Potential Efficacy of Targeting MLL1 in Breast Cancer

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POTENTIAL EFFICACY OF TARGETING MLL1 IN BREAST CANCER

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF MASTER OF SCIENCE

PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

AUSTIN GABLE HOLMES
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ABSTRACT

In 2017, it is estimated that breast cancer will be the most prevalent newly diagnosed cancer in females, at 30% in the U.S. alone\textsuperscript{1}. From the early 1990s to 2014 the death rate in females from breast cancer has dropped by 10% but still stands as the second highest cancer related death in females in the U.S.\textsuperscript{2}. One of the biggest hurdles in breast cancer research is disease heterogeneity. New mechanisms of disease development and progression are encountered frequently. One mechanism studied in the past decades is epigenetics. It has been theorized that the cancer epigenome can maintain an abnormal state and possibly cooperate with genetic mutations\textsuperscript{3} and/or epigenetic modulators can be mutated which may become critical for cancer survival\textsuperscript{4}. MLL1 (KMT2A), an epigenetic modulator that regulates gene expression by trimethylation of H3K4 near gene promoters, has recently been implicated in cancer. Data mining of publicly available primary patient databases revealed that high MLL1 expression is associated with decreased probability of recurrence-free survival in triple negative breast cancer (TNBC). In breast cancer cell lines that represent different breast cancer subtypes we tested the efficacy of pharmacological MLL1 inhibition and MLL1 knockdown. Our data suggests that MLL1 is crucial for anchorage independent growth of the MDA-MB-468 cell line and this is not dependent on high MLL1 expression. The use of an MLL1-specific inhibitor in combination with standard of care chemotherapeutical agents was shown to be more effective for all breast cancer cell lines tested; the drugs could be used at much lower doses when combined. Some of the many MLL1 specific gene targets (ABCG2, AMIGO2 and EMP1) were interrogated for
changes in gene expression with MLL1 inhibition. To our surprise the observed changes varied between the cell lines suggesting that MLL1 inhibition differentially affects expression of specific genes in different cellular contexts. In conclusion, we demonstrate that MLL1 may be a viable target for breast cancer therapy in combination with standard of care agents. We also suggest that MLL1-dependent gene expression mechanisms could decrease drug resistance or impact cell survival.
CHAPTER ONE
LITERATURE REVIEW

Breast Cancer and Epigenetics

About 12% of women in their lifetime will develop invasive breast cancer\(^5\). In the U.S alone it is estimated that in 2017 nearly 250,000 women will be diagnosed with breast cancer and over 40,000 women will die because of the disease\(^5\). A major challenge for breast cancer is that the tumors are highly diverse which possibly renders treatment difficult. Classification systems have been discovered through DNA microarray gene expression analysis of primary patient tumor samples that identifies hormone receptor status and many other gene expression profiles\(^6\). This data gave rise to gene expression profiles with specific clinical prognosis and more optimized treatment modalities\(^7\). Initially 4 breast cancer subtypes were identified which include estrogen receptor (ER) positive or luminal type, basal-like, normal-like and HER2 positive\(^6\). Each subtype poses a different clinical probability with regard to overall and/or relapse-free survival, with basal-like and HER2 positive having the worst and luminal type having the best\(^7\). Even with major advancements in understanding breast cancer over 500,000 women died in 2011 worldwide, and the year after that, more than 1.7 million were newly diagnosed with breast cancer\(^8\).

Recently epigenetics has been implicated in breast cancer. One potential benefit of epigenetic therapy is based on the premise that epigenetic changes are reversible and can potentially recover cells from an abnormal state\(^9\). These epigenetic abnormalities involve DNA methylation, chromatin remodeling and histone modifications which all can have effects on gene expression. One example is that abnormal histone
modifications at H3K4 could be critical for breast cancer growth and were driven by missense p53 mutations\textsuperscript{10}.

**Wild Type MLL1**

The first discovery of MLL1 was in 1991 through analysis of chromosomal translocations at chromosome breakpoint 11q23\textsuperscript{11}. Two of the critical functional domains of the MLL1 protein are the plant homeodomain (PHD) zinc fingers and the Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain. The PHD fingers are involved in chromatin recognition including protein interaction with histones and the SET domain has methyltransferase activity specific for lysine 4 on histone 3 (H3K4). MLL1 is over 4000 amino acids in length and in cells it is cleaved by taspase 1 to generate a 300 kDa N-terminal and a 170 kDa C-terminal fragment\textsuperscript{12}. The fragments heterodimerize and complex with critical functional components WD repeat domain 5 (WDR5), retinoblastoma binding protein 5 (RBBP5), absent, small, or homeotic-like (ASH2L) and multiple endocrine neoplasia 1 (MEN1)\textsuperscript{13}. More specifically, at the C-terminal domain of MLL1 it has been shown that RBBP5 and ASH2L dimerize and bind weakly to MLL1 and WDR5 acts as a bridging molecule that stabilizes the interaction and complex formation\textsuperscript{14}. MEN1 binds to the N-terminal domain of MLL1 and has shown to be critical for transcriptional regulation of the complex in MLL-fusion\textsuperscript{15}. Overall, MLL1 regulates gene transcription through methylation specifically at H3K4. MLL1 binds the promoter region of many genes and regulates their expression, including some of the *HOX* genes which have been tightly associated with development\textsuperscript{16}.
**MLL1 and Cancer**

The majority of studies of MLL1 function to date have been focused on MLL1 fusion leukemias. MLL1 fusion proteins drive leukemogenesis by the dysregulation of certain genes, but wild type MLL1 in leukemia has become of interest as it has distinct roles in transcriptional programs that contribute to the leukemia gene expression program\(^7\). Outside of leukemia, MLL1 has been implicated in many cancer pathways in many different cancer types. MLL1 has been shown to contribute to the tumorigenic potential of glioblastoma stem cells specifically through *HOXA10* gene activation\(^8\). Also, it has been observed that *MLL1* expression levels are high in cervical carcinoma and a consequence of high *MLL1* expression in cervical carcinoma cell lines is transactivation of *CCND1*, which promotes cell proliferation\(^9\). In addition, *MLL1* genetic knockdown greatly reduced tumor growth *in vivo* of a cervical cancer xenograft and decreased key proangiogenic factors\(^10\). In an oncogene-induced senescence (OIS) model of lung fibroblast cells and MCF7 breast cancer cells, MLL1 was shown to repress the expression of some genes required for DNA replication and DNA damage response (DDR) which greatly inhibits cells to undergo the senescence-associated secretory phenotype (SASP)\(^11\). SASP has positive implications on proliferation of malignant cells\(^12\). Another report associates MLL1 with metastasis of hepatocellular carcinoma (HCC) through increased expression of MLL1 target genes *MMP1* and *MMP3*\(^13\). MLL1 has also been shown to regulate kinesin family proteins in breast cancer cell lines that drive proliferation and tamoxifen resistance\(^14\).

All together MLL1 may have critical roles contributing to the cancer phenotype of many different cancers. These roles seem to include the epigenetic gene regulatory
function MLL1 at certain target genes that then drive critical cancer cell survival mechanisms.

**Some MLL1 Targets and Cancer**

MLL1 is an epigenetic regulator that controls the expression of many genes. This study set out to study three that were shown to be specific MLL1 targets through ChiP-seq of MLL-AF9 leukemia cells\(^\text{17}\). These three genes listed below have been greatly changed in expression with MLL1 inhibition as shown by RNAseq in MLL-AF9 leukemia cells\(^\text{17}\).

**ABCG2.**

*ABCG2,* also known as the breast cancer resistance protein, is an ABC transporter of which the ABC family are strictly substrate exporters\(^\text{24}\). ABCG2 has been tightly associated with multi-drug resistance (MDR) and more specifically chemoresistance through study of ABCG2 substrates\(^\text{24}\). The list of known substrates includes anti-cancer agents such as: topoisomerase inhibitors, anthracyclines, camptothecin (CPT) analogs, tyrosine kinase inhibitors (TKI), and antimetabolites\(^\text{24}\). Since the initial discovery of *ABCG2* in 1998\(^\text{25}\), *ABCG2* has been implicated in other cancer types, an extensive list MLL1 targets *ABCG2* in MLL-AF9 transduced leukemia cells and has been shown to positively regulate its expression\(^\text{17}\). This would suggest that high MLL1 expression may increase the *ABCG2* expression and that inhibiting MLL1 may decrease *ACBG2* expression.
AMIGO2.

Adhesion molecule Ig-Like 2 (AMIGO2) is a transmembrane protein involved with the PI3-AKT signaling pathway, angiogenesis and cell endothelial cell survival\textsuperscript{27}. AMIGO2 functions as a scaffold for 3-phosphoinositide dependent kinase 1 (PDK1) membrane localization and subsequent activation of AKT\textsuperscript{27}. Knockdown of AMIGO2 in endothelial cells greatly reduced migration and capillary network formation\textsuperscript{27}. In an osteosarcoma cancer cell model upregulation of AMIGO2 increase attachment to liver endothelial cells which increased metastasis to the liver\textsuperscript{28}. In MLL-AF9 transduced leukemia cells MLL1 was shown to be a direct target of AMIGO2 and specifically activated transcription\textsuperscript{17}.

EMP1.

Epithelial membrane protein 1 (EMP1) is an MLL1 target in MLL-AF9 transduced leukemia cells\textsuperscript{17} and its expression increased 7-fold upon MLL1 inhibition suggesting MLL1 may have a suppressive gene transcription role at this gene\textsuperscript{17}. Gastric carcinoma tissue from primary patients showed decreased EMP1 expression, and this decrease was associated with tumor invasion, lymph node metastasis, clinical stage and histological grade\textsuperscript{29}. In breast cancer, EMP1 is decreased in patient samples and correlates negatively to overall survival\textsuperscript{30}. Increased expression of EMP1 in the MCF7 breast cancer cell line caused increased apoptosis and decreased invasion and migration\textsuperscript{30}.
Targeting Epigenetics in Cancer

Epigenetic-targeting drugs such as DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors have been shown to be effective in breast cancer cells\textsuperscript{31-32}. Studies have shown that these epigenetic drugs in combination with cytotoxic agents may be effective\textsuperscript{32}. Arce et al (2006) demonstrated that treatment of breast cancer patients with DNMTi and HDACi along with doxorubicin or cyclophosphamide was well-tolerated and important proapoptotic genes like \textit{TP53} and \textit{CDKN1A} (\textit{p21}) were upregulated while drug resistant genes like \textit{ABCB5} were downregulated\textsuperscript{33}. Another clinical trial with HDAC inhibitor vorinostat with paclitaxel, a tubulin targeting molecule, and bevacizumab, an anti-VEGF-A antibody, results in 50% of patients achieving a partial or complete response for those with recurrent metastatic breast cancer\textsuperscript{34}. An epigenetic drug that disrupts the interaction of MLL1 and menin, MI-2, has been shown to abrogate MLL acute leukemia development in a murine model system \textit{in vivo}\textsuperscript{35}. To our knowledge, use of MI-2 in breast cancer cells has only been reported in the context of missense \textit{p53} mutation status\textsuperscript{10}. Targeting MLL1 specifically in breast cancer could alter gene expression programs that are necessary for specific aspects of the breast cancer cell survival. It also is possible that the epigenetic-targeting drug MI-2 could be efficacious in combination with chemotherapeutic agents for breast cancer therapy.

In this study, I mined gene expression databases to determine whether MLL1 expression was correlated with clinical subtypes, prognosis, or treatment status in breast cancer. Also in this study, by investigating a panel of breast cancer cell lines for
proliferation and anchorage independent growth with the use of MI-2 and MLL1 knockdown, I aimed to elucidate the potential of using MLL1 specific inhibitors for breast cancer therapy. Furthermore, I determined whether breast cancer cells were more sensitive to combinatorial treatment with MLL1 inhibition plus standard of care chemotherapy agents. Several genes that are known direct MLL1 targets that may have roles in drug resistance, proliferation and metastatic potential were interrogated for gene expression changes in breast cancer cells upon MLL1 inhibition. Our results indicate that MLL1 could be a viable target in specific types of breast cancer and combinatorial treatment is more efficacious, requiring less drug for effective inhibition.
CHAPTER TWO

METHODS

Cell Lines and Culture Conditions.

Six breast cancer cell lines (MDA-MB-468, MDA-MB-231, BT-549, T47D, MCF7, ZR-75-1) were generously donated by Dr. Clodia Osipo (Loyola University Chicago) and thawed from low passage cultures stored in liquid nitrogen. All the cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (4.0 mM L-Glutamine, 4500 mg/L Glucose). Cells were cultured in 10 cm² plates with passage every 3-5 days. Trypsin (0.05%) was used to detach cells.

RNA and cDNA Preparation.

RNA was isolated using 500µL to 1mL of Trizol (SIGMA; catalog # T9424), depending on cell number isolated, using manufacturer’s protocol. cDNA synthesis was competed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (catalog #: 4368814)

qRT-PCR Analysis.

Applied Biosystems Taqman Fast Advanced Master Mix protocol was followed. The Quant Studio 6 Flex machine and software were used and analysis was done using the Comparative Ct Method\(^{37}\). For all analysis, the \(2^{-\Delta\Delta Ct}\) method was utilized to compare cell lines and gene expression following protocol\(^{37}\).
Taqman primer probes sourced from Integrated DNA Technologies were used for the following genes listed below in Table 1. Taqman Master Mix (Applied Biosystems) (Ref #:4444557) was used in each reaction following manufactures protocol.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RefSeq Accession #</th>
<th>Exon Location</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>NM_001257386 (2)</td>
<td>6-7</td>
<td>Hs.PT.58.1283359</td>
</tr>
<tr>
<td>AMIGO2</td>
<td>NM_001143668 (1)</td>
<td>1-2</td>
<td>Hs.PT.58.333002</td>
</tr>
<tr>
<td>β2M</td>
<td>NM_004048</td>
<td>2-4</td>
<td>Hs.PT.58v.18759587</td>
</tr>
<tr>
<td>EMP1</td>
<td>NM_001423 (1)</td>
<td>1-2</td>
<td>Hs.PT.58.3020024</td>
</tr>
<tr>
<td>ISL1</td>
<td>NM_002202 (1)</td>
<td>5-6</td>
<td>Hs.PT.58.2143768</td>
</tr>
<tr>
<td>KMT2A(MLL1)</td>
<td>NM_005933 (2)</td>
<td>11-13</td>
<td>Hs.PT.58.22285060</td>
</tr>
<tr>
<td>MEN1</td>
<td>NM_130799 (7)</td>
<td>3a-4</td>
<td>Hs.PT.58.22752253</td>
</tr>
</tbody>
</table>

Table 1. List of taqman primer probe sets
**shRNA Mediated Knockdown.**

pGFP-C-shLenti plasmids containing *MLL1* specific 29mer knockdown short hairpin sequences were obtained from ORiGene (Catalog #: TL311462). 293T cells were transfected using Clontech calcium phosphate transfection reagents following manufacturer's protocol (Catalog #: 631312). Plasmids and reagents were incubated for 3 days before harvesting virus containing media on the 4th and 5th day post transfection. Two constructs within similar knockdown efficiency (determined in 293T cells) were used and are listed in Table 2 below.

<table>
<thead>
<tr>
<th>29mer ID</th>
<th>shRNA target <em>MLL1</em> sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL311462A</td>
<td>CCACTCCATTCTGGAACAAGGTTTGATAT</td>
</tr>
<tr>
<td>TL311462D</td>
<td>TGTCTCATCACGACTTACATTCCTTC</td>
</tr>
</tbody>
</table>

**Table 2. shRNA expression vector pGFP-C-shLenti plasmids**

**FACS.**

Fluorescence Activated Cell Sorting was performed on by BD Aria Illu using FACSDiva (version 6.1.3) according to manufacturer’s protocols. Knockdown constructs co-expressed GFP, therefore GFP+ cells were sorted and used for functional assays following shRNA mediated knockdown.
Cell Viability Assay.

Promega Cell Titer-Glo 2.0 reagent was used to assess ATP content as an indicator of metabolically active cells. Greiner Bio One 96-well clear bottom white walled plates (Lot#: E16023C5) were used for cell culture. Cell Titer-Glo 2.0 reagent was dispensed into the 96-well plate following manufacturer's protocol. The plates were read on BMG Labtech POLARstar Omega readers using BMG Labtech Omega software version 1.02. Luminescence was determined and quantification and analysis was completed using Microsoft Excel.

Colony Formation Assay.

Stem Cell Technologies Methocult H4100 was used for colony suspension in 12-well non-tissue culture treated plates (Cyto-One). Cells were subjected to single-cell suspension by using 0.125% trypsin and vigorous pipetting. Once in single cell suspension cells were plated in triplicate (2,500-5000 cells/well) and allowed to incubate for 8-14 days. High quality images (1792x1374) using AmScope MU100 camera and AmScope software were captured and colonies were counted using 7 images per well (21 images per triplicate) and was used to represent the entire plate.

Drugs.

Carboplatin from Sigma Aldrich (C2538: Lot # SLBL7058U), 4-hydroxy-tamoxifen (4OHT) (Sigma Aldrich, Catalog #: H7904) and MI-2 from Selleckchem (Cat No. S7618) were used alone and in combination to determine IC$_{50}$ for each cell line. Greiner Bio One 96-well clear bottom white walled plates were used and IC$_{50}$ calculations were
completed using GraphPad Prism 7. Cells were treated with a range of 6-7 different concentrations of each drug to determine an IC$_{50}$. Once an IC$_{50}$ was established the combinatorial experiments treated cells with standard-of-care drugs and MI-2 alone and together at each cell lines respective IC$_{50}$ and an additional set of wells used drugs in combination at $\frac{1}{2}$ the IC$_{50}$.

**Primary Patient Databases.**

*KMT2A* (aka *MLL1*) (AffyID: 226981_at and 220546_at) was interrogated using the Kaplan Meier Plotter software (www.kmplot.com) which includes microarray data from 1809 cancer patients$^{37}$. For each specific analysis breast cancer patient microarrays were separated into either triple negative breast cancer (ER$^{-}$/PR$^{-}$/HER2$^{-}$) as selected individually in the restriction analysis menu box, luminal A (ER$^{+}$/HER2$^{-}$/Ki67$^{\text{low}}$) and luminal B (ER$^{+}$/HER2$^{-}$/Ki67$^{\text{high}}$) as selected with the intrinsic subtype selector. Analysis of recurrence-free survival probability of triple negative and luminal A or B breast cancer was utilized as well as isolating treated and untreated patient samples. Untreated patients are categorized by excluding chemotherapy, adjuvant and neoadjuvant therapy. Treated patients included endocrine therapy and chemotherapy. The high and low expression data was split at the median expression level.
CHAPTER THREE

RESULTS

Aim 1: Determine whether MLL1 expression levels correlate with clinical outcomes in breast cancer

Gene expression analysis and clinical outcomes from primary patient samples are readily available through multiple different online repositories. I searched for MLL1 (KMT2A) expression level and clinical outcomes in breast cancer using the Kaplan Meier plotter software\textsuperscript{37} that integrates information regarding microarray data and clinical outcomes from GEO (Affymetrix microarrays only), EGA and TCGA. Figure 1 demonstrates the relationship between MLL1 expression (divided from the median) in Luminal A/B and triple negative breast cancer (TNBC) plotting the probability for recurrence-free survival. Both Luminal A and B (figures 1A and B) show a positive correlation; higher MLL1 expression is associated with increased probability of recurrence-free survival. In contrast TNBC (figure 1C) shows the opposite: higher MLL1 expression is associated with a decreased probability of recurrence-free survival. Interestingly, if you divide the samples in this study between treated and untreated patients, the negative correlation only lies within the samples isolated from post-treated patients. Figure 1D represents TNBC patients that are classified as untreated and there is no correlation with the probability of recurrence-free survival. If you limit the analysis to only Luminal A and B primary patient samples (data not shown) there still is a positive correlation between recurrence-free survival and higher MLL1 expression. These results suggest that MLL1 may have different roles in different breast cancer subtypes with regard to breast cancer recurrence and that higher MLL1 expression in treated TNBC patients results in decreased probability of recurrence-free survival.
Figure 1. *MLL1* expression in primary breast cancer patient samples with correlation to recurrence free survival. *MLL1* (Affy ID: 226981_at and 220546_at) was assessed in Luminal A, B and TNBC patient samples and correlated with recurrence-free survival. The x-axis represents time (months) and red and black bars represent high and low expression, respectively, of *MLL1*. (A, B, C) excludes untreated patients and (D) is only untreated patients with TNBC. TNBC is defined as ER⁻/PR⁻/HER2⁻. Luminal A and B is defined by the 2013 St Gallen criteria (Luminal A: ESR1⁺/HER2⁻/MI Ki67 low; Luminal B: ESR1⁺/HER2⁻/MI Ki67 high). Data was obtained from Breast Cancer Res Treatment, 2010 123:725-31.
Aim 2: Determine the effect of MLL1 knockdown and pharmacological inhibition on breast cancer cell lines.

Recently MLL1 has been implicated in breast cancer and to a broader extent in some other cancers. MLL1 was shown to drive breast cancer growth in \textit{in vivo} breast cancer cell line xenograft models and \textit{in vitro} breast cancer cell lines\textsuperscript{10}. MLL1 specific inhibitors showed some promise in breast cancer cell lines in a missense mutant p53 dependent manner\textsuperscript{10}. Also, MLL1 has been associated with cervical carcinoma and may have roles in promoting tumorigenesis and metastasis directly through its interaction with $\beta$-catenin\textsuperscript{19}. In addition, studies identified specific genes regulated by MLL1 that were associated with the cancer phenotype. Dirks et al. (2012) suggest that MLL1 can specifically regulate expression of \textit{HOXA10} and this process contributes to tumorigenicity of glioblastoma stem cells. In the MLL1 fusion leukemia model MLL-AF4, the fusion protein upregulates expression of \textit{MDR1} and contributes to chemoresistance\textsuperscript{38}.

With this information in mind I hypothesized that MLL1 is critical for \textit{in vitro} proliferation and growth of breast cancer cell lines. To test my hypothesis, I determined the effect of \textit{MLL1} knockdown and MLL1 pharmacological inhibition on 6 breast cancer cell lines that represent either the TNBC or luminal breast cancer subtypes. I have measured the effects of manipulating MLL1 expression or function on the breast cancer cell line proliferation, anchorage independent growth, migration, and response to standard-of-care drugs. Table 3 describes the breast cancer cell lines used in this study. Subtype and p53 status are included because the clinical information suggests a subtype bias to \textit{MLL1} dependence and a recent report suggested that some missense
p53 mutations drive breast cancer cell line growth \textit{in vitro} and \textit{in vivo} through increasing \textit{MLL1} expression\textsuperscript{10}.

<table>
<thead>
<tr>
<th>Breast Cancer Cell Line</th>
<th>Breast Cancer Subtype*</th>
<th>TP53 Status**</th>
<th>Additional mutations and abnormalities ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{MDA-MB-468}</td>
<td>Basal-like</td>
<td>missense R273H</td>
<td>\textit{PTEN, RB1, SMAD4}</td>
</tr>
<tr>
<td>\textit{MDA-MB-231}</td>
<td>Basal-like</td>
<td>missense R280K</td>
<td>\textit{BRAF (G464V), CDKN2A (M521I), RAS (G13D), p16, p14ARF}</td>
</tr>
<tr>
<td>\textit{BT-549}</td>
<td>Basal-like</td>
<td>missense R249S</td>
<td>\textit{PTEN}</td>
</tr>
<tr>
<td>\textit{T47D}</td>
<td>Luminal A/B</td>
<td>missense L194F</td>
<td>\textit{PIK3CA (H1047R), p16\textsuperscript{meth}}</td>
</tr>
<tr>
<td>\textit{MCF7}</td>
<td>Luminal A/B</td>
<td>WT</td>
<td>\textit{CDKN2A, PIK3CA (E545K), p16, p14ARF, AKT\textsuperscript{amp}, HDM2\textsuperscript{amp}}</td>
</tr>
<tr>
<td>\textit{ZR-75-1}</td>
<td>Luminal A/B</td>
<td>WT</td>
<td>\textit{HDM2\textsuperscript{amp}, CyclinD1\textsuperscript{amp}}</td>
</tr>
</tbody>
</table>

\textbf{Table 3. Breast cancer cell lines used in this study}

Basal MLL1 expression.

I determined the relative MLL1 expression between the cell lines used in this study to see whether MLL1 was differentially expressed in unmanipulated cell lines that represent luminal or basal breast cancer subtypes. Figure 2 represents the relative basal expression of MLL1. As shown in Figure 2 the expression differs between the cell lines and shows that luminal breast cancer cell lines have ~ 3-10-fold more expression of MLL1 than the basal breast cancer cell lines assayed. The relative MLL1 expression levels did not correlate with TP53 mutation status.

Figure 2. Basal MLL1 expression in breast cancer cell lines. Five independent samples of each cell line were harvested at 70% confluency within a span of 2 months culture time. RNA was isolated using Trizol, cDNA synthesized and duplex qRT-PCR performed normalized to β2M. For analysis, the 5 independent replicates were combined. The 2^{-ΔΔCt} was utilized and the lowest MLL1 expressing cell line (MDA-MB-468) was set to one.
shRNA-mediated MLL1 knockdown.

*MLL1* knockdown was mediated by specific shRNA lentivirus. Isolation of transduced cells was achieved through FACS sorting of GFP+ cells 48 hours post transduction. Figure 3A represents the forward and side scatter gates indicating live cells (left) and the gates set for GFP+ (virally transduced) cells (right). *MLL1* knockdown was confirmed through qRT-PCR analysis (Figure 3B). shRNA construct # 2 achieved ~50% knockdown reproducibly within all the cell lines and shRNA construct #1 achieved on average 40% knockdown.

Figure 3C represents the anchorage independent growth (BT-549 is not included as it did not form colonies in our system, see methods) of all breast cancer cell lines used in this study. MDA-MB-468 cell line was the only cell line affected by *MLL1* knockdown with almost no detectable colonies. As shown in figure 4A with both shRNA constructs there are no detectable colonies after incubation in methylcellulose for 14 days. The T47D cell line seems to have a dramatic effect only with construct # 2 that is reproducible. This could be explained this cell line needing a critical amount of MLL1 or it could be off target effects of the shRNA construct. Nonetheless our data suggests conclusively that MLL1 is necessary for anchorage independent growth of the MDA-MB-468 cell line.
Figure 3. Effect of *MLL1* KD on anchorage independent growth and cell proliferation. (A) FACS forward and side scatter (left) and GFP* cells that were sorted and collected (right). (B) *MLL1* knockdown confirmed by qRT-PCR. Analysis was carried out by the 2^ΔΔCt method setting scramble shRNA-infected cells *MLL1* expression to one. RNA was isolated 3 days post infection which include two days of viral incubation and one day post sorting. Numbers above MB-468 and MB-231 sh#1 represent the p value. (C) Anchorage independent growth as assayed by colony number per field. Each field contained seven images with each experiment completed in triplicate. Number within the scramble bar represents the number of independent replicates combined in the data. (D) ATP content as assayed with Cell-Titer Glo 2.0 reagent. Numbers within bars represent the independent replicates combined in the data. (p < 0.05 *, p < 0.01 **)
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Figure 4. Images of breast cancer cell lines grown in methylcellulose and in liquid culture following shRNA-mediated MLL1 knockdown. (A) GFP⁺ colonies (top) after 8-14 days in methylcellulose medium with corresponding bright field images (bottom) for each transduced construct. (B) GFP⁺ breast cancer cells grown in tissue culture treated plates 4 days after sorting (6 days post infection) (top) and corresponding bright field images (bottom) for each transduced construct.
Another property of breast cancer cell lines is their ability to rapidly proliferate endlessly. Proliferation requires metabolically active cells which produce ATP. I measured the ATP content of breast cancer cell lines following MLL1 knockdown. In figure 3D, I demonstrate that shRNA-mediated MLL1 knockdown did not have any effect on the ATP content of breast cancer cells in culture 4 days post isolation of transduced cells (6 days post infection). Figure 4 shows cells in culture 4 days post sorting and 6 days post infection indicating GFP+ directly before isolation and analysis. In conclusion, MLL1 expression level may not affect the metabolic activity of these breast cancer cell lines, which suggests it has no effect on proliferation.

Pharmacological MLL1 inhibition.

I hypothesized that inhibition of MLL activity would inhibit the proliferation of the breast cancer cell lines used in this study as measured by ATP content after incubation with MI-2, a menin-MLL1 inhibitor that disrupts the activity of MLL1. Prior to use of the menin inhibitor MI-2, I had to determine sensitivity of each of the cell line in this study to the inhibitor. To determine the cell line sensitivity, I determined an IC_{50} for each cell line as determined by measuring ATP content after 3 days of exposure to MI-2. An IC_{50} is calculated by using a range of at least six concentrations of any given inhibitor and generate a dose-response curve that can be interrogated for a concentration of drug at which cell viability can be inhibited by 50%. Figure 5A represents IC_{50} data of each cell line. The MDA-MB-468 cell line was the most sensitive, with an IC_{50} of 11.3 ± 4.5 μM, and the ZR-75-1 cell line was least sensitive with an IC50 of 40.6 ± 4.7μM. Figure 5B demonstrates an example of the IC_{50} sigmoidal curves generated for each cell line.
The basal expression level of MEN1 was relevant to determine because the MI-2 inhibitor binds to menin and disrupts its interaction with MLL1. Figure 5C demonstrates the basal MEN1 expression of the cell lines used in this study. I found that all cell lines expressed MEN1. However, there were differential levels of MEN1 expression between the breast cancer subtypes. The luminal subtype cell lines expressed a higher level of MEN1 and the triple negative subtype cell lines expressed less MEN1. The expression of MEN1 did not correlate with MI-2 sensitivity and breast cancer cell lines tested have a varying amount of sensitivity to MI-2. These data suggest that MLL1 inhibition has an effect on proliferation of one breast cancer cell line and does not correlate with breast cancer subtype or overall expression of MLL1 (Figure 1) or MEN1 as determined by the IC₅₀ and anchorage independent data.
Figure 5. IC$_{50}$ for each breast cancer cell line using MI-2. (A) IC$_{50}$s for each cell line. Each point represents an independent replicate IC$_{50}$ and the middle bar is the mean. (B) Shows examples of IC$_{50}$ calculations with the generated sigmoidal curve fit line. (C) (pink = TNBC; blue = luminal) Represents the MEN1 (menin) expression of each cell line. The lowest MEN1 expressing cell line was set to 1 and therefore fold expression is displayed (n = 3). (p < 0.05 *, p < 0.01 **, p < 0.001 ***
Next, I investigated if MI-2 could have an effect on anchorage independent growth of the cell lines used in this study. Figure 6A represents the anchorage independent growth and colonies formed. Figure 6B is the calculation of colonies formed represented as a percent of vehicle control. Similar to what was seen with the cell viability IC$_{50}$ calculations, the MDA-MB-468 cell lines is the most sensitive. In contrast, the other cell lines are relatively insensitive to MI-2 treatment, with an inhibitory effect only observed at the highest concentration tested, 50uM. One cell line, ZR-75-1, actually had an increase in the colony forming activity following MI-2 treatment at intermediate concentrations (12.5uM and 25uM) (Figure 6B).
Figure 6. Anchorage independent growth of breast cancer cell lines following MI-2 treatment. (A) (pink = TNBC; blue = luminal) Relative colony forming ability, calculated from combining at least 3 independent experiments. (B) Images of colonies formed 8-14 days post plating in methylcellulose with 4X objective. (p < 0.05 *, p < 0.01 ***)
Overall MLL1 and its interaction with menin is critical for the MDA-MB-468 cell line’s anchorage independent growth but not the other breast cancer cell lines tested.

**Gene expression changes in cells treated with MI-2.**

I hypothesized that treatment of breast cancer cell lines with the menin inhibitor MI-2 would alter expression of MLL1 target genes, particularly those targets that could impact proliferation, drug resistance and anchorage independent growth of breast cancer cell lines following treatment with the MLL1/menin inhibitor. Recently, in an MLL-AF9 transduced leukemia cells MLL1 direct targets were assayed by ChIP-seq, and RNA-seq analysis was done on cells treated with MI-2\textsuperscript{17}. MLL1 has many different target genes, and has been shown to both positively and negatively affect expression of its targets. To our knowledge, this is the first exploration of changes in gene expression following MI-2 treatment of some MLL1 target genes in the breast cancer cell lines used in this study. *AMIGO2, ABCG2, EMP1* and *ISL1* were assayed by RNA isolation followed by qRT-PCR analysis after treating cells with MI-2 at their respective IC\textsubscript{50} concentrations for four days. First, it was necessary to examine the relative gene expression of each gene selected within the cell lines. I hypothesized that cells with higher MLL1 expression (luminal type) should have higher *ABCG2*, higher *AMIGO2*, and lower *EMP1*. This hypothesis was driven by gene expression changes observed in MLL-AF9 transduced cells treated with MI-2\textsuperscript{17}. Figure 7A-C represents the relative basal expression of each gene assayed. Figure 7A represents *ABCG2*, an ABC transporter, also known as the breast cancer resistance protein, known to function as an efflux component, pumping target molecules from the cytoplasm to the extracellular space. Fold expression changes should keep in mind the relative expression of each gene
within cell lines. As expected cell lines with higher MLL1 expression showed higher ABCG2 expression (Figure 2; Figure 7A). For example, Figure 7A shows that MCF7 and ZR-75-1 have nearly 200-fold more expression of ABCG2 than the other cell lines. The observed 0.8 and 0.5-fold reduction (MCF7 and ZR-75-1, respectively) is much greater in absolute number than the 0.5-fold reduction in BT-549 cell line (Figure 7D). Low EMP1 (epithelial membrane protein 1) expression has recently been reported to correlate with poorer 5-year overall survival in colorectal cancer patients\(^2^9\) and has been shown to increase more than 7-fold upon treatment of MLL-AF9 transduced leukemia cells treated with MI-2\(^1^7\). In only one cell line, T47D, do we see this similar trend to the MLL-AF9 transduced cells (Figure 7E). It was also expected that cells with higher MLL1 expression should have lower EMP1 because MLL1 was recently shown to negatively regulate EMP1 expression\(^1^7\) (Figure 7B). Figure 7C shows basal AMIGO2 expression within the breast cancer cell lines used in this study. A 3.66-fold decrease in expression in MLL-AF9 transduced cells has been reported\(^1^7\). I anticipated a decrease in expression upon MI-2 treatment but observed the opposite (Figure 7F). In osteosarcoma cell lines, decreased AMIGO2 has been reported to decrease migration and tumorigenicity and upregulation has been shown to facilitate attachment to liver endothelial cells\(^2^8\).

Specifically, for ABCG2 the cell lines BT-549, MCF7 and ZR-75-1 demonstrated a decrease in expression upon MI-2 treatment. The relative of expression of MLL1 correlates with the basal level expression of ABCG2 as the cell lines with the highest expression of MLL1 had the highest expression of ABCG2. Inhibition of the menin/MLL1 interaction was variable within the cell lines with regards to changes in ABCG2
expression. In conclusion *MLL1* may activate gene expression of *ABCG2* but inhibition in the breast cancer cell lines used in this study does not always yield a decreased expression of *ABCG2*.

Basal *EMP1* expression correlated with basal *MLL1* expression in the breast cancer cell lines used in this study with regards to its suggested function in MLL-AF9 leukemia cells\(^1\) as a repressor. High *MLL1* expressing cells had the lowest expression of *EMP1*. Changes in expression of *EMP1* upon menin/MLL1 inhibition varied greatly and was only seen in the T47D cell line. This suggest that the menin/MLL1 interaction may not be critical for EMP1 gene repression in the other breast cancer cell lines in this study.

Lastly, *AMIGO2* expression did not correlate with relative *MLL1* gene expression. High expression of *MLL1* did not demonstrate high expression of *AMIGO2*. Also, inhibition of the menin/MLL1 interaction increased expression of *AMIGO2* in multiple cell lines suggesting its role as a gene repressor of *AMIGO2*.

The varied expression data of these cell lines upon menin/MLL1 inhibition demonstrate that MLL1 function may vary in these breast cancer cell lines and that this function may be different than what has been observed in MLL-AF9 transduced leukemia cells.
Figure 7. Changes in gene expression of validated MLL1 target genes ABCG2, EMP1 and AMIGO2 in breast cancer cell lines treated with MI-2. Breast cancer cell lines were treated at IC50 concentrations for 4 days. (A, B, C) Fold changes in genes expression as compared to vehicle control (VC) and normalized to β2M. (D, E, F) Basal level expression of each target gene in all the cell lines relative to BT-549 cell line. (n = 2; triplicate experiments) (p < 0.05 *, p < 0.01 **, p > 0.001 ***
Aim 3: Determine the combined effect of an MLL1 inhibitor with standard of care chemotherapeutic agents on breast cancer cell lines.

I hypothesized that combined treatment of breast cancer cell lines with standard of care chemotherapeutic agents plus MI-2 would be more efficacious than treatment with the chemotherapeutic agents or MI-2 alone. I first generated IC$_{50}$ data measuring ATP content for cells treated with carboplatin, a molecule that cross-links guanine bases, in the TNCB cell lines (MDA-MB-468, MDA-MB-231 and BT-549) and 4-hydroxytamoxifen, an estrogen antagonist, for the luminal breast cancer cell lines (T47D, MCF7 and ZR-75-1). I co-treated cells with the IC$_{50}$ of each drug and then half the IC$_{50}$ and assayed for cell viability 4 days post treatment (Figure 8A). In all cell lines, combinatorial treatment was more effective than either alone and most interestingly, using combinatorial treatment at half the IC$_{50}$ was just as effective and in some cases more effective than either treatment alone at the IC$_{50}$ (Figure 8A). This suggests that combinatorial treatment can be effective at lower doses of both drugs. Figure 7B shows the percent viability as compared to vehicle control of either drug at ½ of its IC$_{50}$ and demonstrates that in most cell lines that half the IC$_{50}$ is not as effective as using combinatorial treatment at half the IC$_{50}$. 
Figure 8. Combinatorial treatment of breast cancer cell lines. Breast cancer cell lines were treated at their IC$_{50}$ concentration of either MI-2 or standard of care chemotherapy agents Carboplatin and 4-OHT. Luminal breast cancer cell lines (right 3) were treated with 4-OHT and triple negative breast cancer cell lines (left 3) were treated with Carboplatin. (A) Cell viability was measured using Cell Titer-Glo 2.0. Significance stars above each bar represent a comparison to vehicle control. (B) Results of cell viability relative to vehicle control of each individual drug using $\frac{1}{2}$ the IC$_{50}$ concentration using the sigmoidal curve function generated when calculating the IC$_{50}$. ($p < 0.05^*,$ $p < 0.01^{**},$ $p > 0.001^{***},$ $p < 0.0001^{****}$)
CHAPTER FOUR
DISCUSSION

The focus of my research was to determine the possible role of MLL1 in breast cancer. I first interrogated publicly available databases of breast cancer patient microarray data that correlates clinical outcomes with MLL1 gene expression levels. My datamining results indicate that there is a correlation between high MLL1 expression levels and decreased probability of recurrence-free survival in triple negative breast cancer patients that have been clinically treated. Therefore, I used a panel of breast cancer cell lines to directly test my hypothesis that MLL1 expression and MLL1 function are critical for multiple properties that likely contribute to breast cancer recurrence in triple negative breast cancer patients.

First, I determined the basal MLL1 expression levels within these cell lines. Initially, the hypothesis was that breast cancer cell lines with missense p53 mutations would have the highest MLL1 expression as it has recently been shown that some missense p53 mutations drive breast cancer growth through missense p53 proteins acting at the promoter of MLL1 as a gene activator\textsuperscript{10}. To our surprise the expression levels of MLL1 were the opposite, those harboring missense p53 mutations (MDA-MB-468, MDA-MB-231, BT-549, T47D) had much lower expression than cell lines with WT p53 (MCF7, ZR-75-1) (Figure 1).

Two cancerous characteristics of these cancer cell lines is unlimited proliferation and anchorage independent growth, both of which were assayed by genetic knockdown or pharmacological inhibition. From these experiments, I concluded that anchorage independent growth and proliferation does not depend on basal MLL1 expression
levels. I found that the cell line with the lowest MLL1 expression, MDA-MB-468, was most affected by manipulating MLL1 mRNA levels by knockdown or functionally with MI-2. No other cell line was affected. Interestingly MLL1 KD in the MDA-MB-468 cell line had no effect on proliferation but MI-2 did have an effect. This could be explained by the KD efficiency of 50% and MI-2 concentration of 10μM having a different effect on overall MLL1 activity. Even with the lowest levels of MLL1 it could be that the MDA-MB-468 cell lines requires a minimal critical amount of MLL1 for proliferation and anchorage independent growth. Also, decreased activity of MLL1 could allow for de-repression or activation of critical proliferation and/or anchorage independent growth genes. Overall the anchorage independent growth of MDA-MB-468 cell line depends on MLL1 and this can be recapitulated with pharmacological inhibition using MI-2.

In addition, breast cancer cell lines with missense p53 mutations (MDA-MB-468, BT-549, HCC70) were shown to be sensitive to MLL1 KD with regards to cell proliferation and anchorage independent growth while WT p53 harboring breast cancer cell lines (MCF7, MDA-MB-175VII) were insensitive to these assays. These data suggest that cells lines with missense p53 mutations are sensitive to MLL1 KD. My data shows the cell line MDA-MB-231 which harbors a missense p53 mutation is completely insensitive to MLL1 KD and inhibition and yet has the same basal MLL1 expression level as the MDA-MB-468 cell line. Also, the cell line T47D, has a missense p53 mutation and shows no sensitivity to MLL1 KD or inhibition with regards to anchorage independent growth which has 3-fold more MLL1 expression than the MDA-MB-468 cell line. Also, it was shown that primary patient samples with missense p53 mutations displayed higher expression levels of MLL1. The trend presented from clinical data.
does not match what is seen in these breast cell lines. Taken together these cell line models could differ from the clinical samples in that different missense p53 mutations act differentially at the promoter of MLL1 or may not associate at all. The function of missense p53 mutations has been shown through KD of missense TP53 (which also included WT TP53) decreases MLL1 expression\textsuperscript{10}. Yet the data shown in my study suggests that cell lines with missense p53 mutations have lower expression of MLL1 as compared to cell lines with WT p53. It is possible that missense p53 mutations bind to the promoter of MLL1 and play a repressive role and the data suggesting that missense p53 mutations act as gene activators could be explained by missense p53 mutations binding tightly (higher affinity) to the MLL1 promoter and by genetic manipulation of missense p53 levels it exposes the MLL1 promoter to a more repressive protein. Further studies need to confirm the role missense p53 mutations have in specific cellular context.

Of the many MLL1 target genes a few candidates were measured that could have an effect with regard to proliferation, metastasis and drug resistance. Emp1 and Amigo2 have been implicated in cancer and are direct MLL1 targets in MLL-AF9 leukemia cells\textsuperscript{17}. Low expression of EMP1 in breast cancer patient samples correlated with poorer clinical prognosis and increased EMP1 in MCF7 cells decreased survival and increased apoptosis\textsuperscript{30}. MLL1 inhibition with MI-2 has been shown to increase EMP1 expression over 7-fold in MLL-AF9 transduced cells and it is the top up-regulated gene\textsuperscript{17}. If EMP1 is a direct target of MLL1 then inhibition could de-repress EMP1 driving expression and potentially decrease the survival of breast cancer cell lines. We hypothesized that MI-2 treatment in breast cancer cells could dramatically
increase the expression of EMP1. The only cell line which showed an increase in EMP1 expression following MI-2 treatment was T47D. Interestingly the cell lines that express the highest amount of MLL1 had the least amount of EMP1 expression (MCF7 and ZR-75-1), supporting the suppressive role MLL1 may have on EMP1. Further studies should focus on the role of EMP1 in breast cancer and understand if increased expression results in decreasing cell survival and increase apoptosis. Another gene interrogated for its role in adhesion was AMIGO2. Specifically, in cancer its upregulation was shown to facilitate attachment to liver endothelial cells and increase metastasis. Amigo2 was shown to be a direct target of Mll1 and downregulation on Amigo2 was observed upon Mll1 inhibition with MI-2. We hypothesized that AMIGO2 would be downregulated in the breast cancer cell lines with MLL1 inhibition but the opposite was observed. This suggest that MLL1 may have a different role at AMIGO2 in these breast cancer cell lines. Further studies should elucidate if AMIGO2 plays a role in liver metastasis in a breast cancer model; liver is the second most common site of metastasis, around 20%. If so, this data should caution MLL1 inhibition in treatment of cells that increase AMIGO2 expression upon MLL1 inhibition.

Looking back to the primary patient data mining results, tumor recurrence could occur as a result of many different factors, one of which could be increased drug resistance. One protein transporter, ABCG2, also known as the breast cancer resistance protein, is an MLL1 target that we hypothesized would decrease upon MLL1 inhibition. ABCG2 functions as an efflux pump that pumps a wide range of molecules from the cytoplasm to the extracellular space. In two cell lines, MCF7 and ZR-75-1, in
which *MLL1* expression is the highest, *ABCG2* was downregulated upon treatment with MI-2. We hypothesized that if MLL1 plays a role in drug resistance, not only by way of decreased *ABCG2*, combinatorial treatment with carboplatin, a DNA damaging agent, and 4-OHT, an estrogen antagonist, could be more effective and less drug would be required in order to achieve a response. In all but the BT-549 cell line, combinatorial treatment was highly effective and when using \( \frac{1}{2} \) the concentration of both drugs it was just as effective as a high concentration alone, indicating a synergistic effect of combinatorial treatment. We cannot conclude that the observed combinatorial results are due to drug resistance as it is not known whether 4OHT or MI-2 are substrates of *ABCG2*. Nonetheless combinatorial therapy can be efficacious for all cell lines regardless of breast cancer subtype, MLL1 expression and missense p53 mutation status. Further studies should look into other MLL1 target genes related to drug resistance and then look to see if those genes play a role in breast cancer cell lines in sensitizing cells to chemotherapy.

In conclusion, we suggest that MLL1 is a viable target in breast cancer and more specifically MLL1 inhibition could enhance the response to standard of care chemotherapeutic agents. Careful analysis of specific traits like anchorage independent growth and proliferation through ATP content revealed that breast cancer cell lines may vary in their utilization of MLL1. Breast cancer cell lines vary by mutation and therefore cell survival, progression, metastatic potential and drug responsiveness could be very different when compared. This would also suggest that not all cell lines depend on MLL1 for any of the above processes or, possibly a worse scenario, MLL1 could be critical for one and increase another. We found that
MLL1 is critical for anchorage independent growth of the MDA-MB-468 cell line. We also observed decreased expression of ABCG2, also known as the breast cancer resistance protein, in MCF7 and ZR-75-1 cell lines upon MLL inhibition suggesting MLL1 in drug resistance and helping to support the observed combinatorial treatment benefits. Gene expression changes in EMP1 could also play a negative role in cell survival and AMIGO2 gene expression changes may forward a caution for some cell lines. Overall cell lines may have different roles for MLL1 and each should be carefully considered. The known MLL1 targets need to be assayed to understand a more complete paradigm of MLL1 as treatment target in breast cancer.
REFERENCE LIST


VITA

The author, Austin Gable Holmes, was born July 23rd, 1989 in Elgin, IL. He attended Wittenberg University in Springfield, OH where he earned a Bachelor’s of Arts, summa cum laude, in Biochemistry and Molecular Biology in 2015. While earning his BA, Austin used computational biochemistry to study binding affinities of glucose/galactose binding protein. After graduation, Austin matriculated into the Loyola University Chicago Stritch School of Medicine Molecular Biology and Biochemistry program and began his graduate education in Cancer Biology under Dr. Zaleznik-Le.

Austin’s Thesis work aims at elucidating the role of MLL1 in breast cancer cells and potential efficacy of using MLL1 specific inhibitors for the treatment of breast cancer. After completion of his Master of Science, Austin will begin his Ph.D. studies at the Driskill Graduate Program at Northwestern University.