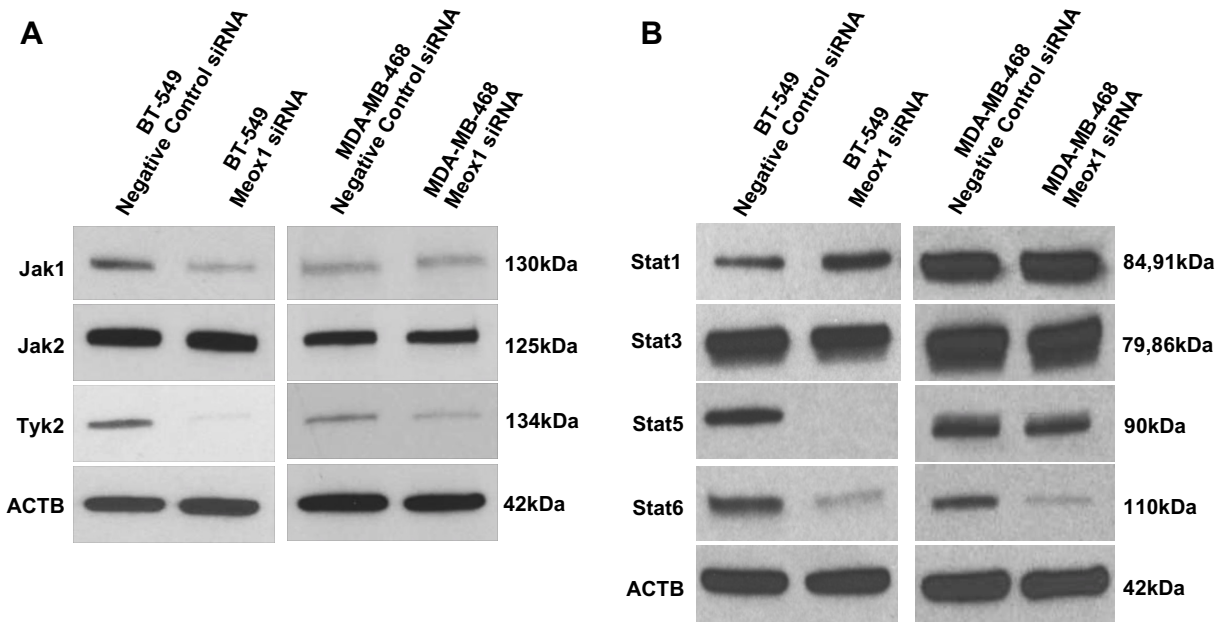

Figure 3.3 - Knockdown of Meox1 effects Jak and Stat family of proteins in p53 and PTEN deficient TNBC. (A) Knocking down Meox1 in claudin-low BT-549 significantly decreases Jak1 and Tyk2 protein levels, however in basal-like MDA-MB-468 only a significant decrease in Tyk2 is observed. (B) Knockdown of Meox1 in claudin-low BT-549 shows a moderate increase in Stat1, complete loss of Stat5, and significant decrease in Stat6 protein levels. In basal-like MDA-MB-468, moderate decrease in Stat5 and significant decrease in Stat6 protein levels are observed.

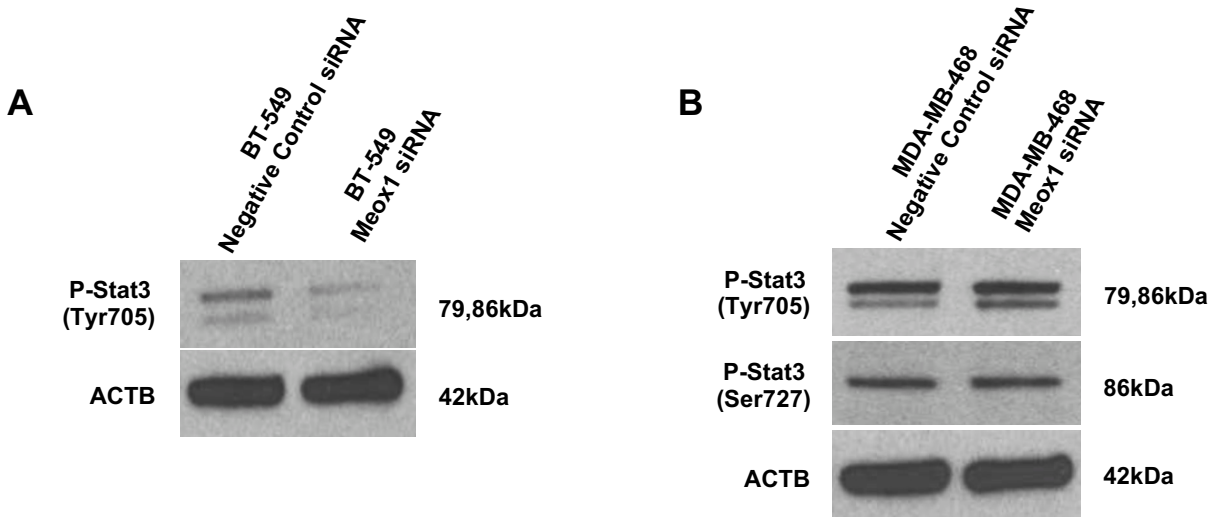

Figure 3.4 - Knockdown of Meox1 effects phosphorylation of Stat proteins in claudin-low p53 and PTEN deficient TNBC. (A) Knocking down Meox1 in claudin-low BT-549 shows a significant decrease P-Stat3 (Tyr705). (B) Knockdown of Meox1 in basal-like MDA-MB-468 shows no change in phosphorylated Stat proteins.

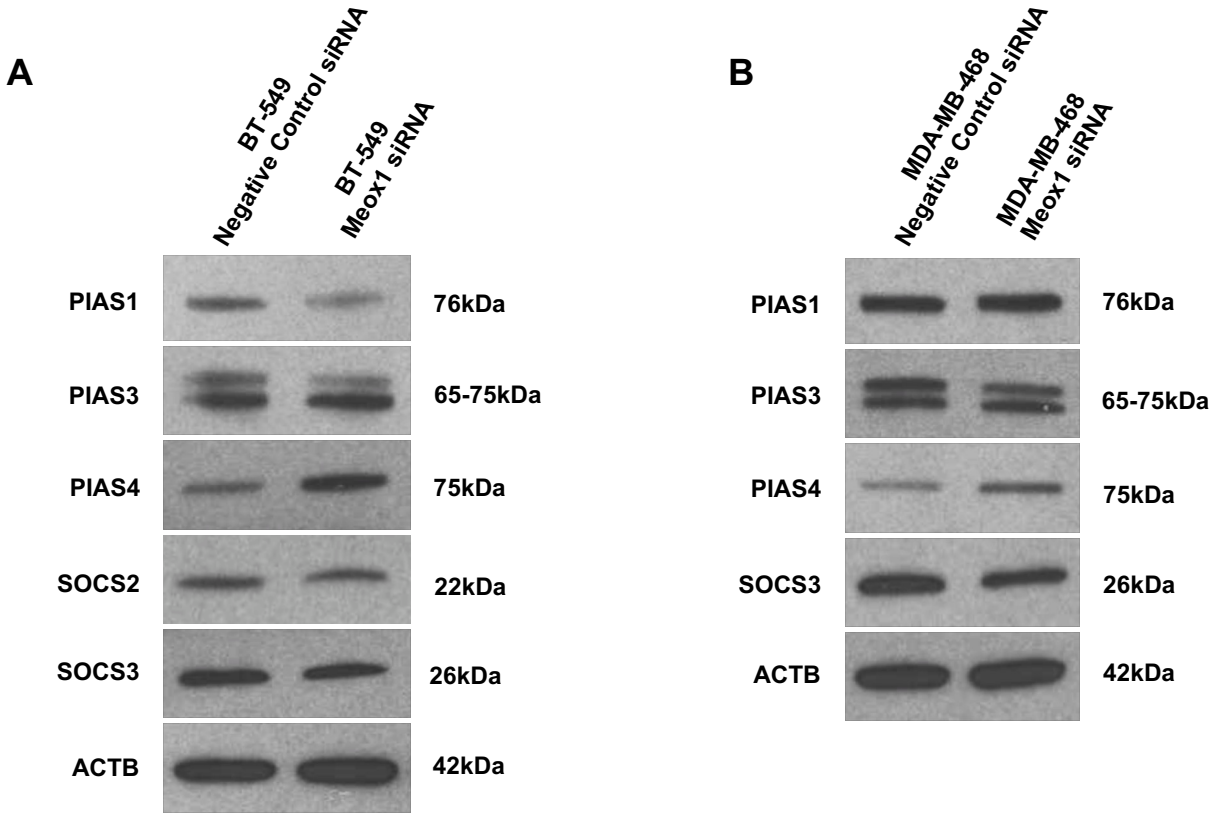

Figure 3.5 - Knockdown of Meox1 moderately effects inhibitors of Jak/Stat proteins in p53 and PTEN deficient TNBC. (A) Knocking down Meox1 in claudin-low BT-549 shows a moderate decrease of PIAS1 and moderate increase of PIAS4. (B) Knockdown of Meox1 in basal-like MDA-MB-468 only shows a moderate increase in PIAS4.

Chapter IV

Meox1 Displays Distinct Regulatory Roles in Different Subtypes of P53 and PTEN Deficient Triple Negative Breast Cancer

Abstract

In performing *in vitro* and *in vivo* assays, previous results have shown knockdown of mesenchyme homeobox 1 (Meox1) significantly decreases cellular proliferation of p53 and PTEN deficient triple negative breast cancer (TNBC). Interestingly, *in vitro* studies with Meox1 knockdown show this decrease in cellular proliferation is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Given the vast heterogeneity of TNBC it is no surprise that decrease in cellular proliferation upon knockdown of Meox1 can be ascribed to distinct mechanistic pathways in two different TNBC intrinsic subtypes. Furthermore, Meox1 may manifest distinct transcriptional roles in the different intrinsic subtypes of claudin-low and basal-like p53 and PTEN deficient TNBCs. Indeed, RNA sequencing results corroborate this hypothesis. RNA sequencing integrative pathway analyses have previously shown not all canonical pathways commonly overlap between the two different intrinsic subtypes upon Meox1 knockdown; canonical pathways show

opposite directions of activation and inactivation between claudin-low and basal-like p53 and PTEN deficient TNBCs. Further RNA sequencing comparison analyses of differentially expressed genes also show not all genes show common overlap between the different intrinsic subtypes following Meox1 knockdown. Results demonstrate only 35% of claudin-low and 53% of basal-like differentially expressed downregulated and upregulated genes commonly overlap. Additionally, a total of 58 genes show downregulation and upregulation in opposite directions in the two different TNBC intrinsic subtypes. Furthermore, Chapter III has shown Jak/Stat signaling is effected differently between claudin-low and basal-like TNBCs after knocking down Meox1, which may be responsible for the diverse biology of apoptosis in claudin-low and cell cycle arrest in basal-like cells. These results elucidate a potential for distinct Meox1 transcriptional regulation of Jak/Stat targets in two different p53 and PTEN deficient TNBCs. Insight of Meox1 transcriptional regulation is offered when comparing RNA and protein levels of Jak/Stats upon Meox1 knockdown; results indicate that RNA and protein levels do not always correspond. As such, Meox1 may be a direct or indirect transcriptional regulator of distinct Jak/Stat targets in different p53 and PTEN deficient TNBC subtypes, manifesting different mechanistic roles.

Introduction

Triple negative breast cancer (TNBC) is vastly heterogeneous and by itself can be considered a separate disease exhibiting distinct biological subtypes. Perou and

Sorlie conducted seminal gene expression profile studies identifying two intrinsic subtypes of TNBC as basal-like and claudin-low [1-7]. Lehmann and Burstein, however, argued that TNBC extensive heterogeneity is more accurately portrayed in four different subtype classifications [8-10]. While Lehmann identifies four TNBC classifications as basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), and luminal androgen receptor (LAR); Burstein identifies four TNBC classifications as basal-like immune-activated (BLIA), basal-like immunosuppressed (BLIS), mesenchymal (MES), and luminal androgen receptor (LAR) [8-10]. TNBC subtype classifications exhibit differences in incidence, biology, survival, and therapeutic response [1-10]. Classification of TNBC into distinct subtypes has important therapeutic applications, as precise characterization of TNBC functional and mechanistic heterogeneity can help ascertain specific therapeutic targets. Given the extensive heterogeneity of TNBC, more than one therapeutic option may be a necessity; different TNBC subtypes may need to be treated as separate diseases.

Lack of defined targets prohibits specified treatment of TNBC and its different subtypes, as such chemotherapy remains the mainstay of treatment for all subtypes of TNBC. Fortunately, patients with TNBC are responsive to chemotherapeutic treatment, exhibiting pathological complete response (pCR) rates higher than non-TNBC patients following neoadjuvant chemotherapy [9, 11]. Unfortunately, only 30% of TNBC patients exhibit pCR, the remaining majority display residual disease and drug resistance, ultimately displaying poorer long-term outcome [11-15]. Thus, there is an important need to identify specific therapeutic strategies against TNBC, in hopes of not only targeting different TNBC subtypes but to also help ameliorate patient's resistance to the

mainstay of chemotherapeutic treatment. Tumor suppressor genes of p53 and PTEN repeatedly manifest as crucial drivers of tumorigenesis and drug resistance in TNBC [12, 16-18]. Frequency of genetic aberrations in p53 and PTEN are highest in TNBC relative to other breast cancer subtypes, with 84% and 35%, respectively [16]. Concurrent aberrations in both tumor suppressor genes of p53 and PTEN are evident in approximately 20-30% of TNBC tumors [19, 20]. *In vitro* and *in vivo* studies show loss of wild-type function for both tumor suppressor genes induces the formation of faster and more aggressive TNBC-like tumors [19-21]. While the importance of p53 and PTEN are well established in TNBC, the molecular biology involved their concurrent loss of function is poorly understood.

In Chapter II, insight is offered into the complex molecular biology of p53 and PTEN deficient TNBC through mesenchyme homeobox 1 (Meox1). Data shows Meox1 is upregulated in TNBC and negatively regulated by tumor suppressor genes of p53 and PTEN. Knockdown experiments with Meox1 show the transcription factor to be an important functional regulator of proliferation and metastasis in p53 and PTEN deficient TNBC. Knocking down Meox1 not only significantly decreases migration and invasion, but it also significantly decreases cell proliferation *in vitro* and *in vivo*. Interestingly, decrease in cell proliferation is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Given the vast heterogeneity of TNBC tumors, it is no surprise that functional decrease in cellular proliferation can be ascribed to distinct mechanistic pathways in the different subtypes of claudin-low and basal-like cells. Meox1 transcriptional regulation may vary between the two different p53 and PTEN deficient TNBCs, governing diverse signaling pathways downstream upon

knockdown. It is important to mechanistically investigate the distinct dominating functional roles involved in decreasing cellular proliferation in the different subtypes of p53 and PTEN deficient TNBC. Evaluating the diverse downstream mechanistic pathways and transcriptional targets altered upon knockdown of Meox1 may help further comprehend the extensive heterogeneity of TNBC and its diverse subtypes, which may help afford more distinct specified targeting.

In this chapter, RNA sequencing is used to analyze how differentially expressed genes upon Meox1 knockdown govern distinct roles of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Results indicate that not all differentially expressed genes commonly overlap between the claudin-low and basal-like intrinsic subtypes following Meox1 knockdown. Upon investigation of downregulated and upregulated genes, only 35% of claudin-low and 53% of basal-like differentially expressed genes show common overlap. Furthermore, a total of 58 genes show downregulation and upregulation in opposite directions between the two different TNBC intrinsic subtypes. Investigating non-overlapping and opposite overlapping distinct genes may offer insight to the different regulatory roles of Meox1 in different TNBC subtypes. Furthermore, a prospective analysis of Meox1 transcriptional targets is also discussed in this chapter when comparing changes in Jak/Stat RNA levels to changes in Jak/Stat protein levels upon Meox1 knockdown. In Chapter III, RNA sequencing and western blot analyses show knockdown of Meox1 effects Jak/Stat signaling. In this chapter, results show that RNA and protein levels of Jak/Stat targets do not always correspond upon Meox1 knockdown, as such Meox1 may be a direct or indirect transcriptional regulator of Jak/Stat signaling. Interestingly, data also shows Jak/Stat

signaling is effected differently in claudin-low and basal-like p53 and PTEN deficient TNBCs. As such, Meox1 may have distinct direct or indirect transcriptional roles for Jak/Stat regulation in different TNBC subtypes, which may be responsible for the diverse biology of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC.

Materials and Methods

In Vitro Cell Culture Growth Conditions

ATCC recommended culture conditions were utilized to grow *in vitro* breast cancer cell lines. Suggested ATCC formulated media could not be used, as such base media from Gibco Life Technologies was purchased. For complete media, necessary supplements required by ATCC were added to Gibco Life Technologies base media in order to match recommended growth conditions. According to ATCC, some cell lines require the use of Leibovitz's L-15 media in an atmospheric gas exchange environment without CO_2, which was suggested for MDA-MB-468. Although these conditions were not possible for accommodation in our lab, other suitable alternatives were substituted.

BT-549 cells were grown in RPMI base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 0.85µg/mL insulin, 1mM sodium pyruvate, and 10mM HEPES. MDA-MB-468 cells were grown in DMEM base media supplemented with antibiotic-antimycotic, 10% fetal bovine serum, and 1mM sodium pyruvate. All cells were grown in a humidified 37^{o}C incubator with 5% CO_2.

Hemagglutinin Tagging and Plasmid Based Cloning by Polymerase Chain Reaction (PCR)

Meox1 was tagged with hemagglutinin (HA) on its C-terminal end using PCR based plasmid cloning. The following primers were designed to tag Meox1 on its C-terminal end by using an Meox1 plasmid template: forward primer 5'-TAAGCAGCTAGCGCCACCATGGATCCCGCGGCCAGCAGCTGC-3' and reverse primer 5'-TGCTTACTCGAGTCAAGCGTAATCTGGAACATCGTATGGGTATCCGCCACCTCCGCCACCCTCTGAACTTGGAGAGGCTGT-3'. New England BioLabs Phusion High-Fidelity DNA Polymerase (#M0530S) was used to amplify and tag Meox1 via PCR from template plasmid Abmgood pLenti-GIII-CMV-hMeox1-RFP-2A-Puro (#LV217710); PCR protocol was followed according to manufacturer's instructions. PCR product with Meox1 C-terminal HA tag was purified using Invitrogen PureLink Quick PCR Purification Kit (#K310001). PCR purified product of Meox1 C-terminal HA and recipient plasmid Abmgood pLenti-GIII-CMV-hMeox1-RFP-2A-Puro (#LV217710) were digested with NheI and XhoI. Digests were run on an agarose gel and gel purified using Invitrogen PureLink Quick Gel Extraction Kit (#K210012). DNA ligation was conducted using New England BioLabs T4 DNA Ligase (#M0202S) to fuse digested PCR purified Meox1 C-terminal HA product with digested recipient plasmid Abmgood pLenti-GIII-CMV-hMeox1-RFP-2A-Puro (#LV217710). Ligated plasmid was transformed into Invitrogen DH5-alpha competent cells (#18265-017) and plated for overnight growth on kanamycin LB agar plates. Colonies were picked and grown overnight in kanamycin LB for plasmid

isolation. Plasmids were isolated and purified using Invitrogen PureLink Quick Plasmid Miniprep Kit (#K210011); after which plasmids were sent for sequencing to verify Meox1 C-terminal HA tag. Sequence verified Meox1 C-terminal HA tag plasmid was used for viral packaging with Addgene pMD2.G (#12259) and Addgene psPAX2 (#12260); viral packaging plasmids were gifts from Didier Trono. Both BT-549 and MDA-MB-468 cells were virally transduced with Abmgood pLenti-GIII-CMV-RFP-2A-Puro Meox1 C-terminal HA and Abmgood pLenti-CMV-RFP-2A-Puro-Blank Control (#LV591), here on after labeled as pLenti Meox1 C-HA and pLenti Control, respectively. Cells were infected with viral particles for 24 hours using 8ug/mL polybrene. After 48 hours from start of infection, selection for infected cells was conducted using 2ug/mL of puromycin.

Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

Approximately 15 million BT-549 and MDA-MB-468 transduced cells were used for chromatin immunoprecipitation. Cell Signaling Technology Simple ChIP Plus Sonication Kit (#56383) was used to perform the experiment. While the entire protocol was followed according to manufacturer's instructions, one extra fixation step was added in the beginning, which was using Diagenode ChIP Cross-Link Gold (#C01019027) before formaldehyde fixation. Formaldehyde fixation is most affective in cross-linking proteins directly bound to DNA; Diagenode ChIP Cross-Link Gold (#C01019027) fixation is commonly used before formaldehyde fixation to ensure proteins in the transcriptional complex not directly bound to DNA are also properly fixed. To perform this additional fixation step, cells were washed twice with 1X PBS, Diagenode ChIP Cross-Link Gold (#C01019027) was used as the first fixative for 30

minutes at room temperature according to manufacturer's instructions, cells were washed twice with 1X PBS, and 1% formaldehyde was used as the second fixative for 10 minutes at room temperature. Protocol was then followed as specified by Cell Signaling Technology Simple ChIP Plus Sonication Kit (#56383).

Sonication was performed using the Covaris S2 System. Instrument program was set as follows: duty cycle 5%, intensity 2, cycles per burst 200, cycle time 60 seconds, cycles 10 or 15, temperature of water bath 4^{o}C, power mode frequency sweeping, and degassing mode continuous. ChIP grade Cell Signaling Technology HA-Tag Rabbit mAb antibody (#3724) was used for immunoprecipitation.

In Vitro Cell Culture Transient Transfection for Small Interfering RNA (siRNA) Knockdown

Transfecting breast cancer cell lines with siRNA was conducted with growth culture conditions specified above. To help increase transfection efficiency and prevent unnecessary toxicity to cells during transfection, antibiotic-antimycotic was not added to the complete media. BT-549 and MDA-MB-468 cells were plated to obtain 50% confluency per well in 6-well plates. Plating of $2x10^5$ cells per well was required for both these cell lines to achieve 50% confluency. Cells were allowed to grow 24 hours after plating, after which cells were transfected using Invitrogen Lipofectamine RNAiMAX Reagent (#13778-150) with 50nM Negative Control siRNA and 50nM Meox1 siRNA Mixture. Protocol to perform transfection was conducted according manufacturer's guidelines. To avoid toxicity, media was removed and fresh media again without

antibiotic-antimycotic was added after 24 hours of transfection with siRNA. Following 72 hours from start of transfection, RNA was extracted for analysis.

To perform experiments, Qiagen siRNA was purchased. Negative Control siRNA (#1027281) contained target sequence 5'-CAGGGTATCGACGATTACAAA-3', sense strand 5'-GGGUAUCGACGAUUACAAAUU-3', and antisense strand 5'-UUUGUAAUCGUCGAUACCCUG-3'. Meox1 siRNA Mixture was used by pooling four different Meox1 siRNAs to a final concentration of 50nM. Combining four different siRNAs together allows the ability to use multiple siRNAs each at low concentrations to ensure minimal off target affects that may be caused when using one siRNA alone. The Meox1 siRNA Mixture pools together 12.5nM of each Meox1 siRNA 1 (#SI00630266) with target sequence 5'-CAGGCTTGACTGGGTGGACAA-3', sense strand 5'-GGCUUGACUGGGUGGACAATT-3', and antisense strand 5'-UUGUCCACCCAGUCAAGCCTG-3'; Meox1 siRNA 2 (#SI00630280) with target sequence 5'-AAGCTAATTGTGCGAGCTCAA-3', sense strand 5'-GCUAAUUGUGCGAGCUCAATT-3', and antisense strand 5'-UUGAGCUCGCACAAUUAGCTT-3'; Meox1 siRNA 3 (#SI03145205) with target sequence 5'-AGCTGGCGACTCGGAAAGTAA-3', sense strand 5'-CUGGCGACUCGGAAAGUAATT-3', and antisense strand 5'-UUACUUUCCGAGUCGCCAGCT-3'; and Meox1 siRNA 4 (#SI04293310) with target sequence 5'-TCCACGATTTCTGGATTGAAA-3', sense strand 5'-CACGAUUUCUGGAUUGAAATT-3', and antisense strand 5'-UUUCAAUCCAGAAAUCGUGGA-3'.

RNA Sequencing (RNA-Seq)

Performing two biological replicates, siRNA transfections were conducted using BT-549 and MDA-MB-468 cells with 50nM Negative Control siRNA and 50nM Meox1 siRNA Mixture. Using Qiagen RNeasy Kit (#74104), RNA was isolated from cells following 72 hours from start of transfection. RNA isolation protocol was followed according manufacturer's guidelines. For statistical significance, two technical replicates of each biological replicate were submitted for library preparation and sequencing RNA at the University of Michigan DNA Core. Libraries were prepared for mRNA, non-strand-specific, using polyA-selection, approximately 120 nucleotide fragment lengths. Illumina Sequencing with the HiSeq-4000 platform was used for sequencing RNA. Samples were multiplexed together and split into two lanes. Single-end sequencing was conducted with 50 nucleotide read lengths. Reads per kilobase per million mapped reads (RPKM) was used to measure gene expression. Genes with RPKM average values of less than 1.0 across all samples were excluded from analysis. To conduct calculations for differential gene expression between Meox1 knockdown versus negative control, edgeR was used to compute $\log_2$(fold change), p-value, and false discovery rate (FDR).

Integrative Pathway Analysis (IPA) was used to generate venn diagrams; differential gene expressions between Meox1 knockdown versus negative control were filtered with $\log_2$(fold change) > 1.5 and FDR < 0.05. Broad Institute Morpheus was utilized to generate heatmaps for differential genes meeting filtering criteria of $\log_2$(fold change) > 1.5 and FDR < 0.05. Values of $\log_2$(1+RPKM) were used for heatmap

generation. Rows were hierarchically clustered using one minus Pearson correlation metric.

Results and Discussion

Meox1 has different regulatory roles in claudin-low and basal-like p53 and PTEN deficient TNBC.

Previous data has shown Meox1 knockdown elicits apoptosis in claudin-low BT-549 but cell cycle arrest in basal-like MDA-MB-468 p53 and PTEN deficient TNBC. To further assess the distinct roles of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC, changes in differentially expressed genes upon Meox1 knockdown are compared between the different intrinsic subtypes. RNA-seq data shows that upon knockdown of Meox1 there are 2,972 claudin-low BT-549 and 1,963 basal-like MDA-MB-468 genes significantly altered with $\log_2$(fold change) > 1.5 and FDR < 0.05 (Figure 4.1A). While differential expression of these genes shows important canonical pathways of proliferation and metastasis to be significantly perturbed in both BT-549 and MDA-MB-468, not all downregulated or upregulated genes show common overlap between the two cell lines. Only 35% of claudin-low BT-549 and 53% of basal-like MDA-MB-468 affected genes show common overlap of downregulation and upregulation (Figure 4.1B and 4.1C). Interestingly, RNA-seq data shows 58 genes to be downregulated and upregulated in opposite directions within the two cell lines (Figure 4.1D and 4.1E, Figure 4.2). Studying the functional and

mechanistic specificity of these opposite overlapping genes may offer mechanistic insight to the different functional dominating roles of apoptosis in claudin-low BT-549 and cell cycle arrest in basal-like MDA-MB-468 observed upon Meox1 knockdown. Additionally, investigating non-overlapping distinct genes in the two different subtypes of p53 and PTEN deficient TNBC may help further discern the diverse mechanistic pathways involved in the different functional roles.

Meox1 may directly or indirectly regulate Jak/Stat signaling in p53 and PTEN deficient TNBC.

Previous data has shown knockdown of Meox1 effects Jak/Stat signaling in p53 and PTEN deficient TNBC. While significant changes in Jak/Stat protein levels are observed upon Meox1 knockdown, it is important to assess whether these changes in protein levels correspond with changes seen at the RNA level. Examining this association can offer insight into potential transcriptional targets of Meox1, as levels of RNA transcription influence levels of protein translation. However, it is also quite common for RNA and protein levels to not match, as multiple separate mechanisms are involved for regulation of their expression.

Previously shown, western blot analysis for the Jak/Stat pathway shows Meox1 knockdown has a significant impact on decreasing protein levels of Jak1, Tyk2, Stat5, and Stat6 in claudin-low BT-549 TNBC, but only has a significant impact on decreasing Tyk2 and Stat6 protein levels in basal-like MDA-MB-468. It is also importantly noted that the moderate increase in Stat1 in claudin-low BT-549 and small decrease in Stat5 in basal-like MDA-MB-468 should not be overlooked upon knockdown of Meox1.

It is evident that knockdown of Meox1 affects Jak/Stat signaling differently in claudin-low BT-549 and basal-like MDA-MB-468. As such, Meox1 may have distinct transcriptional regulatory roles of Jak/Stat targets in the different p53 and PTEN deficient subtypes of TNBC. Thus, differences in transcriptional regulation of Jak/Stat signaling upon Meox1 knockdown can potentially contribute to the distinct mechanistic pathways of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC.

Comparing changes of protein levels and RNA levels upon Meox1 knockdown, RNA-seq data shows that not all Jak/Stats may be direct transcriptional targets of Meox1. In claudin-low BT-549 significant decrease in Jak1 RNA levels are evident, no significant change of Tyk2 RNA levels are seen, and a small but significant decrease in Stat5B and Stat6 RNA levels are observed (Figure 4.3A). In basal-like MDA-MB-468 significant decreases in both Tyk2 and Stat6 RNA levels are evident (Figure 4.3B). Furthermore, both cell lines show significant increase in Stat1 RNA levels (Figure 4.3A and 4.3B). These results indicate that Meox1 may directly or indirectly regulate these Jak/Stat proteins. Aside from being a direct transcriptional regulator, Meox1 can either physically interact with Jak/Stats to regulate function and protein stability, or Meox1 can transcribe expression of other targets that may regulate Jak/Stat protein expression (Figure 4.4A, 4.4B, and 4.4C). ChIP-seq analysis is further required to obtain conclusive results for Meox1 transcriptional regulation in different subtypes of p53 and PTEN deficient TNBCs.

<u>Meox1 transcriptional targets can be enriched with C-terminal HA tagging.</u>

Unfortunately, ChIP-seq results are unavailable for this dissertation as sequencing results did not make it in time. However, optimized protocol shows the experiment can be successfully conducted to ascertain Meox1 transcriptional targets with C-terminal HA tagging. A ChIP grade antibody for Meox1 is unavailable to perform ChIP-seq, as such the transcription factor is tagged with HA on the C-terminal end. HA tagging using plasmid based cloning shows both claudin-low BT-549 and basal-like MDA-MB-468 cells successfully express HA at the appropriate size of 28kDa, which is the protein size of Meox1 (Figure 4.5A).

Next to optimization of fixation time, one of the most difficult parameters for ChIP-seq is optimizing settings for sonication. Both fixation and sonication times can affect transcription factor target enrichment. Agarose gels show both 10 and 15 minute sonications produce DNA fragments of which a great majority are below 500bp (Figure 4.5B). To ascertain which sonication time improves percent input, as well as confirm the validity of ChIP, real-time quantitative PCR (RT-qPCR) is performed. Results from the positive control histone H3 immunoprecipitation followed by RT-qPCR using RPL30 primers show 10 minute sonication produces higher levels of percent input, especially in BT-549 and MDA-MB-468 cells transduced with pLenti Meox1 C-HA (Figure 4.6A and 4.6B). As such, 10 minute sonication will likely improve enrichment of Meox1 transcriptional targets. Unfortunately, there are no known transcriptional targets of Meox1 in breast cancer, consequently RT-qPCR validation of ChIP cannot be performed for Meox1 target enrichment. However, results from both the positive and negative controls confirm the validity of the ChIP protocol. Thus, successful sequencing

data can be obtained using BT-549 pLenti Meox1 C-HA and MDA-MB-468 pLenti Meox1 C-HA with ChIP grade HA-Tag Rabbit mAb.

Conclusion

TNBC is a vastly heterogeneous disease that can be divided into several different subtypes [1-10]. Each subtype of TNBC displays differences in incidence, biology, survival, and therapeutic response [1-10]. Accurate classification of TNBC subtypes presents important therapeutic implications, as comprehensive characterization of their functional and mechanistic heterogeneity can help ascertain specific targets for therapy. Given that each subtype manifests vast distinctions, different treatment options may be a necessary to treat individual TNBC subtypes as separate diseases.

Lack of distinct targeted therapies prevents specified treatment of TNBC and its individual subtypes, consequently chemotherapy is the main form of treatment for all TNBC subtypes. While some TNBC patients are responsive to chemotherapy exhibiting pCR rates of 30% after neoadjuvant treatment, a great majority are unable to achieve pCR displaying residual disease and drug resistance [9, 11, 12]. Targeted therapeutic options are in dire need to not only treat TNBC and its specific subtypes, but to also treat patients resistant to the mainstay of chemotherapeutic treatment. Tumor suppressor genes of p53 and PTEN exhibit frequent genetic aberrations in TNBC, conferring a selective advantage for tumorigenesis and drug resistance [12, 16-18]. The

underlying molecular biology for concurrent loss of function for both tumor suppressor genes is not well known.

Homeobox transcription factor Meox1 offers insight into the complex molecular biology of p53 and PTEN deficient TNBC. In Chapter II Meox1 function for regulating cell proliferation and metastasis is explained in the context of p53 and PTEN deficient TNBC. Interestingly, data shows knockdown of Meox1 manifests different mechanistic pathways of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Since TNBC is a vastly heterogeneous disease, it is no surprise that decrease in cellular proliferation upon Meox1 knockdown manifests distinct pathways in different intrinsic subtypes of claudin-low and basal-like. Additionally, regulation of transcription by Meox1 may vary between the different p53 and PTEN deficient TNBC subtypes, thus exhibiting distinct mechanistic signaling downstream upon knockdown. Analyzing Meox1 regulation of different mechanistic pathways and transcriptional targets in different TNBC subtypes will offer better insight into the vast heterogeneity of TNBC and its distinct subtypes, which in turn will help improve specified targeting.

In Chapter III, RNA-seq results demonstrate potential mechanistic pathways involved in Meox1 function to regulate proliferation and metastasis. While RNA-seq analysis shows important canonical pathways of proliferation and metastasis to be significantly perturbed, it is evident that not all canonical pathways or differentially expressed genes overlap upon knockdown of Meox1 in BT-549 and MDA-MB-468. Interestingly, a few canonical pathways showed opposite directions of activation and inactivation between claudin-low and basal-like intrinsic subtypes. Continued analysis of differentially expressed genes shows only 35% claudin-low and 53% basal-like

differentially expressed genes show common overlap of downregulation and upregulation. A total of 58 genes are also downregulated and upregulated in in opposite directions between the two cell lines. It is evident that while claudin-low BT-549 and basal-like MDA-MB-468 share common canonical pathways and common overlapping genes, the two cell lines also significantly manifest diverse mechanistic signaling and gene expressions following knockdown of Meox1. Investigating opposite activated/inactivated canonical pathways, opposite overlapping genes, and non-overlapping distinct genes may help further offer insight to the different regulatory roles of Meox1 in different TNBC subtypes, including ascertaining the different functional roles of apoptosis in claudin-low and cell cycle arrest in basal-like intrinsic subtypes observed upon Meox1 knockdown.

While in Chapter III RNA-seq and western blot analyses have shown knockdown of Meox1 effects Jak/Stat signaling, it is evident that Jak/Stat signaling is regulated differently between the intrinsic subtypes of claudin-low and basal-like p53 and PTEN deficient TNBCs. This suggests that Meox1 may have different transcriptional roles and targets for Jak/Stat proteins in different subtypes of TNBCs. Different transcriptional regulation of Jak/Stats by Meox1 may govern the distinct roles of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Previous results show Meox1 knockdown decreases protein levels of Jak1, Tyk2, Stat5, and Stat6 in claudin-low BT-549 TNBC, but only decreases Tyk2 and Stat6 protein levels in basal-like MDA-MB-468. When comparing protein and RNA levels following Meox1 knockdown, it is evident they do not always correspond. As such, Meox1 may be a direct or indirect transcriptional regulator of Jak/Stat targets. Through indirect regulation,

Meox1 can transcribe other targets that can regulate Jak/Stats or it can physically interact with Jak/Stats to regulate function and protein stability. Further analyses with ChIP-seq will offer more profound insight into the mechanistic and transcriptional regulatory roles of Meox1 in different intrinsic subtypes of p53 and PTEN deficient TNBC.

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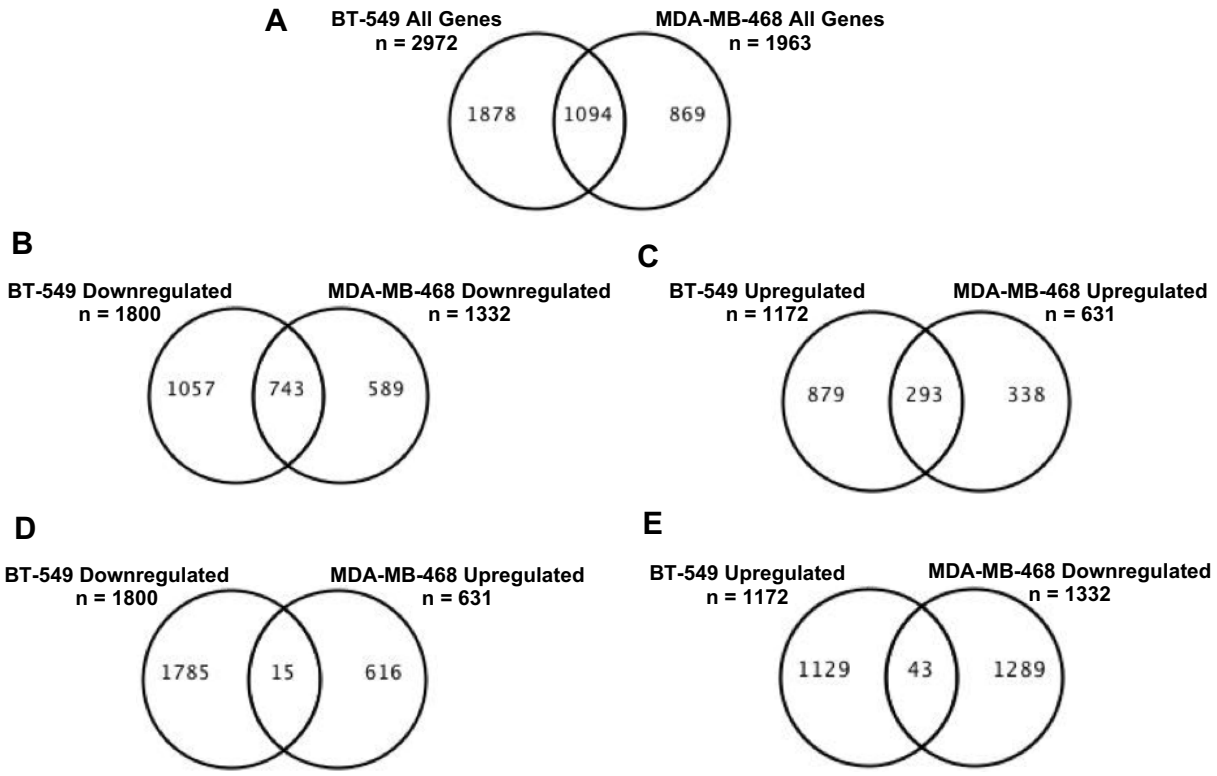


Figure 4.1 - Venn diagrams representing common overlapping, non-overlapping, and oppositely overlapping genes upregulated and downregulated after Meox1 knockdown in claudin-low and basal-like p53 and PTEN deficient TNBC. (A) Total number of genes significantly downregulated and upregulated following Meox1 knockdown with $\log_2$(fold change) > 1.5 and FDR < 0.05 in claudin-low BT-549 and basal-like MDA-MB-468. (B and C) A total number of 743 downregulated and 293 upregulated genes overlap between BT-549 and MDA-MB-468. However, common overlap of these downregulated and upregulated genes only represents 35% [(743+293) / 2972] of claudin-low BT-549 and 53% [(743+293) / 1963] of basal-like MDA-MB-468 genes affected. (D and E) Interestingly, 58 genes (15+43) are upregulated and downregulated in opposite directions in BT-549 and MA-MB-468. Further research into oppositely overlapping as well as non-overlapping genes may offer insight into the diverse mechanistic pathways involved in different Meox1 dominating roles of apoptosis in claudin-low BT-549 and cell cycle arrest in basal-like MDA-MB-468.

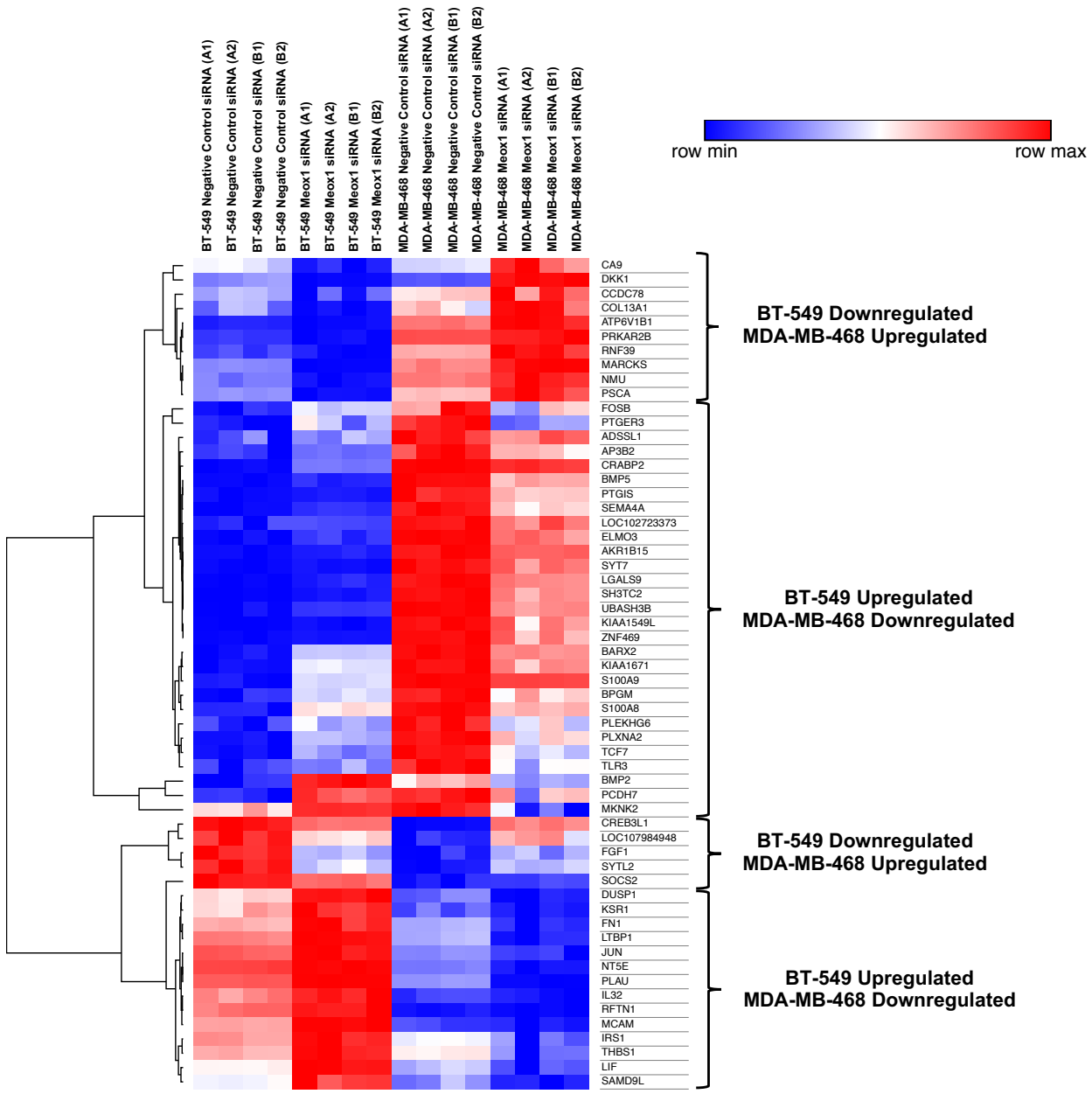
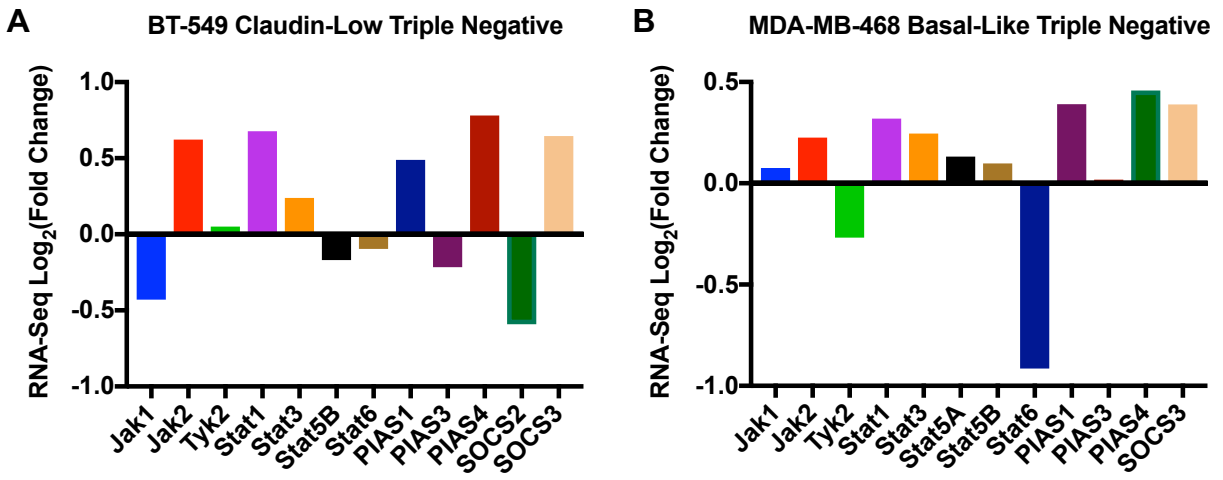


Figure 4.2 - Heatmap representing the 58 genes upregulated and downregulated in opposite directions following Meox1 knockdown in claudin-low BT-549 and basal-like MDA-MB-468 p53 and PTEN deficient TNBC. Hierarchical clustering was applied to rows, with one minus Pearson correlation metric. Genes represented have $\log_2$(fold change) > 1.5 and FDR < 0.05, heatmap was generated using $\log_2$(1 + RPKM) values. Analysis of of these 58 oppositely regulated genes may help ascertain the functional and mechanistic specificity involved in the largely diverse roles of apoptosis in claudin-low BT-549 and cell cycle arrest in basal-like MDA-MB-468 seen after Meox1 knockdown.



BT-549	Log_2(Fold Change)	p-value	FDR
Jak1	-0.428783136	1.03E-48	8.29E-48
Jak2	0.622764926	5.45E-40	3.59E-39
Tyk2	0.050613496	0.276094213	0.324608227
Stat1	0.677406764	1.49E-122	4.16E-121
Stat3	0.238379003	1.45E-15	4.33E-15
Stat5B	-0.169525681	0.000350423	0.000577816
Stat6	-0.096133902	0.00348515	0.005259431
PIAS1	0.488925344	9.15E-23	3.54E-22
PIAS3	-0.215974045	5.71E-10	1.34E-09
PIAS4	0.780779449	3.52E-55	3.28E-54
SOCS2	-0.590059476	3.80E-27	1.71E-26
SOCS3	0.645241101	2.33E-48	1.86E-47

MDA-MB-468	Log_2(Fold Change)	p-value	FDR
Jak1	0.075242359	0.382891703	0.486518525
Jak2	0.225331061	0.038031743	0.07199119
Tyk2	-0.267732592	0.004558469	0.011516685
Stat1	0.318879073	4.13E-05	0.000171427
Stat3	0.245250736	0.003476932	0.009076362
Stat5A	0.132398074	0.174839201	0.259213026
Stat5B	0.098329085	0.271149424	0.369909858
Stat6	-0.914075691	3.25E-28	1.81E-26
PIAS1	0.39120132	4.77E-05	0.000194574
PIAS3	0.013306874	0.902594574	0.934063233
PIAS4	0.458447316	0.000102655	0.000389586
SOCS3	0.388942408	0.001366171	0.003957186

Figure 4.3 - RNA-seq data showing differential gene expression of the Jak/Stat family upon Meox1 knockdown versus negative control. As previously shown at a protein level, knockdown of Meox1 effects Jak/Stat signaling in p53 and PTEN deficient TNBC. Looking at corresponding changes in RNA and protein levels in claudin-low BT-549 and basal-like MDA-MB-468 may offer insight into potential Jak/Stat transcriptional targets of Meox1 in different p53 and PTEN deficient TNBC subtypes.

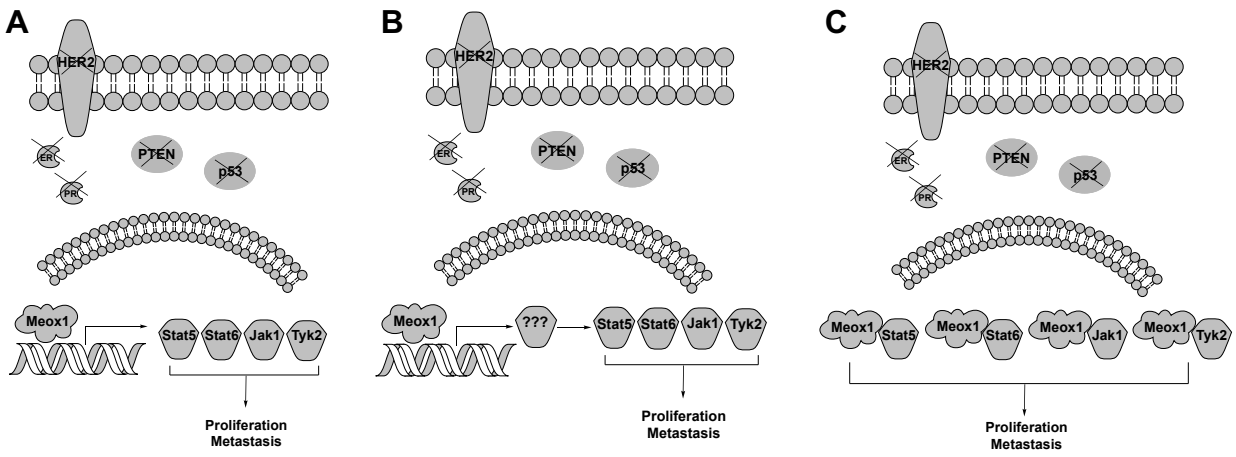


Figure 4.4 - Meox1 may directly or indirectly regulate Jak/Stat signaling in p53 and PTEN deficient TNBC. (A) Meox1 may serve as a direct transcriptional regulator for Jak/Stats. (B) Meox1 may transcribe expression of other targets that regulate Jak/Stats. (C) Meox1 may physically interact with Jak/Stats to regulate function and protein stability.

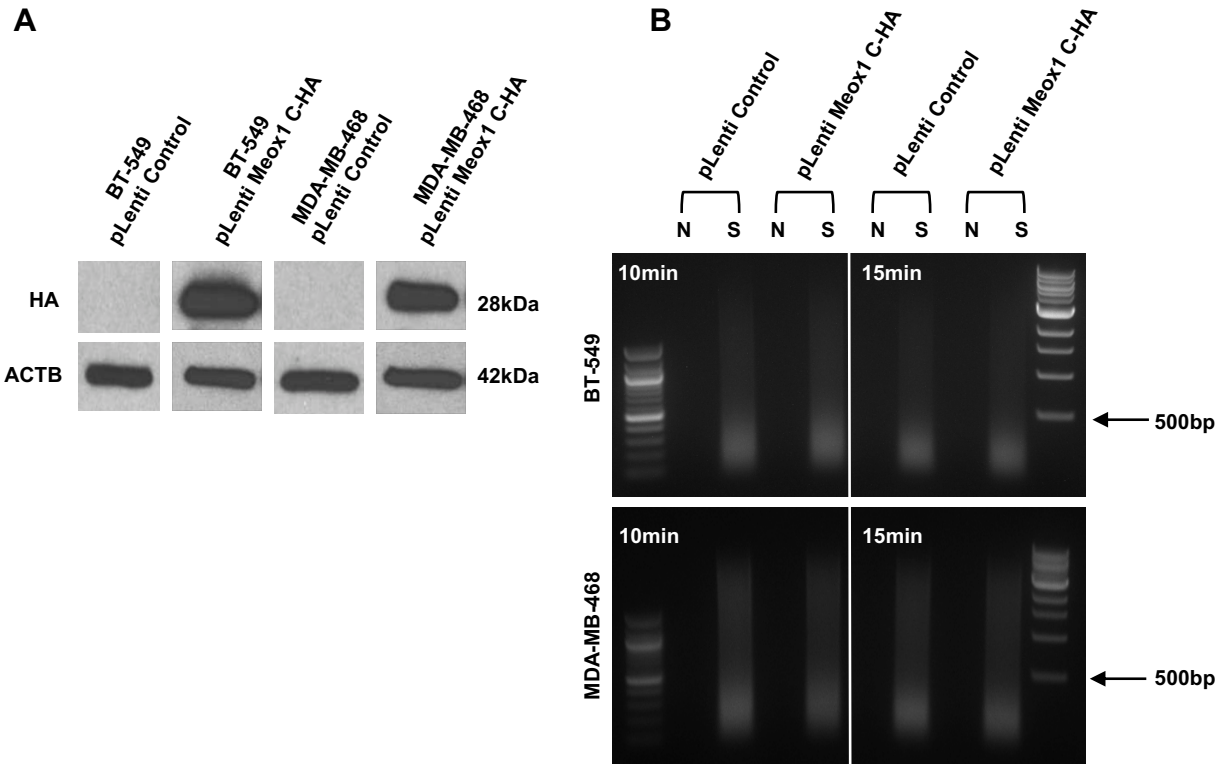


Figure 4.5 - Meox1 transcriptional targets can be enriched with C-terminal HA tagging. (A) Both p53 and PTEN deficient TNBC cell lines of claudin-low BT-549 and basal-like MDA-MB-468 successfully express HA at 28kDa, which is the size of Meox1 protein. As such, western blot analysis shows Meox1 is successfully tagged with HA in p53 and PTEN deficient TNBC cell lines. (B) Optimization of sonication for p53 and PTEN deficient TNBC cell lines of claudin-low BT-549 and basal-like MDA-MB-468. Both sonication times of 10 minutes and 15 minutes are able to successfully fragment the majority of DNA below 500bp in size.

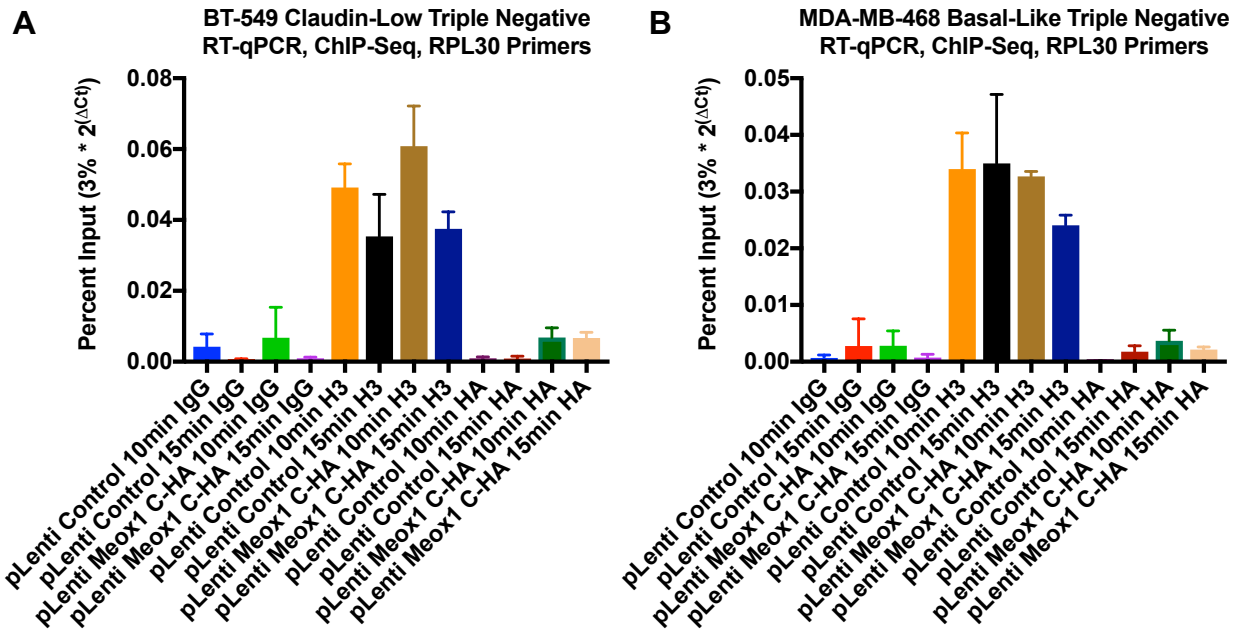


Figure 4.6 - RT-qPCR analysis is performed to verify ChIP protocol and ascertain optimal sonication time. ChIP protocol was performed using Normal Rabbit IgG as a negative control, Histone H3 Rabbit mAb as positive control, and HA-Tag Rabbit mAb as a test. Only positive control Histone H3 Rabbit mAb should enrich for the RPL30 gene. BT-549 pLenti Control and MDA-MB-468 pLenti Control are transduced control cell lines with no Meox1 C-HA expression. BT-549 pLenti Meox1 C-HA and MDA-MB-468 pLenti Meox1 C-HA are transduced cell lines overexpressing Meox1 tagged with HA on its C terminal end. Sonication times of 10 and 15 minutes are conducted to ascertain optimal sonication time required for increased ChIP target enrichment. (A and B) Both p53 and PTEN deficient TNBCs of claudin-low BT-549 and basal-like MDA-MB-468 show 10 minute sonication produces higher levels of percent input, especially in cells transduced with pLenti Meox1 C-HA. Validity of the ChIP protocol is verified, all negative controls show little to no enrichment for the RPL30 gene and positive controls for Histone H3 ChIP show specific enrichment for RPL30.

Chapter V

Conclusion

Triple negative breast cancer (TNBC) constitutes approximately 10-20% of the total breast cancer subtype population [1]. Compared to other breast cancer subtypes, TNBC displays more aggressive clinical behavior and worse patient prognosis [2, 3]. TNBC tumors are characterized as highly proliferative, displaying larger overall mean size and higher grade upon diagnosis [2, 3]. Lacking overexpression for all three biomarkers of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER2), TNBC is the only subtype that lacks targeted therapy to improve long-term patient outcome of overall survival. Consequently, chemotherapy is the mainstay of treatment for patients with TNBC. Fortunately, TNBC patients are sensitive to chemotherapeutic treatment, exhibiting pathological complete response (pCR) rates higher than non-TNBC patients following neoadjuvant chemotherapy [4, 5]. Unfortunately, only 30% of TNBC patients achieve pCR, the remaining vast majority exhibit residual disease and drug resistance [6]. Due to the aggressive nature of TNBC, patients displaying residual disease drug resistance inevitably experience recurrence, distant metastasis, and mortality within 5 years of diagnosis [3, 4]. As such, there is not only a dire need to develop specified therapeutic

options for patients with TNBC, but there is also a dire need to apply these therapeutic strategies to patients who display resistance to the mainstay of chemotherapeutic treatment.

Genetic aberrations that repeatedly manifest as key drivers of TNBC tumorigenesis and drug resistance are tumor suppressor genes of p53 and PTEN [6-9]. Relative to other breast cancer subtypes, TNBC tumors exhibit the highest frequencies of p53 and PTEN genetic aberrations, with 84% and 35%, respectively [7]. Combined aberrations in both tumor suppressor genes of p53 and PTEN are displayed in approximately 20-30% of TNBC tumors [10, 11]. *In vivo* studies show loss of wild-type function for p53 and PTEN in mammary epithelia induces the formation of TNBC-like tumors, exhibiting fast and aggressive cancer hallmark characteristics [10, 11]. Mice containing tumors with combined loss of p53 and PTEN exhibit a gene set enrichment profile for increased proliferation and migration [10, 11]. Additionally, human TNBC tumors show patients with decreased p53 and PTEN expressions display worse prognosis with poorer metastasis-free survival compared to patients with normal p53 and PTEN levels [10]. Moreover, Balko and colleagues show genetic aberrations in p53 and PTEN are frequent in TNBC drug resistant residual disease tumor samples [6].

No actionable targets exist for TNBC patients harboring combined loss of p53 and PTEN wild-type function. While the importance of p53 and PTEN have been established in TNBC, the molecular biology involved in their concurrent loss of function is poorly understood. In this dissertation, the functional and mechanistic roles of mesenchyme homeobox 1 (Meox1) are elucidated in the context of p53 and PTEN deficient TNBC, offering a specific therapeutic option.

RNA expression analysis using a panel of *in vitro* breast cancer cell lines shows Meox1 expression is upregulated in TNBC, no expression is seen in luminal or HER2-enriched subtypes. Furthermore, RNA expression analyses also show Meox1 is negatively regulated by both tumor suppressor genes of p53 and PTEN. *In vitro* p53 and PTEN small interfering RNA (siRNA) knockdown experiments demonstrate that only with combined loss of p53 and PTEN does expression of Meox1 significantly increase. Continued experiments with siRNA knockdown show decrease in Meox1 expression significantly decreases *in vitro* cellular proliferation of claudin-low and basal-like p53 and PTEN deficient TNBC. However, no effect on proliferation is seen in normal-immortalized, luminal, or HER2-enriched breast cancer cells upon knockdown of the transcription factor. Additionally, *in vivo* tumor xenograft mouse models using doxycycline inducible short hairpin RNA (shRNA) show knockdown of Meox1 significantly decreases tumor growth of basal-like p53 and PTEN deficient TNBC in an adjuvant and neoadjuvant setting. *In vitro* cell based assays of apoptosis and cell cycle arrest demonstrate this decrease in cellular proliferation is largely attributed to apoptosis in claudin-low but cell cycle arrest in basal-like intrinsic subtypes. Further Meox1 siRNA knockdown experiments show decrease in Meox1 expression also decreases migration and invasion of both claudin-low and basal-like p53 and PTEN deficient TNBCs, attributing to its functional role in regulating metastasis.

RNA sequencing (RNA-seq) was utilized to ascertain the mechanistic signaling pathways involved in Meox1 function to regulate proliferation and metastasis in p53 and PTEN deficient TNBC. Results show knockdown of Meox1 in claudin-low and basal-like p53 and PTEN deficient TNBC inactivates important canonical pathways involved in

growth and survival as well as migration and invasion. Accordingly, RNA-seq results corroborate *in vitro* functional data seen with Meox1 knockdown to induce apoptosis in claudin-low but cell cycle arrest basal-like intrinsic subtypes. Integrative pathway analysis shows knockdown of the transcription factor significantly activates canonical pathways associated with cell death in claudin-low and significantly perturbs canonical pathways associated with cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Further examination of RNA-seq shows important canonical pathways inactivated in both claudin-low and basal-like p53 and PTEN deficient TNBCs are Stat3 and Jak/Stat signaling. Investigation of the Jak/Stat pathway using western blot analysis shows knocking down Meox1 decreases Jak1, Tyk2, Stat5, Stat6, and P-Stat3 (Tyr705) protein levels in claudin-low, but decreases Tyk2 and Stat6 protein levels in basal-like intrinsic subtypes. While these preliminary results demonstrate Meox1 has the ability to regulate Jak/Stat signaling, more mechanistic research is necessary to assess how change in Jak/Stat protein levels upon knockdown of Meox1 effect functions of proliferation and metastasis.

Interestingly, results suggest Meox1 may have different mechanistic and transcriptional roles in the different intrinsic subtypes of claudin-low and basal-like p53 and PTEN deficient TNBC. Not only does knockdown of Meox1 show distinct roles of apoptosis in claudin-low and cell cycle arrest in basal-like TNBC, but it also effects Jak/Stat signaling differently in the two different intrinsic subtypes. Since TNBC is a vastly heterogeneous disease, it is no surprise that functional decrease in cellular proliferation can be attributed to distinct mechanistic pathways in the two different intrinsic subtypes. Indeed, when looking at RNA-seq results of activated/inactivated

canonical pathways and differentially expressed genes, not all canonical pathways and differentially expressed genes show common overlap between the claudin-low and basal-like intrinsic subtypes following Meox1 knockdown. Only 35% of claudin-low and 53% of basal-like intrinsic subtypes show common overlap of downregulation and upregulation. Furthermore, 58 genes are downregulated and upregulated in opposite directions between the claudin-low and basal-like p53 and PTEN deficient TNBCs. As such, further investigation of opposite activated/inactivated canonical pathways, opposite overlapping genes, and non-overlapping distinct genes may offer insight into the distinct biological roles of apoptosis in claudin-low and cell cycle arrest in basal-like p53 and PTEN deficient TNBC. Additionally, it is important to further ascertain how these activated/inactivated pathways and differentially expressed genes are transcriptional regulated by Meox1. The future use of chromatin immunoprecipitation sequencing (ChIP-seq) will offer more profound insight into the mechanistic and transcriptional regulatory roles of Meox1 in different intrinsic subtypes of p53 and PTEN deficient TNBC. A small perception into the transcriptional regulation of Meox1 is evident when comparing changes in Jak/Stat RNA levels to changes in Jak/Stat protein levels upon knockdown of the transcription factor; interestingly results show that RNA and protein levels of Jak/Stat signaling do not always correspond. As such, Meox1 may be a direct or indirect transcriptional regulator of Jak/Stat targets. Through indirect transcriptional regulation, Meox1 may transcribe other target genes that in turn may regulate Jak/Stats or Meox1 may directly interact with Jak/Stat proteins to regulate function and protein stability.

Studying Meox1 has offered insight into the complex and poorly understood molecular biology of p53 and PTEN deficient TNBC. The transcription factor presents an opportunity for specified targeting against the aggressive tumorigenic and drug resistant disease of p53 and PTEN deficient TNBC, which lacks specific actionable targets. Given the strong functional role of Meox1 to decrease proliferation and metastasis, targeting Meox1 may help decrease the aggressive proliferative and metastatic potential of TNBC. Since genetic aberrations in tumor suppressor genes of p53 and PTEN are frequent and common 'drivers mutations' for tumorigenesis as well drug resistance in TNBC, targeting Meox1 may further help ameliorate patients' resistance to the mainstay of chemotherapeutic treatment. Potential modes of therapy include Meox1 specific inhibitors designed through structure relationship analysis or through the use of emerging technologies in gene therapies such as siRNA delivery. Since Meox1 is expressed during development and not known to be present in adult tissues, therapies targeting Meox1 are suspected to be highly specific with limited adverse effects. Taken together, this dissertation identifies a specific transcription factor, which can be targeted by utilizing the latest treatment modalities in the clinic.

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