THE ROLE OF EZH2 IN GENOMIC STABILITY AND TUMORIGENESIS IN BREAST CANCER

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ABSTRACT:

Enhancer of Zeste Homolog 2 (EZH2) is a protein which over the years has come to be recognized as a *bona fide* oncogene. Originally discovered to be overexpressed in prostate cancer, my research has focused on the role of EZH2 in breast cancer. Preliminary data showed that increased EZH2 expression is associated with higher tumor malignancy and increased invasiveness in the estrogen receptor (ER) negative sub-type of breast cancer. My research initially probed possibilities for EZH2 overexpression in this type of breast cancer. Associated with my work, our lab published data showing that EZH2 overexpression leads to increased proliferation in breast cancer cells, and that this effect is mediated through the tumor suppressor BRCA1. Our studies then moved to further explore the relation between EZH2 and BRCA1 proteins. We show that increased EZH2 expression causes breast cells to sequester BRCA1 in the cytosol, and that this localization is mediated through the phophatidylinositol 3-kinase/AKT (PI3/AKT) pathway. My research specifically showed that increased EZH2 expression leads to an increase in aneuploidy in CAL51 breast cancer cells and in MCF10A benign breast cells, and that in the presence of AKT pathway inhibitors aneuploidy rates return to wild-type levels. Our work in elucidating the mechanisms of EZH2-driven oncogenesis is intimately related to many of the current debates surrounding cancerous transformation as a more general phenomenon. In this thesis, I will highlight my work investigating the relation between EZH2 and genomic stability in the context of our lab's work in investigating the role of EZH2 in breast cancer cells. I shall end with a discussion of the implications of cancer stem cell theory and aneuploidy-driven tumorigenesis theory as possible models of how EZH2 may lead to cancer, as well as the therapeutic implications.

INTRODUCTION:

Breast cancer is the most common cancer among women and is a leading cause of cancer death in the United States and worldwide (1-2). The term "breast cancer" refers to a malignant tumor that has developed from cells in the breast. Breast cancer may occur in both men and women, although male breast cancer is rare (3). Usually breast cancer either begins in the epithelial cells of the lobules and ducts, which are the milk-producing glands and the passages that drain milk from the lobules to the nipple, respectively. Less commonly breast cancer can begin in the stromal tissues, which include the fatty and fibrous connective tissues of the breast. Over time, cancer cells can invade nearby healthy breast tissue and make their way into the underarm lymph nodes, small organs that filter out foreign substances in the body (4). If cancer cells get into the lymph nodes, they then have a pathway into other parts of the body. The cancer cells may attach to other tissues and grow to form new tumors that may damage those tissues. The spread of cancer is called metastasis. For breast cancer, the most common sites for metastatic tumors are the bones, liver, lungs, and brain (5).

Based on rates from 2004-2006, 1 in 8 women will be diagnosed with cancer of the breast during their lifetime. It is estimated that 192,370 women will be diagnosed with and 40,170 women will die of cancer of the breast in 2009 (6). Despite advances in breast cancer treatment, once metastases develop there is no cure (7). This provides great incentive for the discovery of biomarkers of breast cancer risk and prognosis that have potential clinical utility.

With the advent and popularization of the DNA microarray in the late 1990's and early 2000's, this process of searching for prognostic biomarkers in cancer was greatly accelerated due to the ability to compare expression levels of a large number of genes between normal and cancerous cells. In 2002, Varambally et al (as part of the Chinnaiyan research group at the

University of Michigan's Comprehensive Cancer Center) used microarray analysis to compare gene expression between localized and metastatic prostate cancer. At the top of the "list" of genes upregulated in prostatic cancer was the protein EZH2 (Enhancer of Zeste, Homolog 2) (Figure 1), a polycomb group protein (PcG) (8). Polycomb group proteins, along with the trithorax (trxG) group proteins, were known to be implicated in maintaining homeotic gene expression (genes determining cellular identity) (9-10). Knowing that a lack of cell differentiation is a hallmark of cancer, it was hypothesized that EZH2 may contribute to the lethal progression of prostate cancer through dysregulating the transcriptional memory machinery. It was found that a decrease in EZH2 expression led to a significant (≈80%) growth inhibition in these cells as compared to the control cells. The work from this paper provided the first line of evidence that the protein EZH2 mediates cell proliferation and transcriptional repression in prostate cancer cells, as well as that EZH2 mis-expression is a key alteration in malignancies of epithelial origin (10).

Collaborating with the authors of the previous study, in 2003 Kleer et al. took a similar approach towards the investigation of EZH2 expression in relation to breast cancer phenotype (11). Comparing past gene expression sets between neoplastic and normal breast tissue, as well as investigating EZH2 expression in new breast tissue samples, showed that EZH2 protein levels are elevated in the more advanced phases of breast cancer as compared to earlier benign-epithelium. Also a strong association was found between EZH2 levels and patient outcome, with higher EZH2 levels being associated with lower overall survival and a high probability of disease specific death (the ten year survival rates for patients with high and low EZH2 expressing tumors was 24.76% and 58.92%, respectively). Overexpressing EZH2 in normal breast cells was found to promote anchorage independent growth and cell invasion, both mechanisms associated with

Figure 1

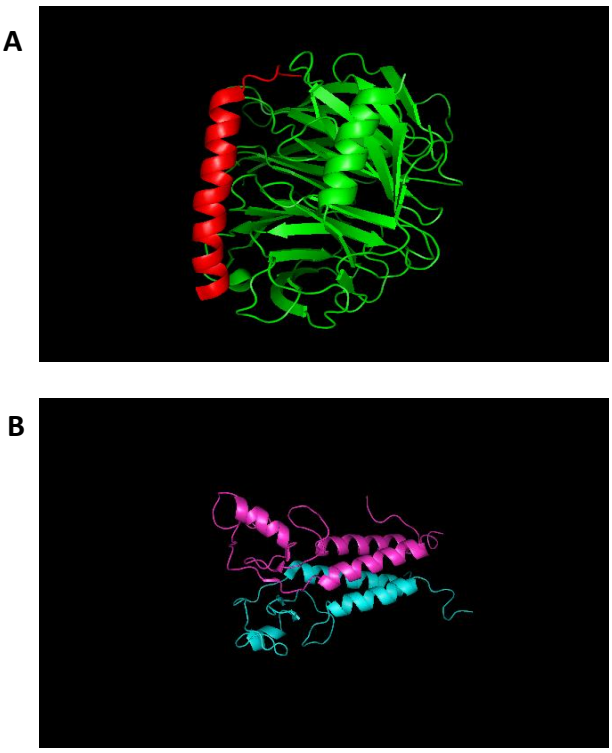


Figure 1: Major proteins of concern. (A) Part of EZH2 (red), bound to binding partner EED (green) as part of its functional complex in the cell. EZH2 contains a SET domain responsible for its methyltranserase activity, as well as two closely spaced SANT domains which are often found in chromatin remodeling enzymes. (73)

(B) BRCA1 (magenta) in complex with BARD1 (teal), forming the heterodimer observed in its main cellular function. BRCA1 forms its heterdimer through its RING domain. It also contains a BRCT domain which binds phosphorylated proteins, and is present in many DNA-damage response proteins. (74)

aggressive cancers. This study thoroughly established EZH2 as an independent predictor of survival of women with breast cancer and a functionally important protein in tumorigenesis (11). Subsequent investigations have confirmed these findings on the prognostic value of EZH2 in breast cancer (12-14). Taken together, these data strongly suggest that EZH2 has a pivotal role in the tumorigenic process in multiple organs and tissues.

These two papers have laid the foundation for our laboratory's ongoing research. In the years since, research has grown exponentially on EZH2, leading to further illustration of its cellular functions, as well as demonstrating its role in tumorigenesis in an assortment of other human malignancies including transitional cell carcinoma of the bladder (15-16), aggressive and

invasive urothelial carcinomas (17), endometrial cancer (18), Wilms tumor (19), and heptocellular carcinoma (20).

To understand the mechanism of EZH2 function in breast carcinogenesis, it is important to first understand how EZH2 functions in development. EZH2 is a polycomb group protein. PcG proteins, along with the Trithorax group (TrxG) proteins, are involved with maintaining "cellular memory" and are intimately involved in the cellular development process. (21) That is to say, these proteins are involved in creating and maintaining the DNA transcriptions patterns that are unique to different cell types. In general, PcG proteins maintain gene silencing while trxG proteins maintain active states. Expression of the PcG proteins is high during embryonic development, where most of the biological mass is made of undifferentiated stem cells. The high expression of these proteins represses the expression of genes related to cell differentiation, giving stem cells their characteristic plasticity. As the organism develops, inherited methylation patterns will appear on genes selected for repression, concomitant with the declining expression of the PcG proteins. This process effects terminal cell differentiation through differential gene expression and methlyation patterns capable of being passed on to daughter cells (21).

PcG proteins effect this chromatin remodeling through two complexes referred to as polycomb repressor complexes 1 and 2 (PRCs) (17, 21). PRC1 includes BMI1, HPC proteins, and RING proteins, whereas the PRC2 complex comprises EZH2, EED, and SUZ 12 (22). EZH2 contains a SET domain, a highly conserved domain found in many proteins with histone methyltransferase (HMT) activity. Functionally, EZH2 is the catalytically active component of PRC2 and is capable of methylating lysine 9 (H3K9) and lysine 27 (H3K27) of histone H3 (24). In addition to the SET domain, EZH2 contains two closely spaced SANT domains, which are often found in chromatin remodeling enzymes (23). Repression is mediated when PRC2 is

recruited to chromatin, where the methyltransferase EZH2 catalyzes H3 trimethylation of lysine 27 (triMeK27-H3) (24). This histone mark then provides a platform to recruit PRC1 (24) which aids in PcG-mediated repression either by chromatin compaction or by interfering with the transcription machinery (21,26,27). Working together, PRC1 and PRC2 are responsible for creating and maintaining gene expression patterns specific to different types of differentiated cells, and their proper function is required to correctly pass these methylation patterns on to daughter cells. Without EZH2 activity, PRC1 cannot be recruited to chromatin, and PcG-mediated repression is not established (24,28).

Non-canonical roles for EZH2 have also been discovered. Shi et al. have reported that EZH2 was able to activate gene transcription through mechanisms that do not involve histone methylation (29). It has also been reported that EZH2 can function by forming transcriptional complexes independent of its methyltransferase activity (30). In addition, cytosolic EZH2 complexes have been found to control cellular signaling via actin polymerization in various cell types (31). With such duplicity in functional roles, it has been difficult to elucidate precise mechanisms through which EZH2 influences breast cancer progression.

Breast and ovarian cancer type 1 susceptibility protein (BRCA1) is a tumor suppressor involved in DNA damage repair, activation of cell cycle checkpoints and maintenance of chromosome stability (32). The *BRCA1* gene mutation was one of the first deregulations correlated with breast cancer occurrence, and accounts for most familial cases of breast and ovarian cancer (33). Heterozygous germ-line mutations in the BRCA1 gene predispose women to breast and ovarian cancer with a lifetime risk of breast cancer up to 80% (34). Although somatic mutations of BRCA1 are not common, expression of its messenger RNA and protein are reduced in approximately 40% of sporadic breast carcinomas (35-37). The vast majority of breast

tumors in these patients display a basal-like phenotype, and BRCA1 dysfunction by downregulation, mutation or other mechanisms has been suggested to have an etiological role in the development of this aggressive breast cancer subtype (38-39).

It has been found that BRCA1 plays a major role in maintaining genome integrity through its ability to form various distinct protein complexes, each of which is dedicated to a specific cellular function in the DNA damage response pathway. To date at least three distinct BRCA1-containing protein complexes have been emerged, identified as BRCA1A, BRCA1B, and BRCA1C. BRCA1A functions to target BRCA1 to double strand breaks in DNA for repair, and also to transiently inhibit entry into mitosis in the presence of DNA damage to avoid aberrant chromosome segregation (40). BRCA1B is serves to inhibit DNA synthesis if DNA damage is present, thereby avoiding the stalling or collapsing of replication forks during the S-phase (41). BRCA1C functions in damage signaling and homologous recombination repair of double strand DNA breaks. BRCA1 has emerged as a master regulator of genome integrity through these diverse protein associations involved in somewhat redundant yet important cellular processes ensuring genomic stability and promoting cell survival.

Our lab's research aim is to evaluate the oncogenic function of EZH2 in the breast and elucidate the relevant mechanisms of this process. This knowledge may be applied to the clinical field as inhibition of EZH2 may halt or prevent cancer progression.

Estrogen receptor (ER) negative breast cancer cell lines exhibit high EZH2 protein expression. Our initial studies involved knocking down EZH2 expression in CAL51 and MDA-MB-231 breast cancer cells using lentivirus-mediated short-hairpin RNA (shRNA) and comparing phenotypes between these cells and control cancer cells. The results of our experiments showed that EZH2 knockdown decreases proliferation of ER-negative breast cancer

cells and causes a delay in the G2-M transition of the cell cycle. Our data revealed that EZH2 modulates BRCA1 protein levels, as well as its phosphorylation at serine 1423, an important modification in the regulation of the G2-M transition. We also show that BRCA1 is required for the proliferative and G2-M effects of EZH2. We then highlighted the relevance of our in vitro and animal findings by demonstrating that ER-negative invasive carcinomas have high levels of EZH2 and concomitant low levels of BRCA1 protein. These initial studies provided the first published functional link between EZH2 and BRCA1 proteins in breast cancer cells, and further proposed that EZH2 knockdown in BRCA1 deficient cancer cells could possibly restore BRCA1 levels and function, as well as providing novel insight as to ER-negative breast cancer tumor treatment.

Our research next aimed to investigate this newly found relationship between EZH2 and BRCA1, as well as its implications in EZH2-mediated tumorigenesis. To do this, we created an inducible expression system in MCF10A normal breast cells, which would overexpress EZH2 protein in the presence of Doxycylcline in the cell media. To complement these studies, we also used the CAL51 cancer cell shRNA system previously described.

In investigating the link between these two proteins, we discovered that EZH2 regulates the intracellular localization of BRCA1 protein in benign breast cells and in breast cancer cells during cell cycle progression. Increased EZH2 expression leads to increased nuclear export of BRCA1 in both MCF10A and CAL51 cell lines, and this is sufficient to trigger aberrant mitosis with extra centrosomes and aneuploidy. EZH2 inhibition in tetraploid CAL51 breast cancer cells induces BRCA1 nuclear accumulation and rescues defects in ploidy and mitosis. Mechanistically, this data shows that EZH2-induced BRCA1 nuclear export, mitotic defects, and aneuploidy require activation of the PI3/Akt signaling pathway.

In all, my research in the Kleer lab has followed the long of this story, beginning in 2007 through the present day. Although beginning with more modest projects, my data now represents significant contributions to our lab's research aim, to illustrate the role of EZH2 in breast cancer and breast cancer transformation. The experiments I have performed are part of two research manuscripts, one published, and the other one in preparation. In this thesis I will highlight the data I personally gathered (while giving credit to collaborating faculty for corroborating data) in the context of our lab's larger story, and through this I will incorporate the future directions and current debate at the cutting edge of breast cancer research.

RESULTS:

EZH2 expression is increased and deregulated in breast cancer cells in the absence of EZH2 gene mutation

A panel of breast cancer cell lines was investigated for EZH2 expression. These cells include the nontumorigenic line MCF10A and breast cancer cells MDA-MB-231, SUM149, CAL51, and MCF7. Western Blot analysis shows that breast cancer cells have higher EZH2 expression than the benign breast cells. (Figure 2)

Figure 2

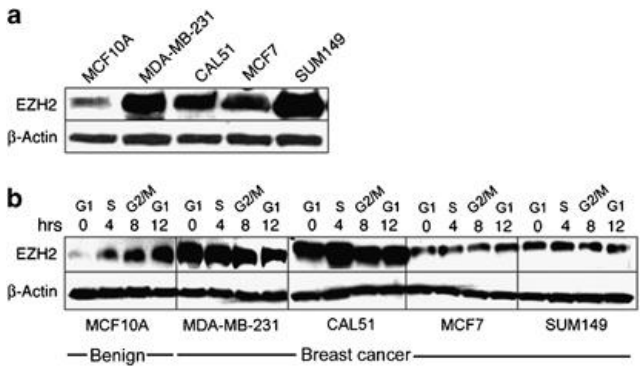


Figure 2: EZH2 is upregulated and deregulated in breast cancer cells. (**a**) Immunoblot for EZH2 in a panel of breast cells shows that EZH2 protein is elevated in breast cancer cells when compared with the nontumorigenic MCF10A cell line. (**b**) EZH2 is deregulated in breast cancer cell lines compared with MCF10A cells. **Data from M.E.G.**

EZH2 expression has been observed to be cell cycle regulated in diploid fibroblasts, and so our lab sought to determine whether EZH2 expression exhibited a similar pattern of regulated expression in both normal and cancerous breast cells (42). To this end, benign and cancerous breast cells were subjected to double thymidine block and subsequently restimulated by their medium to enter the cycle. Figure 2 shows that EZH2 protein expression is cell growth regulated

and accumulates at the G1/S transition in benign MCF10A cells, similar to the expression seen in fibroblasts (42). This regulation of EZH2 expression is lost in cancer, as seen in the four breast cancer lines studied which exhibit unregulated, high expression of EZH2.

To determine whether this deregulation of EZH2 expression in breast cancer cells was due to an activating mutation in the *EZH2* locus, I sequenced the locus for MCF10A and CAL51 cell lines by direct sequencing. The *EZH2* locus contains 2,695 base pairs, so sequencing involved designing numerous forward and reverse primers to cover this length. After obtaining the data, the experimentally determined sequences were then compared to that of the normal sequence found in the NCBI database, checking for any possible mutations between the cell lines. The results of this sequencing showed that there were no mutations in the CAL51 *EZH2* locus (Figure), ruling out the possibility of an activating mutation in the *EZH2* reading frame being the cause of its deregulated expression in breast cancer.

Increased EZH2 levels increase cyclin transcription levels and proliferation rates in breast cancer cells

To study the effects of EZH2 expression on cell proliferation, we used shRNA interference to stably knockdown EZH2 in breast cancer cells. EZH2 knockdown in MDA-MB-231 and CAL51 cells significantly decreased proliferation (Figure 4). In searching to determine how EZH2 increases proliferation in cells, we hypothesized that it may be due to upregulating the expression of key cell cycle proteins, such as members of the cyclin family. Cyclins are proteins which are expressed at varying levels during the cell cycle, and their association with Cyclin dependent kinases (Cdks) is what drives cells through mitosis. To investigate if EZH2

expression regulated mRNA levels of key cyclins, I performed Real time PCR (RT-PCR) in CAL51 shVector and shEZH2 cell lines. My results show that EZH2 increases the mRNA expression of these key cyclins, therefore consistent with the observed increased proliferative phenotype of EZH2 overexpressing cells (Figure 4).

This data was supported by other techniques performed in our lab. We found that EZH2 knockdown significantly increased the number of cells at the G2-M transition, while also decreasing the phosphorylation of many key proteins necessary for entry into mitosis. Using flow cytometry, we found that EZH2 knockdown caused over a 50% reduction in the percentage of breast cancer cells undergoing mitosis. Collectively, these data show that EZH2 is important in the regulation of the G2-M transition and the number of cells undergoing mitosis, which have major influence in tumor growth. Our lab complemented these studies with in vivo experiments, where MDA-MB-231 breast cancer cells (both shEZH2 and shVector) were injected into immunocompromised mice and tumor growth was examined. We found that EZH2 knockdown mice had decreased tumor growth rate, decreased tumor size, and better survival rates when compared to control mice. It was also observed that mitotic activity also significantly decreased in shEZH2 tumors as compared to control.

Figure 4

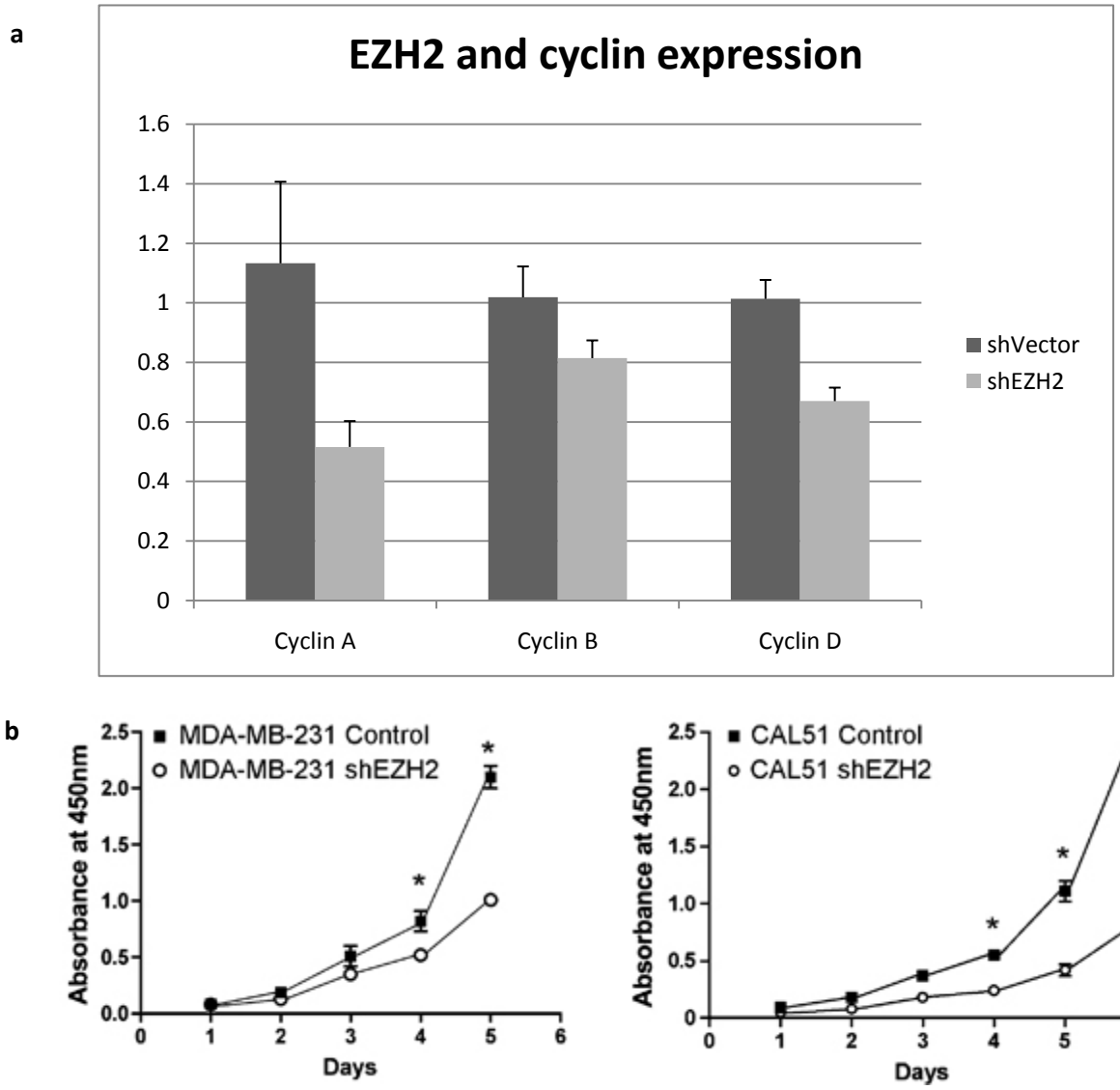


Figure 4: EZH2 inhibition decreases proliferation of breast cancer cells (**a**) Increased EZH2 expression is associated with increased cyclin mRNA levels, providing link to its observed effect on breast cell proliferation. Quantitative real-time PCR (qRT-PCR) measured the mRNA levels of cyclins A, B, and D in both CAL51 vector cells and EZH2 knockdown cells. Cyclin A expression is reduced by half, cylin B is reduced by a fifth, and cyclin D expression is reduced by a third in CAL51 shEZH2 as compared to shVector cells. This data provides a possible functional insight into how EZH2 overexpression in breast cancer cells increases proliferation EZH2 inhibition decreases proliferation of breast cancer cells. (**b**) Time course of proliferation determined using the Wst-1 assay. shEZH2 in CAL51 and MDA-MB-231 cells significantly decreases their doubling time when compared with vector control-transduced cells (Student's *t*-test, $P<0.001$ for both cell lines). **Proliferation data from M.E.G.**

EZH2's influence on proliferation is mediated through BRCA1

The rest of our first paper gave first light to the relation between EZH2 and BRCA1 proteins. Although I did not contribute to this section of our paper, I will summarize our findings to illustrate the scope and significance of our findings. Studies have demonstrated that EZH2 overexpression in human breast carcinomas is associated with the ER-negative basal-like phenotype, characterized by low BRCA1 protein expression (11,14). From this, it was hypothesized that EZH2 may promote breast tumorigenesis by regulating BRCA1 in this subtype of breast cancer. Studies in ER-negative breast cancer cells showed that EZH2 downregulation increased the protein levels of total BRCA1 and BRCA1 phosphorylated at serine 1423 in the nuclei. Consistantly, overexpression of EZH2 in nontumorigenic MCF10A cells resulted in a 75% decrease in nuclear BRCA1 protein and a 67% decrease in nuclear pBRCA1 s1423.

To investigate whether the effects of EZH2 expression on cell proliferation and transition from the G2 phase to mitosis requires BRCA1 protein, the expression of EZH2 and BRCA1 were both knocked down using shRNA in CAL51 breast cancer cells. We observed that BRCA1 knockdown in shEZH2 CAL51 cells was sufficient to completely rescue the reduction in proliferation and the G2-M cell cycle arrest caused by EZH2 downregulation. Inhibition of BRCA1 alone in CAL51 cells had no effect on proliferative activity. We also found that BRCA1 knockdown abolished the effect of EZH2 inhibition on important mitotic proteins. Taken together, these data provide strong evidence supporting the hypothesis that the decrease in cell proliferation and prolongation of G2 caused by EZH2 knockdown is mediated through BRCA1.

To investigate the relevance of these previous in vitro and in vivo studies, a large sampling of invasive breast carcinoma tissue was probed for EZH2 and pBRCA1 expression and

possible correlation. It was found that in 76% of ER-negative tumors (associated with high EZH2 expression) were negative for pBRCA1 s1423, further strengthening this link between EZH2 and BRCA1.

The functional connection between EZH2 and BRCA1 proteins was our next area of investigation.

Inducible EZH2-expressing MCF10A System Engineered

To further our investigation into the effect of EZH2 on cell proliferation and tumorigenesis, as well as the relationship between EZH2 and BRCA1, we wanted to create a cell-line system in which we could overexpress EZH2 in benign breast cell. I was put in charge of the project to engineer this system which would overexpress EZH2 in the presence of Doxycycline in the cell media. Greater detail as to how this system works and how it was engineered to express our protein is found in the methods section. MCF10A cells naturally express very low concentrations of EZH2, and Western blot analysis confirmed that EZH2 concentrations significantly increased in the cell when exposed to Dox.

EZH2 regulates the nuclear-cytoplasmic shuttling of BRCA1 in benign and in breast cancer cells.

EZH2 upregulation in the MCF10A cells resulted in nuclear export of BRCA1 protein and increased BRCA1 cytoplasmic concentrations, as evinced by both Western blot and immunofluorescence analysis (Figure 5). Similar results were found in CAL51 shVector and shEZH2 cells, which showed that CAL51 breast cancer cells exhibit predominantly cytoplasmic

and perinuclear BRCA1 protein whereas CAL51 shEZH2 cells had accumulation of BRCA1 in the nucleus. This data reveals that EZH2 influences the intracellular localization of BRCA1 protein in non-tumorigenic breast cells and in breast cancer cells.

Figure 5

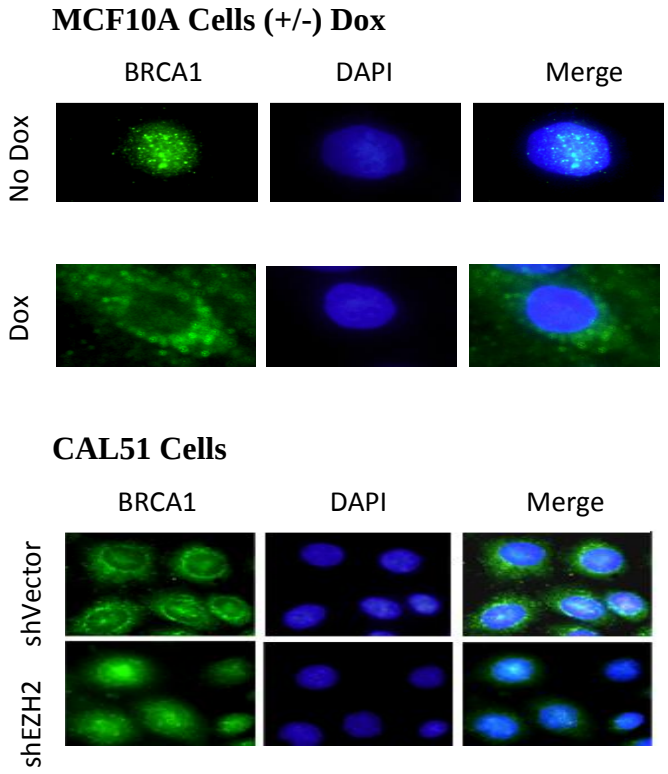


Figure 5: EZH2 regulates nuclear/cytoplasmic shuttling of BRCA1. DAPI is a fluorescent stain which strongly binds DNA, identifying the nucleus. The top picture shows fluorescence data gathered from the Tet-On EZH2 overexpressing MCF10A cell line. BRCA1 and DAPI are independently detected in the first two columns, and the images are merged in the third column. The MCF10A data shows that there is increased cytosolic BRCA1 concentration with increasing EZH2 concentration. The bottom pictures show similar data for CAL51 breast cancer cells. In vector CAL51 cells (with high EZH2 expression), there is observed a high cytosolic BRCA1 concentration. Knocking down EZH2 expression using shRNA shows increased nuclear BRCA1 concentrations. Together this shows that EZH2 plays a significant role in BRCA1 subcellular localization. **Pictures from M.E.G.**

Overexpression of EZH2 protein induces centrosome amplification and abnormal mitosis

Most malignant tumor cells have been found to have an abnormal number of chromosomes, or aneuploidy, and frequently contain multiple centrosomes, which are the microtubule-organizing centers required for proper chromosome segregation (43). Seeing as EZH2 expression is elevated in many ER-negative invasive breast carcinomas, which display many of these malignant characteristics, we hypothesized that EZH2 overexpression may induce alterations in mitosis and centrosome number. Immunofluorescence analysis of mitotic nuclei with α-tubulin and p-H3 (SER10) antibodies revealed that doxycycline-induced EZH2 upregulation in MCF10A cells led to mitotic defects characterized by the presence of multiple mitotic spindles and extra centrosomes (Figure 6A). This contrasted with the absence of mitotic defects in control MCF10A cells.

Aurora A is a key protein involved in centrosome maturation, separation, and spindle formation. We hypothesized that the defective mitotic spindles observed upon EZH2 overexpression in MCF10A cells resulted from centrosome abnormality. We found that Aurora A properly localizes to the centrosome during metaphase, as evidenced by the two distinct foci that colocalized to the spindle poles and MCF10A control cells had two Aurora A foci. However, although Aurora A was properly localized, EZH2 overexpression caused a greater than 6 fold increase in the percentage of mitotic cells with more than two Aurora A foci, indicating increased Aurora A expression and suggesting centrosome amplification. These cells contained either a single giant nucleus or multiple multilobed nuclei. EZH2-induced extra centrosomes were associated with multipolar mitosis (more than three and up to six spindle poles) as compared with control MCF10A cells (example pictures given in Figure 6).

We next investigated the effect of EZH2 downregulation on mitotic spindle formation and Aurora A containing centrosomes in CAL51 breast cancer cells (Figure 6B). CAL51 breast cancer cells are diploid but contain a tetraploid population with centrosome amplification, multiple mitotic spindles, and aberrant mitoses. Thus, they constitute a good model to test the effect of EZH2 knockdown on the number of centrosomes, mitotic spindle formation, and mitotic defects. 13.6% of CAL51 cells transduced with scrambled shRNAs have aberrant mitoses characterized by increased number of centrosomes. In contrast, CAL51 shEZH2 cells exhibit a 5-fold reduction in the number of cells with aberrant mitoses and supernumerary centrosomes. Collectively, these data show that EZH2 may play a role in mitosis by regulating the number of centrosomes in benign and breast cancer cells.

Further data our lab collected also showed that EZH2 expression is correlated with the protein kinases Aurora A and B expression, suggesting further that EZH2 may mediate its influence on mitosis through the regulation of these proteins.

Figure 6

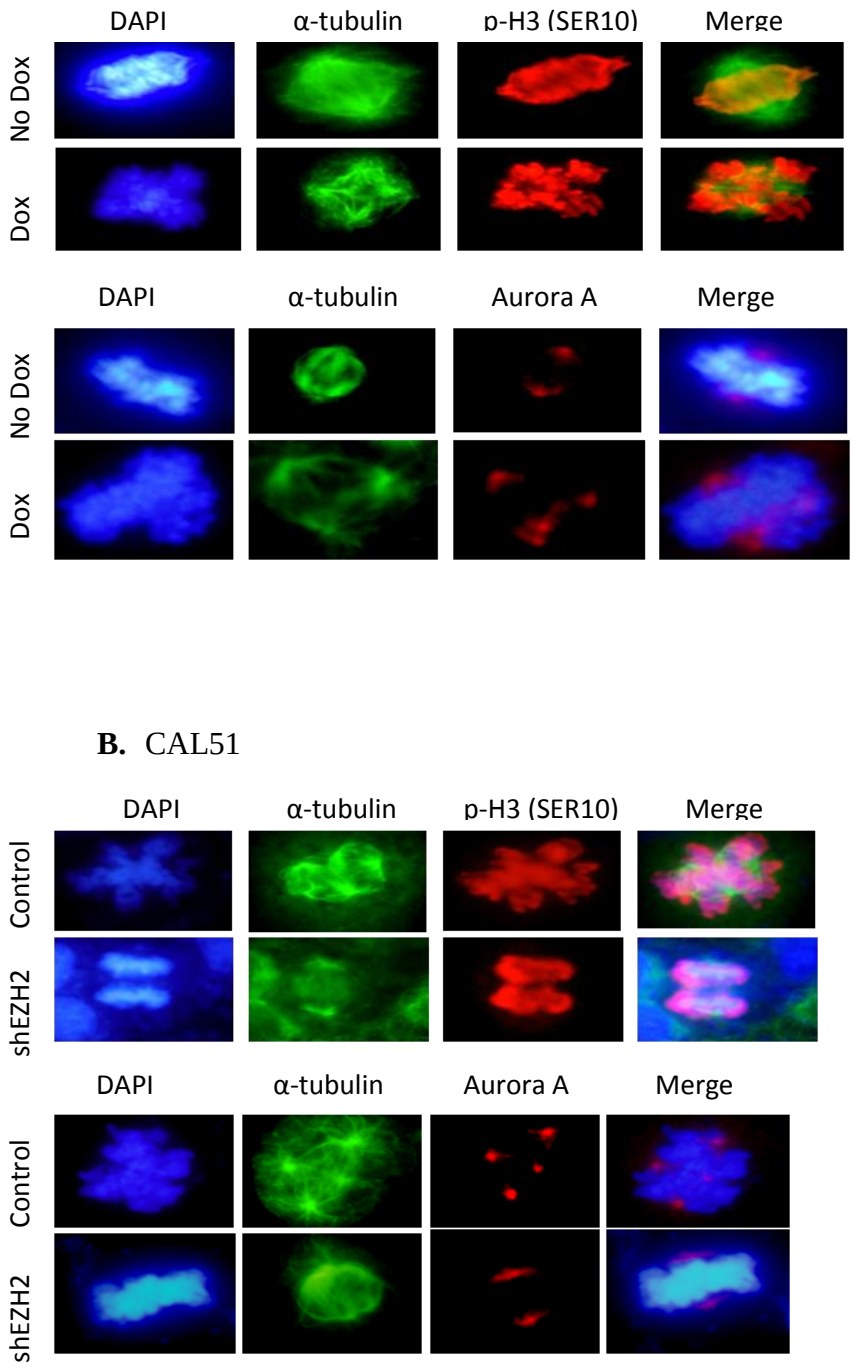


Figure 6: EZH2 overexpression increases frequency of mitotic aberrations. Confocal fluorescence imaging was used to visualize the localization of mitotic machinery in high and low concentrations of EZH2 in MCF10A and CAL51 cells. DAPI stains the nucleus, α-tubulin is involved in the mitotic spindle connecting centrosomes and chromosomes, p-H3 is a histone indicative of chromosomal alignment, and Aurora-A is involved and localizes on the centrosome structures. (**A)** shows the experiments for the MCF10 Tet-On System. With no dox added, the normal bipolar mitotic alignment can be seen (two spindle poles with actin extending from poles and connecting to the chromosomes, which are a linearly aligned in middle). The merge in the top section shows this proper alignment. With dox added, mitotic aberrations are more frequently observed. In this example, actin can be seen to come from many directions, and the chromosomes are much more disorganized. Also seen is an increase in Aurora A foci observed, indicative of centrosome amplification, known to cause aberrant mitosis. **(B)** shows complementary data in the CAL51 system. In control cells the mitotic spindle is more frequently disorganized, although knocking down EZH2 expression decreases mitotic aberrations in these cells. **Pictures from M.E.G.**

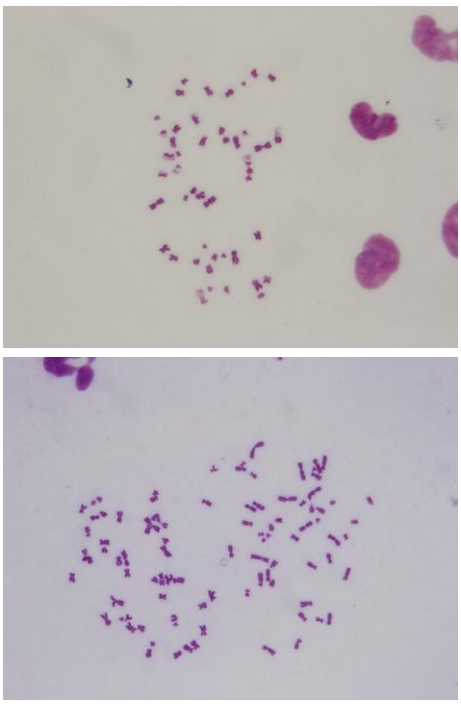
EZH2 overexpression causes genomic instability

Because errors in mitosis can lead to genomic instability and the development of cancer, we next investigated whether EZH2 exerts a role in the maintenance of genomic integrity. To investigate aneuploidy in our target cell lines I used a metaphase spread technique, a method which fixes cells on a microscope slide and with the proper staining renders the chromosomes of each individual cell visible and quantifiable (Figure 7A). EZH2 expression was found to be correlated with increased prevalence of aneuploidy (Figure 7B). Tet-On MCF10A cells were exposed to Dox for 1, 3, and 5 days before performing the metaphase spread technique. Significant increases in aneuploidy were not observed until the third day of EZH2 overexpression. Giemsa stained metaphase spreads revealed that while 95% of untreated MCF10A controls are diploid, 19.2% of cells became aneuploid after 72 hours of exposure to Dox. We did not observe structural chromosomal abnormalities. Since CAL51 cells are diploid with a tetraploid population, we reasoned that they constitute a good model to test if EZH2 can rescue ploidy abnormalities. Giemsa stained metaphase spreads showed that EZH2 knockdown decreased the percentage of tetraploid CAL51 cells from 23.2% to 9.2%, indicating that EZH2 downregulation can revert tetraploidy (Figure 7C). Taken together, this data reveals that besides its ability to regulate the number of centrosomes and mitotic spindle formation, EZH2 plays a role in the maintenance of genomic stability.

Figure 7

Figure 7: High EZH2 expression associated with increased aneuploidy. The metaphase spread technique allows for the visualization of the chromosome count of individual cells. Around 25 cells were counted on each slide, and three slides were counted for each cell line. (**A**) An example of what is visualized with this technique. The top picture shows a normal cell, while the bottom picture displays an aneuploid cell (84 chromosomes). (**B**) The percentages of anueploidy counted in MCF10A cells for 3 days and 5 dayls with added Dox. Aneuploidy significantly increases upon increase in EZH2 expression. When Dox is added aneuploidy jumps from 3% to 19%(**C**) The rates of aneuploidy were counted for these four CAL51 cell lines. It can be seen that when EZH2 expression is decreased using shRNA in CAL51 cells, aneuploidy levels drop significantly from 23% to 9%. Also interestingly, when BRCA1 expression is abrogated as well as EZH2 using shRNA, aneuploid levels almost revert back to the normal phenotype, suggesting that BRCA1 may be responsible for the EZH2's effect on genomic stability.

A



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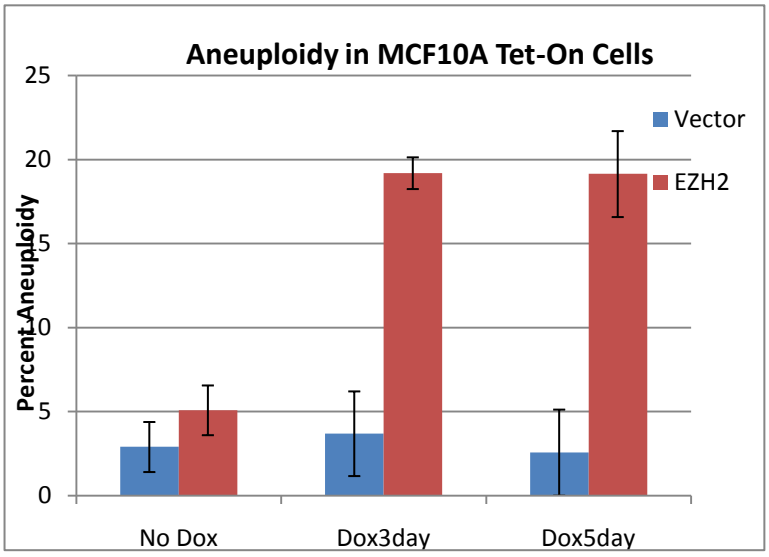

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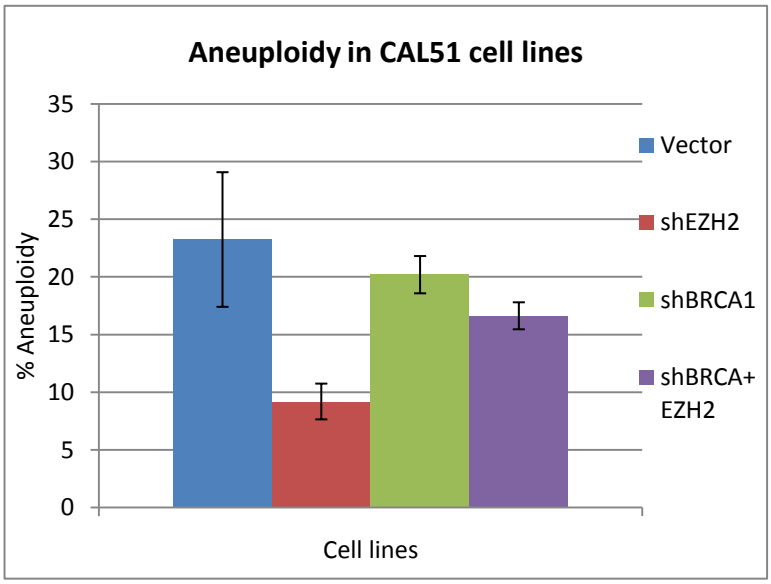


EZH2-induced BRCA1 nuclear export, mitotic defects, and aneuploidy require activation of PI3K/Akt

Although the mechanism that regulate the intracellular localization of BRCA1 protein are not fully elucidated, recent studies suggest that the membrane serine/threonine protein kinase Bα (AKT1) may play a role. In 2008 Plo et al. reported that AKT1 activation by phosphorylation of serine 473 results in cytoplasmic localization of BRCA1 (44). This led us to hypothesize that EZH2 may regulate the intracellular distribution of BRCA1 in normal breast cells and in breast cancer cells through the PI3/AKT1 pathway.

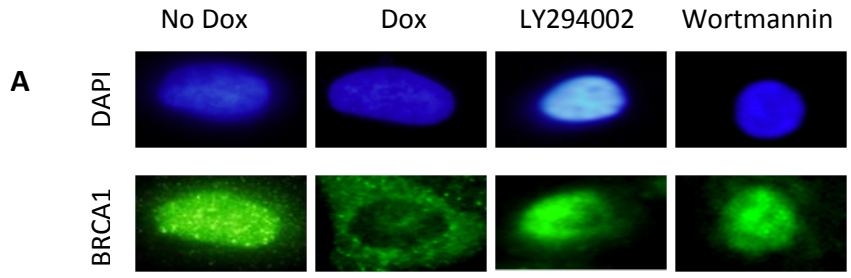
Our laboratory shows that overexpression of EZH2 in MCF10A cells increases AKT and AKT phosphorylation at serine 473, which is required to promote its maximal activation. The levels of total and phosphorylated at SER473 AKT1 were also upregulated when EZH2 was overexpressed. These results were similarly observed in CAL51 breast cancer cells. CAL51 EZH2 knockdown cells exhibited a drastic decrease in both AKT and AKT1 phosphorylation at serine 473.

The significance of the PI3K/AKT pathway for BRCA1 intracellular localization in EZH2 overexpressing MCF10A cells was next evaluated in a series of time course experiments. BRCA1 protein was detected by immunofluorescence and by Western blot in the nuclear and cytoplasmic-enriched fractions in the presence or absence of the PI3K inhibitor LY294002 or Dox (Figure 8A). In the absence of the PI3K inhibitor, induction of EZH2 overexpression with Dox led to high levels of pAkt SER473 and pAkt1 SER 473. Following LY294002 treatment, pAKT and pAKT1expression dropped to almost undetectable levels. While Dox-induced EZH2 upregulation decreased BRCA1 and pBRCA1 proteins in the nuclei of MCF10A cells, inhibition

of PI3/AKT with LY294002 rescued BRCA1 and pBRCA1 nuclear expression. Similar results were observed using Wortmannin to inhibit PI3K/AKT pathway. Immunofluoresence analysis supported the immunoblot results as LY294002 or Wortmannin increased the expression of BRCA1 in the nuclei reverting EZH2-induced nuclear export of BRCA1 (Figure 8A).

To investigate if EZH2 requires activation of the PI3/AKT pathway to trigger mitotic defects and aneuploidy, I analyzed chromosome numbers and mitotic aberrations utilizing metaphase spreads and immunofluorescence in the absence and presence of doxycylcline and the PI3K/AKT inhibitors, LY294002 or Wortmannin (Figure 8B). As shown previously, doxycycline-induced EZH2 overexpression causes aneuploidy in MCF10A cells after 72 hours. LY294002 or Wortmannin treatment rescues EZH2-induced aneuploidy (figure). It was also found that LY294002 or Wortmannin treatment also reverted the percentage of cells with extra centrosomes back to near wild-type levels. Altogether, these results demonstrate that EZH2 upregulation increases AKT and AKT1 phosphorylation at serine 473 and that activation of the PI3K/AKT is essential in the EZH2-induced BRCA1 nuclear export, aneuploidy, and mitotic defects.

Figure 8



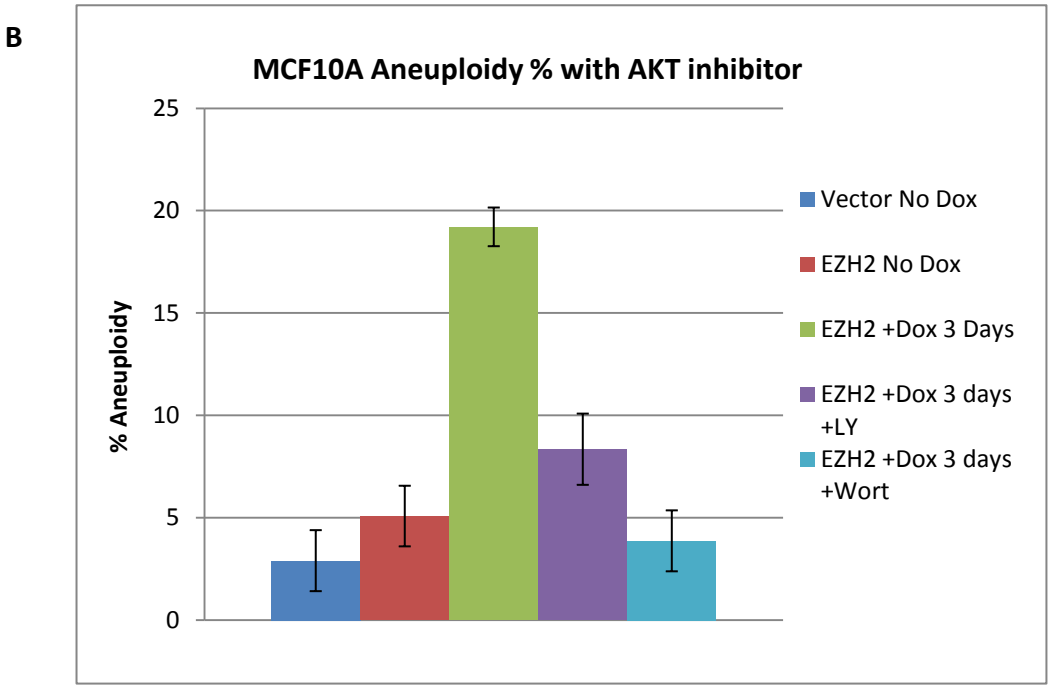


Figure 8: EZH2 mediates BRCA1 localization and effects on mitosis through PI3K/AKT pathway. (**A**) Fluroescence data of MCF10A Tet-On cells. With low EZH2 (no dox) BRCA1 is found predominantly in the nucleas. With the addition of dox, EZH2 protein levels increase and BRCA1becomes predominantly found in cytosol. Adding the AKT inhibitors LY and Wortmannin abrogates the cytosolic shuttling of BRCA1. **Pictures from M.E.G.** (B) Aneuploidy counting from MCF10A cell line again, this time in presence of AKT inhibitors. Adding Akt inhibitor decreases aneuploidy to near wild-type levels in these cell lines. This graph shows that the addition of the PI3/Akt pathway inhibitors LY and Wortmannin decrease the prevalence of aneuploidy in MCF10A cells.

DISCUSSION:

Our research significantly contributes to our modern understanding of the function of EZH2 in breast cancer. We have first shown that EZH2 overexpression in ER-negative breast cells increases cell proliferation and tumor growth both *in vitro* and *in vivo*. Next we have shown that EZH2 plays a role in regulating the sub-cellular localization of BRCA1 through the PI3K/AKT pathway. Finally, we have shown that EZH2 overexpression increases the frequency of mitotic aberrations and aneuploidy in breast cells. This brings us to the pertinent question of if and how these newfound functions of EZH2 contribute to breast cancer initiation or predisposition. I will discuss the implications and interpretation of our data in the context of current cancer models later in the discussion. The bottom line of our research is that in providing a greater illustration of how EZH2 functions in ER-negative breast cancers, we are providing essential information for the guidance of novel treatments and cures that could target EZH2 and mitigate its tumorigenic effects.

EZH2 has been found to be significantly overexpressed in certain subtypes of breast cancer (basal-type tumors, characterized by estrogen receptor (ER), progesterone receptor (PR) and Her-2/neu negative status as well as low levels of BRCA1 protein) (14), and is associated with larger tumor development, increased tumor proliferation rates, increased invasiveness, and worse prognosis in patients (11). While EZH2 overexpression leads to this litany of negative tumor phenotypes, knowledge of this fact has allowed EZH2 to become an independent marker of prognosis, recurrence and metastasis in women with breast cancer (11). My first research project delved into investigating potential mechanisms of EZH2 overexpression in breast cancer. My data showed that the deregulated expression is not due to genetic mutations, and other groups have come to show other possible mechanisms of EZH2 deregulation independent of mutation.

Varambally et al. has shown that microRNA-101 regulates EZH2 expression levels in prostate cancer cells by binding to the 3' untranslated region of its mRNA (45). Bracken et al. 2003 have shown that the EZH2 promoter is a target of E2F transcription factors (42), and that EZH2 expression is therefore regulated through the pRb-E2F pathway. Other groups have traced back EZH2 regulation to p53 control (46).

EZH2's effect on proliferation has also been well documented in the past, and my data has helped shed more light on the mechanisms through which EZH2 influences proliferation in breast cancer cells. I found that increased EZH2 expression is associated with an increase in cyclin levels, while others in our lab have found that increased activation levels of other essential cell-cycle proteins are also associated with higher EZH2 expression. Another major novel finding presented in our first paper was that EZH2 downregulation in aggressive ER-negative breast cancer cells greatly decreases their proliferative capacity and rate of progression through the cell cycle, causing a prolonged doubling time of ER-negative breast cancer cells and caused an arrest at the G2/M transition of the cell cycle. These studies were then complimented with previously unexplored *in vivo* experiments; targeted downregulation of EZH2 in aggressive ER-negative breast cancer cells resulted in significant reduction of mammary tumor size as well as improved survival.

One of the most important conclusions of our first paper was to show that EZH2 mediates its proliferative effects through BRCA1. BRCA1 is known to play a significant role in breast cancer, and in addition to its function in hereditary breast cancer, BRCA1 expression is reduced in up to 40% of sporadic breast carcinomas (Wilson, Yoshikawa, Turner). Although BRCA1 promoter methylation is responsible for 10-15% of sporadic breast carcinomas, it does not explain BRCA1 deficiency in the remainder of the tumors, leaving the door open to other

possible alternatives of BRCA1 modulation. We demonstrate that EZH2 knockdown in ER-negative breast cancer cell lines causes upregulation of BRCA1 protein levels with a concomitant increase in pBRCA1 s1423, the total amount of the latter being crucial for G2/M arrest (47-48). Consistantly, ectopic overexpression of EZH2 in benign breast cells decreases nuclear BRCA1 and pBRCA1 s1423 protein levels. Our data demonstrate that the observed effects of EZH2 downregulation on breast cancer proliferation and G2/M transition require BRCA1, as BRCA1 inhibition was sufficient to completely rescue the decrease in cell proliferation and the delay in G2 caused by EZH2 downregulation.

Our experiments show that EZH2 knockdown is sufficient to increase BRCA1 levels in breast cancer cells. We propose that EZH2 knockdown in breast cancer cells reduces their growth by enhancing the cell-cycle regulatory effects of BRCA1, slowing the transition from G2 to M phases, and allowing more time for DNA repair to occur. This hypothesis is supported by our experiments showing that breast cancer cells with EZH2 knockdown have increased levels of pBRCA1 s1423 protein, which is essential for G2/M arrest and is activated during the DNA damage response (48). EZH2 knockdown had no significant effect on BRCA1 messenger RNA. We have no evidence by co-immunoprecipitation of a direct interaction between EZH2 and BRCA1 proteins.

The relevance of the association between EZH2 and BRCA1 proteins to human breast cancer is highlighted by the finding that 76% of ER-negative invasive carcinomas overexpress EZH2 and are negative for pBRCA1 s1423. Our work demonstrated a previously undescribed function of EZH2 during ER-negative breast cancer progression by showing that EZH2 knockdown decreases tumor proliferation and growth in vivo and in vitro, and influences the transition from G2 phase to mitosis. Our first paper revealed the first link between EZH2 and

BRCA1 proteins and showed that EZH2 knockdown depends on BRCA1 upregulation to decrease breast cancer proliferation and progression through G2. We could hypothesize that restoration of BRCA1 function by EZH2 knockdown may effectively decrease tumor progression enabled by BRCA1 deficiency, informing future strategies tailored to restore BRCA1 levels and function, and for possibly aiding in the treatement of ER-negative breast cancer tumors.

Continuing, our research ventured further into investigating the functional connection between EZH2 and BRCA1 proteins. Engineering the inducible expression system in MCF10A benign breast cell line was essential to our continued investigation into this relation, providing a stably overexpressing system with which to complement our studies in the CAL51 breast cancer cell line. It was from investigations in both these cells lines that most of our data was obtained.

Knowing that EZH2 and BRCA1 protein expressions were inversely correlated in basal-like ER-negative breast cancer, and with our previous research showing that EZH2 overexpression leads to decreases in nuclear BRCA1, our research provided new insight into the development of this phenotype. Using confocal fluorescence microscopy and western blot analysis, our lab discovered that EZH2 regulates the distribution of BRCA1 protein between the cytoplasm and the nucleus in benign breast cells and in ER negative breast cancer cells. By measuring the nuclear and cytoplasmic expression of BRCA1 protein at different timepoints after release from cell cycle synchronization, we concluded that EZH2 overexpression in MCF10A induces nuclear export of BRCA1 protein. Consistent with this observation, BRCA1 protein was translocated from the cytoplasm to the nucleus upon EZH2 downregulation in the high-EZH2 expressing CAL51 breast cancer cells.

To further the links between EZH2, BRCA1, and genomic stability, our studies go on to demonstrate that transient EZH2 overexpression in MCF10A cells causes aberrant mitosis with extra centrosomes, contrasting with control MCF10A cells showing organized mitotic spindles. Further strengthening the function of EZH2 in mitosis, we observed that EZH2 overexpression increased the levels of important centrosome protein kinases Aurora A and Aurora B as well as dysregulating their expression during cell cycle. The effect of EZH2 on mitosis was also analyzed in CAL51 breast cancer cells. While CAL51 cells transduced with scrambled shRNAs exhibited aberrant mitosis with supernumerary centrosomes and mitotic spindles, EZH2 knockdown with the proper shRNA mitigated these abnormalities. EZH2 knockdown also led to downregulation of Aurora A and Aurora B kinases.

These described effects of EZH2 on centrosome amplification and mitosis underscore a very important connection between EZH2 and tumorigenesis. Regulation of mitosis is a major cell cycle control mechanism that guards against production of aneuploid daughter cells and tumorigenesis. We hypothesized that the mitotic defects induced by EZH2 overexpression may lead to aneuploidization of MCF10A cells. These cells constitute a good model to study ploidy alterations since they are diploid. Conditional EZH2 upregulation caused aneuploidy in MCF10A cells as early as 72 hours after addition of doxycycline. EZH2 knockdown in CAL51 cells diminished the number of tetraploid cells compared to scrambled shRNA controls. Importantly, we observed that knocking down both EZH2 and BRCA1 expression in CAL51 cells lead to the reemergence of wild-type aneulploidy levels, giving further credence to the conclusion that it is through regulating BRCA1 that EZH2 exerts its effect on genomic stability and tumorigenesis.

While the mechanism responsible for the nuclear-cytoplasmic shuttling of BRCA1 protein is not fully elucidated, recent literature suggests that the membrane serine/threonine protein kinase B (AKT) plays a role. Our data provides compelling evidence that the effect of EZH2 on BRCA1 intracellular localization requires the activation of PI3K/Akt. Pharmocological inhibition of the PI3K/Akt pathway with LY294002 or Wortmannin reverted EZH2-induced cytoplasmic retention of BRCA1 and promoted its nuclear translocation.

The PI3K/Akt pathway has been reported to play a role in the maintenance of genomic stablility. We observed that the effect of EZH2 overexpression on mitotic defects and aneuploidy requires activation of PI3K/Akt. Inhibition of this pathway using LY294002 or Wortmannin was sufficient to rescue the increased number of centrosomes, abnormal mitosis, and aneuploidy caused by EZH2 overexpression. Our data not only reveal a novel function of EZH2 in maintenance of genomic stability, but strongly suggest that the promotion of aberrant mitosis and aneuploidy may be important for the oncogenic role of EZH2 in breast cancer.

Our results demonstrate a previously undescribed function of EZH2 in breast tumorigenesis. We show that EZH2 overexpression influences BRCA1 nuclear-cytosolic shuttling and is sufficient to promote aberrant mitosis and aneuploidy of benign breast cells. In breast cancer cells, EZH2 knockdown induces nuclear localization of BRCA1, decreases mitotic aberrations and reverts tetraploidy. Our results enable us to pinpoint one mechanism by which EZH2 controls BRCA1 intracellular localization and genomic stablility, implicating the PI3K/Akt-1 pathway. We show that Akt activation is indispensable for driving EZH2-mediated BRCA1 nuclear export, mitotic defects and aneuploidy of mammary epithelial cells. In view of our results and based on the profound effects of EZH2 knockdown to breast cancer cells, we propose that modulation of EZH2 expression levels may be a valid strategy to prevent or halt

neoplastic progression in the breast. Our data also provides an answer to observation of why it is that although BRCA1 gene mutation is very infrequent in sporadic breast cancer, its expression is still found to be dysregulated in many cases. Whereas mutations in tumor suppressor genes such as BRCA1 or p53 can clearly lead to cancer, there do exist examples of tumor suppressor inactivation independent of their genetic mutations. Such an example is seen with p53, where wild-type p53 protein is found mislocated in the cytoplasm in breast cancer cells, while mutant p53 remains in the nucleus (49). Our research suggests that this observed BRCA1 dysregulation in sporadic cancers may be a result of such a mechanism mediated by EZH2 and AKT1.

My research, along with that of my colleagues, cuts to the heart of the question surrounding the oncogenic mechanism underlying EZH2 overexpression. Over the course of my time in the lab, we have highlighted two novel proteins associated with EZH2, BRCA1 and AKT1. In looking at the large picture again, our data now allows for the more comprehensive analysis of the role of EZH2 in breast cancer tumorigenesis.

The question of the role of EZH2 in breast cancer, and my main research aim in the Kleer lab, sits at the crux of two large debates currently taking place in breast cancer research: that of the role of aneuploidy in tumorigenesis, and the cancer stem cell theory. Pursuing future directions in our research has led down both of these paths, and these theories play a large role in the further interpretation of our data as well as the development of further clinical utility.

Our data has shown that EZH2 overexpression can cause anueploidy in breast cells, *but can this aneuploidy leads to tumor formation?* This hypothesis has been debated since it was first proposed over one-hundred years ago (50), and research since this time has only revealed the intrinsic complexity inherent in tumorigenesis and anuploidy formation. Aneuploidy is the

most common characteristic of human solid tumors, yet it is still unclear whether it is aneuploidy is driving tumorigenesis or if it is merely a product of other malignant mutations in the cell (51). By investigating in more detail the observed anuploidies in MCF10A and CAL51 cells, we have constructed a more detailed image of how EZH2 overexpression may lead to aneuploidy and tumorigenesis.

In performing the metaphase spread technique and counting chromosomes of CAL51 and MCF10A cells, I noticed that the aneuploid nuclei I counted tended to display drastic alterations in chromosome number (around near triploid or tetraploid karyotype, around 69 and 92 chromosomes respectively) instead of perhaps more subtle, single chromosome addition or subtractions. Of all the cells I counted, I quantified not just the prevalence of aneuploid and non-aneuploid cells, but also the total chromosome numbers from each cell. Figure 9 shows the results, supporting my observation that EZH2-driven aneuploid cells tended to have near tetraploid chromosome numbers. This data, along with the known relation between EZH2 and BRCA1, provides further insight into possible mechanisms of EZH2-driven aneuploidy and tumorigenesis.

Figure 9

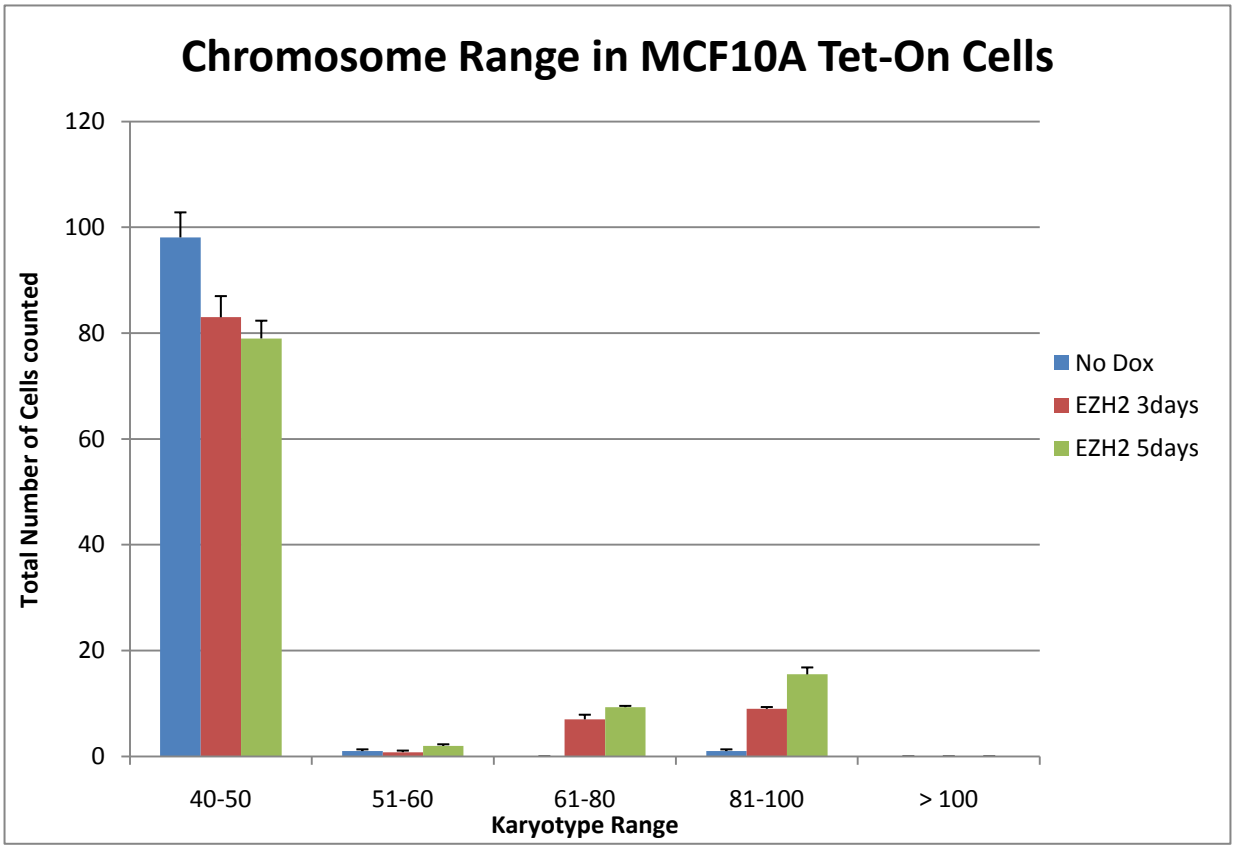


Figure 9: Near-tetraploid phenotype is the most commonly observed aneuploidy in the EZH2 overexpressing MCF10A system.

Tetraploidy in cells usually arise from aberrations in mitosis (52). During mitosis, chromosomes attach via their kinetochores (proteinaceous structure on the DNA) to spindle microtubules from the centrosomes, with this formation favoring even chromosome segregation between the two daughter cells. Cells have natural checkpoints which delay anaphase until all kinetochores are properly attached. In the presence of a persistant error however, mitotic cells can escape the checkpoint arrest and exit mitosis without undergoing anaphase or cytokinesis, thereby producing a tetraploid cell with a single nucleus and two centrosomes (53). Abnormal

spindle positioning and movements are known to interfere with cytokinesis and lead to the accumulation of tetraploid cells (54). Recalling our earlier work, we have shown that EZH2 overexpression leads to centrosome amplification and mulitipolar spindle formation, so it is likely though these aberrations in mitosis that EZH2 favorably yields more tetraploid cells.

Important in the discussion of aneuploidy and tumorigenesis is also the characterization of chromosomal instability (CIN), where cells display an increase in the rate of gain or loss of whole chromosomes during cell division, which is not synonymous with aneuploidy (55). Tetraploidy has been observed to instigate chromosomal instability (CIN) in yeast and mammalian cells (56-57). Supernumerary centrosomes are proposed to be the major source of CIN (58-59). Extra centrosomes can lead to the formation of multiple spindle poles during mitosis, resulting in the unequal distribution of chromosomes and the production of aneuploid daughter cells. Multipolar mitoses have been shown to result in high CIN owing to unsynchronized sister-chromatid separation, a high frequency of non-disjunction and the occurrence of diplochromosomes (60). Our data again suggest that EZH2 may induce CIN through its role in centrosome amplification.

Of greater importance now is asking how these characteristics may lead to tumor formation. It can be reasoned that the appearance of CIN in a diploid cell would quickly lead to cell death due to aberrant mitosis, however CIN in tetraploid cells would have a higher chance of survival due to greater chromosomal redundancy, hinting at a selection method to pair CIN and aneuploidy (52). Pairing CIN and aneuploidy together would lead to the production of progeny that may be grossly aneuploid, though viable, and able to acquire further mutations. Chaotic multipolar mitosis may break chromosomes directly, abnormal mitosis and prolonged mitotic arrest can lead to the accumulation of DNA breaks, the double amount of DNA leads to twice the

amount of spontaneous DNA damage, coupled with the fact that DNA repair is probably much less efficient; all of these events may lead to increased genetic mutation and therefore increased likeliness for malignant transformation.

Tetraploidy has been identified in early stages of cancers, in which it precedes the development of CIN and aneuploidy (62-64). Overexpression of Aurora A in the mammary gland of mice leads to an increase in the generation of tetraploidy, CIN, and the formation of mammary tumors, providing a further detailed picture of EZH2's possible effects in tumorigenesis (65-66). Although aneuploidy has been shown to lead to tumor formation in some studies, others have shown that aneupolidy can also play a neutral and even a tumor-suppressing role in cancer (67). What hinders the hypothesis of aneuploidy-driven tumorigenesis is that to date, there has been no found direct correlation between the level of aneuploidy and the incidence of spontaneous tumor development. A comprehensive review on the role of aneuploidy across a spectrum of cancers and models has shown that aneuploidy can increase the risk of neoplastic transformation in some tissue lines, although a predisposed background or some other activating mutation is usually required for tumorigenesis. The effect of aneuploidy might not be driven by a particular combination of chromosomes per se, but rather by the specific interaction of the karyotype with the various genetic contexts and microenviroments found in different tissues. In relation to our research, a clear goal for the future could be to investigate further whether aneuploidy promotes tumorigenesis specifically in the genetic context of ER-negative breast cancers.

Another interesting model of tumorigenesis to consider in light of our data is the cancer stem cell theory of breast cancer. This model posits that cancers develop from a subset of malignant cells that possess stem cell characteristics (called cancer stem cells) and has been

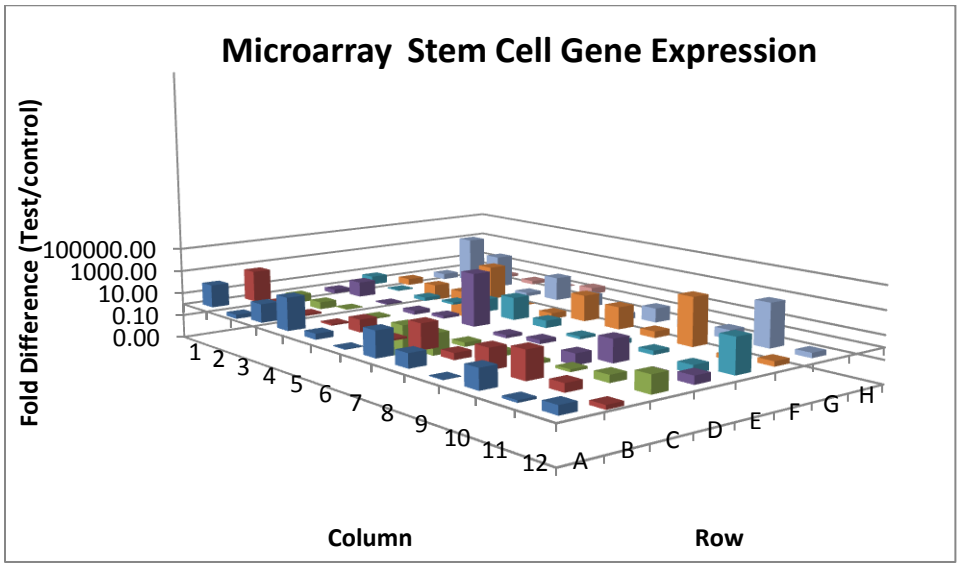
proposed to account for the development of a variety of malignancies, including breast cancer. These cancer stem cells originate from either mutated stem cells or from the dedifferentiation of a lineage committed cell that has acquired stem cell characteristics through mutation. This theory holds special relevance to our work seeing as how striking parallels can be found between our studied subset of ER-negative breast cancer cells and stem cells. As brought up in the introduction, EZH2 is an epigenetic repressor found in high concentrations in embryonic stem cells to keep them proliferating and in an undifferentiated state. This is a very similar situation as observed in our studied breast cancer cells, where EZH2 overexpression in breast cells is associated with both increased proliferation and cell dedifferentiation, both hallmarks of more malignant forms of breast cancer.

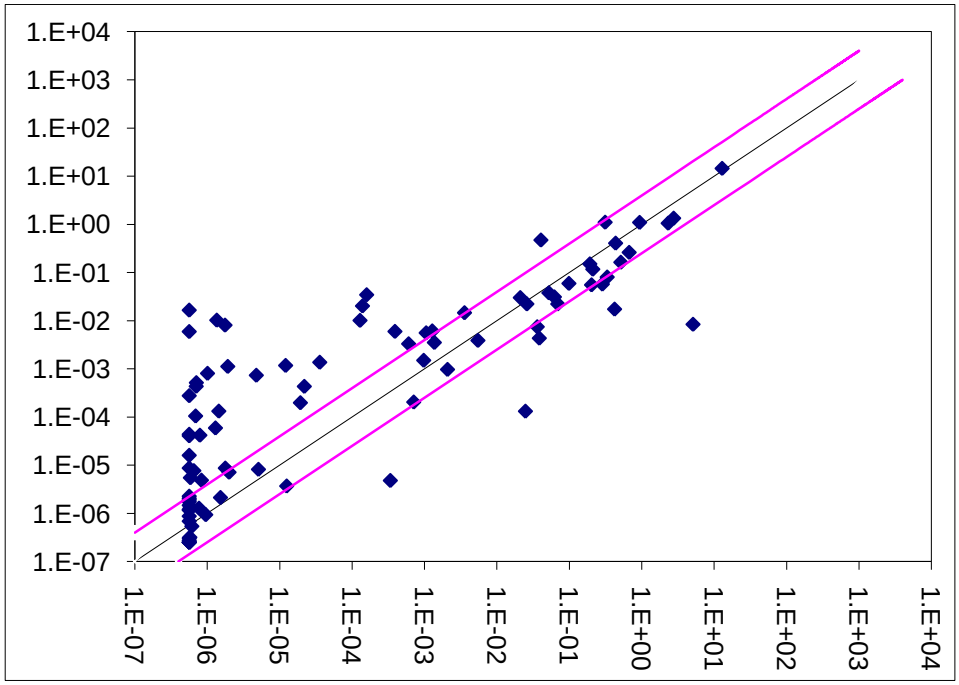
This cancer stem cell model of oncogenesis, first proposed for haematopoietic stem cells in 2001 (68) and then for breast cancer in 2003 (69) (by the Wicha group at the University of Michigan) details, implies a different mechanism of tumor progression and would therefore recommend a different type of treatement in patients. Whereas the anueploidy-driven model suggests that tumors become more homogenous masses of aneuploid cells, and so treatement would need to be directly targeted against the entire tumor, the cancer stem cell model implies that only a small subset of cells within a tumor possess the ability to self-renew and possibly metastasize. This cancer model would then recommend that specifically these stem cells must be targeted for treatement, and the rest of the tumor would soon die off (70). Implicit in designing treatement then is identifying these cancer stem cells from the other tumor cells. In breast cancer, cancer stem cells have been found to typically have the CD44+/CD24- phenotype (the CD proteins are cell surface proteins), while the presence of ALDH-1 has also been used to distinguish breast cancer stem cells. Our lab investigated into the possible relevance to our

cancer model by performing a cDNA microarray to test differential gene expression between SUM149 cancer cells, both ALDH + and ALDHA -, as well as having EZH2 expression knocked down using shRNA (Figure 10). The genes assayed in the microarray specifically pertained to stem cell development pathway. Of the many observed difference, of note was the differential expression of the Notch signaling proteins, which varied as a function of EZH2 expression between the ALDH+ and ALDH- populations. Notch signaling plays a role in self renewal in both normal and malignant stem cells, and finding differential expression could support the theory that it is a small subset of breast cancer stem cells that are responsible for tumor growth and invasion (71). This stem cell model would be an interesting model to look into, however its restrictions of its applicability have been already heavily decried by critics. While this model of tumorigenesis may explain the observed heterogeneity in tumors, there still has been a marked lack of correlation between stem cell marking and cancer severity (72). This model is still currently contested, however the great amount of research put into this investigation has left its indelible mark on the future of cancer research.

Figure 10

Figure 10: Microarray comparing SUM149 shEZH2 ALDH+ cells with SUM149 parental ALDH- cells. These figures are given as illustration as to the power of microarray analysis for widespread gene analysis. The top figure shows the differential gene expression as observed in the differing well locations on the microarray plate, while the bottom scatter plot provides another view with overexpressed genes above the purple lines and repressed genes below the purple line.





Both of these models of EZH2 driven tumorigenesis provide interesting yet varying ideas as to how best treat these types of breast cancers. In reviewing these hypotheses, I have brought us to the forefront not just of our research of EZH2 in breast cancer, but also of the current state of cancer research. While in the end, the data I have collected while part of the Kleer lab has helped us form a more comprehensive view of the role of EZH2 in breast cancer tumorigenesis, there still remains many more questions and untreaded directions with which to continue our research. We have discovered novel associations between EZH2 and BRCA1 expression in breast cancer through AKT. We have provided potential in targeting any one of these relations to increase breast cancer survival in patients. As quoted from Max Wicha himself, we are within five years of being able to sequence patients' entire genome to search for the activating mutations and genetic relations that we are elucidating today. The future of breast cancer research is evolving through the beautiful amalgamation of advancing technology and intense effort. Older models of breast cancer tumorigenesis are being questioned, and treatment advances along with every new development. Though many questions remain, our work continues.

MATERIALS AND METHODS:

Cell Lines

Breast cancer cell lines and immortalized human mammary epithelial cell lines (MCF10A) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown under recommended conditions.

DNA Direct Sequencing

Direct Sequencing performed through the DNA sequencing core at the University of Michigan. Primers were designed to cover entire EZH2 locus and ordered through Applied Biosystems. Data received from the core was analyzed with FinchTV (Geospiza). The nucleotide sequences were then compared using the NCBI BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program. Samples were compared to the NCBI reference sequence for the Homo sapiens enhancer of zeste homolog 2 (Drosophila) (EZH2), transcript variant 1, mRNA, locus NM_004456. If a mutation was found in the forward strand, then the reverse stand was then consulted. A mutation would be considered valid if it was found in both the forward and reverse strands.

Western immunoblots

Immunoblot analysis was performed as described in Kleer et al., 2003 using 100 mg of nuclear enriched fractions extracted with NE-Per kit (Pierce, Rockford, IL, USA), or whole cell extract as indicated in the legends. The following antibodies were used: mouse anti-b-actin (1:10 000),

mouse anti-a-tubulin (1:1000), goat anti-rabbit:horseradish peroxidase (HRP) secondary antibody (1:10 000) and goat antimouse HRP (also 1:10 000). These antibodies were purchased fromSigm a (St Louis, MO, USA). In addition, we used anti-EZH2 (1:1000; BD Biosciences, San Jose, CA, USA); anti-BRCA1 (D-9) (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-BRCA1 (Ser1423) (1:1000; Abcam, Cambridge, MA, USA); anti-cdc25C (5H9); anti-cdc2; anti-phosphor-cdc2-tyr15, used at 1:1000 dilution, fromCell Signaling Technology, Danvers, MA, USA; anti-Cyclin B1 (1:1000 dilution, Calbiochem; EMD Chemicals, La Jolla, CA, USA) and anti-phospho-Cyclin B1 (Ser126) (1:1000 dilution; Novus Biologicals, Littleton, CO, USA). Control immunoblots using preimmune immunoglobulin G (IgG) confirmed the specificity of the antibodies. Semiquantitative protein expression levels were determined by densitometry using IMAGE J 1.38x software.

Cell synchronization

Cells were synchronized with double thymidine block as described previously (Fan et al., 2000). Briefly, cells were incubated in medium containing 2mM thymidine for 12 h, released into their normal medium for 8–10 h and then incubated for 12 h in medium containing 2mM thymidine (see Supplementary methods for details).

Knockdown of shEZH2 and shBRCA1 in breast cancer cells

To generate stable short-hairpin interfering RNA-EZH2 and RNA-BRCA1 in MDA-MB-231 and CAL51 breast cancer cells, cells were transduced with lentivirus and selected for antibiotic resistance in ATCC-recommended media with puromycin (100 mg/ml, Sigma), at 37 1C under

10% CO2. Lentivirus was purchased fromthe Vector Core, University of Michigan. Background vector control was Lenti- PuroEMPTY-VSVG. For targeting EZH2 (NM_152998 NCBI) and BRCA1 (NM_009764 NCBI), the shRNA oligos ID used were as follows: V2LHS_17507 targeting EZH2 and V2LHS_254648 targeting BRCA1, corresponding to these catalog numbers RHS4430-99139126 and RHS4430-99157192, respectively, fromOpen Biosystems, Huntsville, AL, USA.

Cell proliferation

Cells were plated at the same density and cultured for 24 h in a 96-well microplate. WST-1 reagent was added and absorbance at 450nmwas measured after 3 h of incubation, following the manufacturer's instructions (Roche Molecular Systems, Pleasanton, CA, USA).

Real-time quantitative PCR

Total RNA was isolated following the manufacturer's instructions with the RNeasy kit (QIAGEN Inc., Valencia, CA, USA). cDNA samples from breast cancer cells were amplified in triplicate from the same starting material of total RNA following the manufacturer's instructions (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). Samples were amplified using TaqMan MGB FAM dye-labeled probes from Applied Biosystems in an ABI7900HT model real-time PCR machine. The following probes were used: Hs99999903_m1 (ACTIN), Hs00173233_m1 (BRCA1) and Hs00544830_m1 (EZH2).

Engineering Tet-On System in MCF10A Cell Line

This project was one of my more significant undertakings in the lab, so I will go into a little more detail about how the system works and how I created our system. Our lab purchased the Lenti-X^{TM} Tet-On$^{®}$ Advanced Inducible Expression System from Clontech. This involved a system which would overexpress EZH2 when Doxycycline (Dox) was added to the cell culture media. The system is derived from *E. coli*'s tetracycline operon. In *E. coli*, the Tet repressor protein (TetR) negatively regulates the genes of the tetracycline-resistance operon. TetR blocks transcription of these genes by binding to the tet operator sequence (*tet*O) in the absence of tetracycline (Tc). In the presence of Tc, TetR dissociates from *tet*O and transcription of resistance mediating genes begins. This interaction between TetR and tetO are the basis for this inducible system

The system comes with two elements, the regulator vector (pLVX-Tet-On Advanced) and the response vector (pLVX-Tight-Puro). The pLVX-Tet-On Advanced vector constitutively expresses the tetracycline-controlled transactivator, rtTA-Advanced. This protein consists of a mutant TetR (rTetR) that posseses a "reverse" tetO binding phenotype in that this protein will bind to *tet*O sequences in the presence of Dox, rather than its absence. The pLVX-Tight-Puro response vector contains P_{tight}, the inducible promoter that controls the expression of our gene of interest EZH2. P_{tight} consists of a modified Tet-Responsive Element (TRE_{mod}), made up of seven direct repeats of an altered *tet*O sequence, joined to a minimal CMV promoter ($P_{minCMV\Delta}$). Upon induction in the presence of Dox, rtTA-Advanced binds to the P_{Tight} promoter on the response vector, activating transcription of the downstream gene (see Fig). These expression vectors where introduced into the cell using viral vectors.

I will outline my strategy to splice the EZH2 locus from our stored plasmid into the P_{Tight} promoter. The pLVX tight-puro vector was opened with a double digestion with the restriction

enzymes BAMHI and EcoRI. The EZH2 locus was cut from its vector using the same enzymes, typical of this type of procedure, since this will allow for a simple mix and anneal step. Complications arise however since EZH2 contains an internal EcoRI cut site, which separates an almost 300bp segment from the rest of the gene. To get around this, I used PCR to magnify and isolate the missing 300bp segment, while also engineering complementary cut site so this small segment would fit in with the main vector fragment. After inserting the large part of the EZH2 locus into the pLVX tight-puro vector, I then double digested the small fragment with EcoRI and BAMHI and then inserted and ligated the small segment into the larger vector construct using viral T4 DNA ligase. Calf Intestinal (CIP) Alkaline Phosphatase was used to dephosphorylate the 5' phosphate groups from the fragments to prevent self annealing.

The vector was then sequenced to confirm that our intended vector was obtained. Western Blot analysis confirmed that EZH2 expression is at normal wild type levels in the absence of Dox and that EZH2 expression increases dramatically upon the addition of Dox into the media.

Metaphase Spead

Cells were grown in culture and colecemid added 24 hours before harvesting at around 70% confluency. Colecemid limits microtubule formation and therefore freezes cells in metaphase. If doxycycline was to be added (as in to collect data from the MCF10A inducible expression cell line), it would be added to cell culture at around 50% confluency, in order to attain the desired 70-80% confluency. This level of confluency is need since colcemid will freeze cells currently going through mitosis, however if the cells are already at 100% confluency then the mitotic

index will be very low and very few metaphases will be visible on the slide. Cells were collected by trypsinization and resuspended in 0.075 mol/L KCl on ice for 30 min. Cells were fixed in a 3:1 mixture of methanol and glacial acetic acid with mild vortexing, dropped onto glass slides, and stained with 544 Ag/mL Giemsa solution. To determine ploidy, the number of chromosomes was counted in at least 25 metaphases for each cell line.

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