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Establishment of CRISPR/Cas-9 Aided Knockout of the ZIC2 Gene in the African-American Prostate Cancer Cell Line E006AA-PR

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ABSTRACT

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ESTABLISHMENT OF CRISPR/CAS-9 AIDED KNOCKOUT OF THE ZIC2 GENE IN THE AFRICAN AMERICAN PROSTATE CANCER CELL LINE E006AA-PR

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The largest U.S. cancer health disparity exists in prostate cancer, with African American men having the highest incidence and mortality rates. The present study evaluated the effects of ZIC2 and the underlying mechanisms in the E006 parental African-American cell line that produces tumors at accelerated growth rates because of the increase of ZIC2 genes in African-American males. We analyzed the experimental research that the overexpression of ZIC2 contributes to progression of prostate cancer. E006AA cells with overexpressed or suppressed ZIC2 were analyzed to determine phenotypic differences, PCR, cell proliferation and immunoblot assays. The expression levels of ZIC2 were analyzed by CRISPR-Cas9, Western blot and proliferation growth curves. We discovered using these experimental techniques to knockout ZIC2, reduced cell proliferation occurred. This research investigated the role of ZIC2 in prostate cancer progression and the effects of the loss or gain of function of ZIC2 by using CRISPR-Cas 9 genome editing technology.

ESTABLISHMENT OF CRISPR/CAS-9 AIDED KNOCKOUT OF THE ZIC2 GENE IN THE AFRICAN-AMERICAN PROSTATE CANCER CELL LINE E006AA-PR

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TABLE OF CONTENTS

CHAPTER

LIST OF FIGURES

Figure

LIST OF TABLES

Table

CHAPTER I

INTRODUCTION

Prostate cancer is one of the most commonly diagnosed malignancies in American men. A more aggressive form of the disease is particularly prevalent among African Americans. It is also the second leading cause of cancer death in American males, exceeded only by lung cancer.[1] Fortunately, the therapeutic success rate for prostate cancer can be tremendously improved if the disease is diagnosed early. In some cases, when prostate cancer is detected at a very early stage, it can be treated effectively and even eradicated. Consequently, much effort is being placed on detecting prostate cancer in an early, curable stage to decrease the rate of mortality from this disease.[2]

The largest U.S. cancer health disparity exists in prostate cancer (PCa), with African American (AA) men having the highest incidence and mortality rates. PCa is the second leading cause of cancer-related death in AA men. AA men have a two-thirds higher PCa incidence rate and two times greater mortality rate than Caucasian Americans (CAs).[2] In addition, they are more frequently diagnosed with higher Gleason grades, advanced tumor stage, higher Prostate specific antigen levels, higher tumor volume, poorer prognosis, and worse overall survival rate than CA men. Screening for elevated PSA levels or an abnormal digital rectal exam (DRE), followed by a prostate biopsy, is used to diagnose early stage prostate cancer. Currently, this is the only way to identify

prostate cancer in high- risk groups, such as African American men, and to reduce their mortality due to this disease. Diagnosis at an early stage when the cancer is still localized to the prostate gland, leads to nearly a 100% 5-year relative survival rate. In contrast, diagnosis at a later stage, when the cancer has spread to distant lymph nodes, bones or other organs, results in only a 28% 5-year relative survival rate. These data indicate that early diagnosis by routine screening can save lives. Once diagnosed, not all cancers will progress to late stage disease. On in 7 men will be diagnosed with prostate cancer, however only 1 out of 38 will die from prostate cancer. While less than half of the patients are diagnosed with potentially lethal disease (Gleason score >/= 7), up to 90% of patients with low-risk disease undergo prostatectomy.[3]

Despite the advances in the discovery of biomarkers for prostate cancer, there remains a need for more clinically reliable biomarkers that will have a high specificity for the diagnosis and prognosis of prostate cancer.[4] Provided herein are cell lines capable of expressing Zic family member 2 (ZIC2) protein or mutant. The cell lines are produced from CRISPR/Cas9 mediated genome editing that comprise transfected host cell lines with a CRISPR/Cas9 plasmid to produce the cell line. The CRISPR/Cas9 plasmid comprises a guide RNA targeting ZIC2 gene. The guide RNA used is capable of guiding CRISPR/Cas9 to ZIC2 gene in the host cells to elicit a double strand break in host gene. In one embodiment, the method further comprising isolating ZIC2 protein from one of the ZIC2 mutant cell lines.[5]

CHAPTER II

LITERATURE REVIEW

2.1 Prostate Cancer

Prostate cancer is the most common type of cancer in men in the United States. Prostate cancer occurs when normal cells begin to grow faster or die slower.[6] Whether cells grow faster or die slower, this causes a tumor to form from abnormal changes which are called mutations in genes. Aging, of men of African-American (AA) descent and having family members with prostate cancer has been linked to a higher chance of getting prostate cancer. Most prostate cancers are adenocarcinomas that start in cells that line glands and make semen.[6] The prostate gland is located in the compartment between the pelvic diaphragm and the peritoneal cavity. It is located posterior to the symphysis pubis, anterior to the rectum, and inferior to the urinary bladder (Figure 1A). Prostate cancer cells do not behave like normal cells because they grow more rapidly and live much longer than does a normal cell. The differences between the normal and prostate cancer cells are that normal cells divide and make new cells and die when they become old or damaged. In contrast, cancer cells make new cells that are not needed, and they do not die quickly when they become old or damaged. Eventually, the abnormal cell forms into a tumor. Cancer cells invade other tissues, making a tumor grow larger and takes over the prostate. Prostate cancer cells can metastasize and merge in blood or lymph creating secondary tumors (Figure 1B). [8-9]

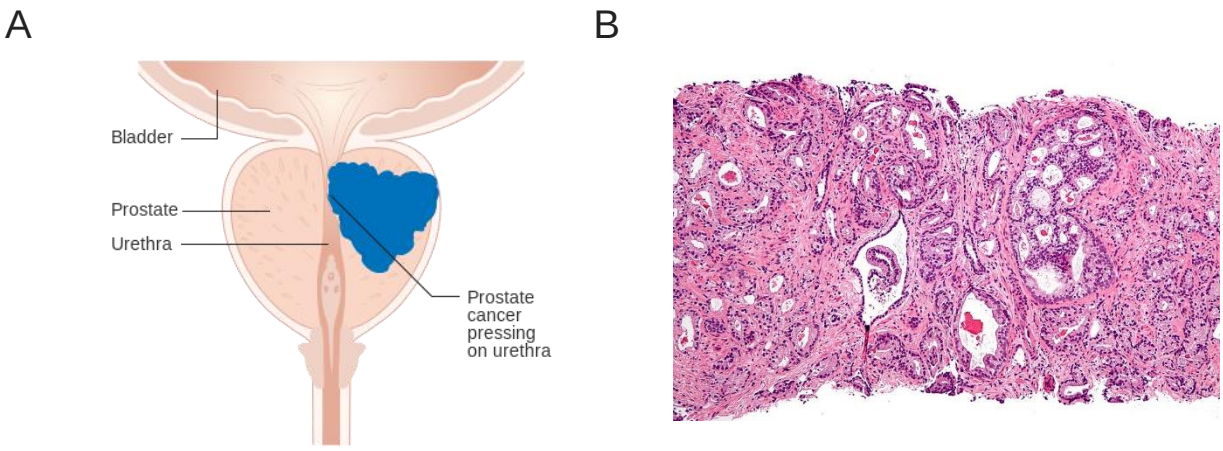


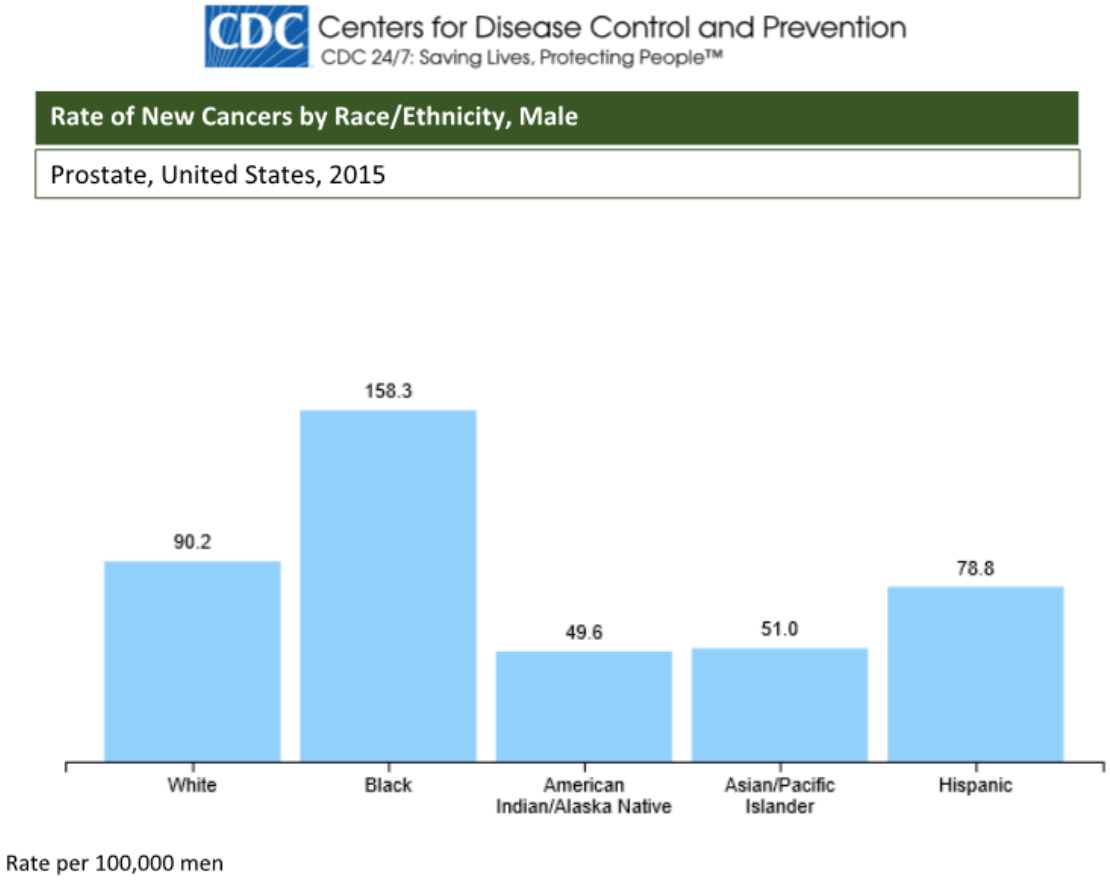
Figure 1. A diagram and micrograph of Prostate Cancer. (A) A diagram of prostate cancer pressing on the urethra, which can cause symptoms. (B) Micrograph of prostate adenocarcinoma, acinar type, the most common type of prostate cancer. Needle biopsy, H&E stain.

2.2 *Incidence and Mortality*

African-American men have a higher prostate cancer incidence rate and two times greater mortality rate than Cas.[10] Prostate cancer affects African-American males in the United States in a disproportionate number compared to white males. African-American males are 1.7 times more likely to develop and 2-3 times more likely to die from prostate cancer than White males. Incidence and mortality rates for African Americans are 1.5 and 2.3 times higher than for Caucasians (CA), respectively. AAs also have a higher incidence earlier in life and, upon diagnosis, present with a more aggressive disease.[11] About 9.7% of cancers in men are due to prostate cancer; in developed parts of the world it increases to 15.3% and in the underdeveloped world it only accounts for 4.3%. Differences in genetics, hormones, environmental exposures, and other factors can lead to differences in risk among different groups of people. For most cancers, though,

increasing age is the most important risk factor.[12-13] We report a rate of new cancers by race/ethnicity identifying incidence and mortality in the United States in Table 1.

Table 1. Rate of New Cancers by Race/Ethnicity



2.3 *Risk Factors*

Age. The occurrence of prostate cancer is mainly age-dependent. Its incidence is increased in men over 40-years of age and the average age at diagnosis in Finnish men is approximately 70 years.[14] The probability of developing prostate cancer is the highest in men over 70 years (11%).[15]

Family history. Prostate cancer cases have been observed as inherited susceptibility, lifestyle habits, and other environmental factors and their interactions. The risk of prostate cancer increases with an increasing number of affected first-degree relatives, with increases of 2- to 3-fold in men with a single affected relative and of over 4-fold in men with two or more affected family members.[16] Furthermore, family history predisposes individuals to early-onset of this disease before age 65.[16]

Other factors. Age and ethnicity are the major prostate cancer risk factors, but other factors have been reported to have potential significance in cancer prevention. With regard to dietary factors, high intake of saturated fat, well-done meat and calcium may increase the risk of advanced prostate cancer, while the influences of total meat, fruit and vegetable intake on this risk are unclear.[17] Hormones, particularly that of testosterone, have reported inconsistent results. Accumulating data suggest that lifestyle factors, such as obesity[17] and smoking, may be associated with aggressive prostate cancer and prostate cancer death, and alcohol consumption has also been correlated with an overall increased risk[18] however, further studies are warranted to support these findings. De Marzo et al., 2007 reported that chronic inflammation has been suggested to contribute to the risk of developing prostate cancer. Prostatic inflammation-promoting factors are largely unknown, but potential causes of prostate inflammation include infectious agents, urine reflux, physical trauma, hormonal changes and dietary habits. The average prevalence of symptoms of prostatitis syndromes, including bacterial infections, both inflammatory and non-inflammatory chronic pelvic pain syndromes and asymptomatic inflammation, in men is 8%. The overall rate is anticipated to be much higher due to asymptomatic

conditions.[20] Few studies have directly examined the association between chronic inflammation and prostate cancer risk, but inflammation of benign prostate tissues has been suggested to predispose individuals including aggressive prostate cancer.[19]

2.4 *ZIC2 Role in Prostate Cancer*

ZIC family member 2 is one of five members that make up the family of ZIC proteins.[26] The entire ZIC family comprises transcription factors that have been reported to play various roles in neuronal and embryonic development. Each ZIC family member has five zinc fingers that is with the C2H2 motif. The zinc finger domains allow for DNA binding and protein-protein interactions. ZIC family members 1-3 have a ZIC/ odd-paired conserved (ZOC) region, which play an essential role in protein-protein interactions. ZIC proteins are hypothesized to regulate transcriptional activity by way of direct DNA binding. ZIC2 is now known to be overexpressed in several cases of prostate tumors[27] and in other tumors and it has been noted recently in a GEP study of oral cancer.[28] Members of the ZIC family of zinc finger transcription factors play a major role in early embryonic development. Although the 5 ZIC genes are highly homologous, they must fulfil different roles, as spontaneous mutations in humans and targeted inactivation of individual ZIC genes in mice give distinct pathologies. ZIC2 mutations affect left–right asymmetry and can cause congenital heart abnormalities.[22] ZIC2 proteins have significant sequence homology to GLI proteins, also known as glioma-associated oncogene, transcription factors that are crucial downstream effectors of the sonic hedgehog (SHH)

pathway. There is evidence that this pathway may be upregulated in ovarian cancer compared with the ovarian epithelium.[26]

Koochekpour et al. 2004, reported the establishment of a new human prostate cancer cell line, E006AA, which was derived from a Gleason 6 localized prostate cancer in a hormone-naïve prostate cancer patient of African American descent.[10] The epithelial origin of the E006AA cells was confirmed by positive expression for cytokeratins 8 whereas the stromal origin of the S006AA cells was confirmed by the positive expression of mesenchymal markers, desmin and alpha-smooth muscle actin and the absence of CK8 and CK18.[10] E006AA is an epithelial cell line with low tumorigenicity derived from cancerous tissue of an African-American patient diagnosed with clinically localized prostate cancer.

Reduction or depletion of mitochondrial DNA (mtDNA) has been associated with cancer progression**.** Reduced mtDNA content may contribute to the higher incidence as well as aggressive prostate cancer phenotype in AA men compared to CA men.[11] In addition, depletion of mtDNA leads to the development of resistance to anticancer agents, including cisplatin and adriamycin.[12] However, increased mtDNA levels are also associated with acquired resistance to docetaxel in head and neck cancer cell.[12] Hence, maintaining a steady-state level of mtDNA in cancer cells is critical for a positive outcome during cancer therapy.[10-12] Studies have shown positive and negative responses due to mtDNA being altered in cancer which is why it is difficult to gauge in research efforts.

2.5 *CRISPR-Cas 9 Genomic Editing*

One of the most recently developed strategies is based on a technology utilizing the clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated genes (cas). The CRISPR-Cas9 system is naturally occurring gene-editing machinery in prokaryotes which operates as an adaptive and heritable immune system enabling bacteria and archaea to defeat invading viruses within the human body.[31] Cas9 is a nuclease that was first discovered as a component of the CRISPR system in Streptococcus pyogenes and has been adapted for utility in mammalian cells. RNA guided Cas9 can efficiently introduce precise double-stranded breaks at endogenous genomic loci in mammalian cells with high efficiencies. Cas9 nucleases can be directed by short guide RNAs (sgRNA) to induce precise cleavage at endogenous genomic loci in human and mouse cells.[32] Cas9 can also be converted into a nicking enzyme to facilitate homology-directed repair with minimal mutagenic activity. Lastly, multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several sites within the mammalian genome.[5] Decreased cell proliferation will be caused by cellular apoptosis. E006AA cell line was the targeted cell line and CRISPR-Cas9 with 114/118 inserted in the plasmids was used to disrupt the androgen-sensitive prostate cancer cells.

The hypothesis is that the overexpression of ZIC2 contributes to the progression of prostate cancer. If ZIC2 expression is silenced, it will attenuate cell proliferation. The following were objectives were used to test this hypothesis:

Aim 1: Establishment of ZIC2 knockout E006AA-Pr cell line

- CRISPR-Cas9 aided ZIC2 knockout in E006AA-PR cell line
- Verification of ZIC2 knockout by genomic and immunological assay.

Aim 2: Functional characterization of ZIC2 knockout E006AA-Pr cell line

- Determination of ZIC2 knockout after cell morphology.
- Determine the proliferation rate of E006AA and validate change of cell growth behavior.

CHAPTER III

MATERIALS AND METHODS

3.1 *Mammalian Cell Line*

E006AA is derived from a 50-year-old African American male patient with organ-confined Gleason 6 tumor. This cell line is not tumorigenic in nude mice and exhibits androgen-dependent growth (EMD Millipore).

3.2 *Cell Maintenance*

E006AA-PR cell line was maintained continuously by routine passaging with trypsin in DMEM supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. In addition, daily maintenance and monitoring of culture condition of E006AA cell line during experimentation showed that they can be grown in serum-free medium for 2 days either in DMEM or in culture medium containing as low as 1% FBS and Penstrep for a period of 2–7 days.

3.3 *Establishment of ZIC2 Knockout Cell Line*

Targeted ZIC2 gene editing was achieved by transfecting $1x10^6$ E006AA cells in OptiMEM media using the DNA 2.0 CRISPR-Cas9 114 & 118 plasmids. Cells were cultured for 72 hours under standard conditions and then sorted by green fluorescent protein (GFP) fluorescence into single colonies by fluorescence activated cell sorting

(FACS) into a 96-well plate. Single cells were cultured for approximately two months to achieve confluency. Individual cell lines were then expanded and used for experimentation.

3.4 PCR Analysis

DNA was obtained from cells using the QIAprep Spin Miniprep Kit (Omega Biotek) according to manufacturer's protocol. A digestion with was used directly in a PCR reaction to amplify the ZIC2 exon using primers. Digested products were analyzed by gel electrophoresis.

3.5 *Western Blot Assay*

Western analysis was performed on whole cell lysate using antibodies against basal cell (cytokeratin 5), epithelial (cytokeratin-8, -18, and c-Met), and stromal (desmin, HGF, and α-SM-actin) markers. E006AA-PR used as a positive and negative control for epithelial and stromal markers, respectively. E006AA-G12 cell line was used as negative control for ZIC2 expression. GAPDH was used as protein loading control.

3.6 *Cell Proliferation Assay*

To evaluate the growth rate, $1x10^4$ cells were plated in 6-well culture dishes. At day 3, 5 and 7, cells were collected in triplicates using 200µl of Trypsin/EDTA and diluted in 300µl of DMEM/F-12 media and counted using a Nexelcom cell counter.

CHAPTER IV

RESULTS

4.1 ZIC2 Knockout after Morphology

While observing the phenotype of the E006AA parental and mutant cell lines (Figure 2) we noticed the mutant cell lines are more translucent than the parental cell line. The nuclei seem to be more visible than the parental cell line and much larger in size.

A **B**

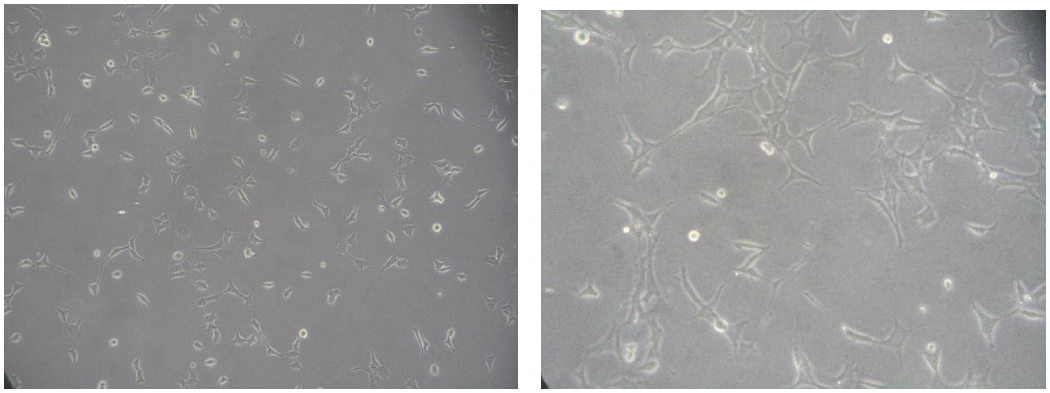


Figure 2. **Phenotype of wild-type and CRISPR-Cas9 mutant cells.** Microscopic brightfield images of E006AA parental cells and E006AA ZIC2 -/- cells. (A) Image of E006AA parental cell line without CRISPR-Cas9. (B) Brightfield image of E006AA-G12 expressing CRISPR-Cs9 ZIC2 knockout.

4.2 ZIC2 Knockout with CRISPR-Cas9

The predicted molecular weight of ZIC2 is 55kDa. Cell lines from the E006AA-PR that is an African American prostate cell line were chosen for experimental analyses. All prostate cancer cells examined by immunoblot express a common ZIC2 band that

runs just above 65 kDa. Androgen receptor (AR) positive cell lines such as E006AA also express a lower ZIC2 band just below 65 kDa. To determine if ZIC2 contributes to aggressive prostate cancer cell growth, we subjected the E006AA cell line model to CRISPR-Cas9 gene editing of ZIC2. CRISPR-Cas9 gene editing was successfully performed with the guide RNA 114 and 118. Clonal cell lines resulting from single cell sorting of edited E006AAcells were evaluated for ZIC2 alterations at both the DNA sequence and protein expression levels. Western blot analysis confirmed that ZIC2 protein levels in E006AA ZIC2 -/- cells were altered with a rabbit monoclonal. This antibody is extremely sensitive indicating that it can detect the potentially low abundance ZIC2 protein. This antibody consistently detects two bands just above and below the 65KD molecular marker in the E006AA-G12 and E006AA-E5, cell lines chosen to do experimental studies. The E006AA G12 114/118 ZIC2 -/- cell line expressed itself as a mutant by not expressing any bands on the western blot when compared to the E006AA Parent cell line and E006AA 14/118 ZIC2 -/- E5 mutant cell line expressed one band and the parent cell line expressing two bands at/ 65 kD.

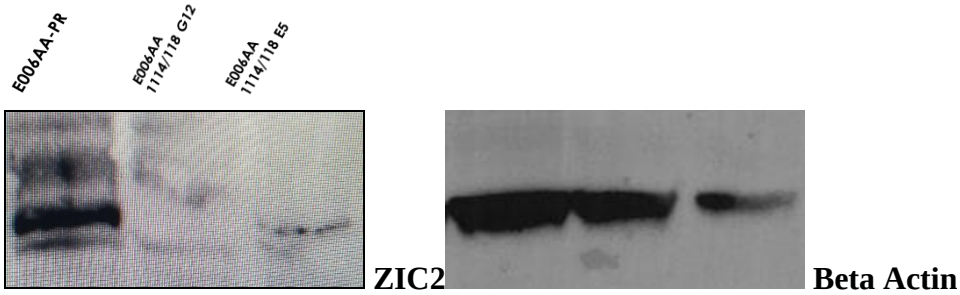


Figure 3. Western blot of ZIC2 protein expression in E006AA parental and E006AA ZIC2 -/- mutant cells.

4.3 Cellular Proliferation is Incapacitated by ZIC2 Knockout

Cell proliferation assays were experimented with the parental cell line and two mutant E006AA cell lines in the form of growth curve analyses. The results showed that E006AA ZIC2 -/- cells exhibited a significant decrease in cell growth when compared to the parental E006AA cells. With these data, it expressed evidence that ZIC2 promotes prostate cancer cell growth. There was a significant difference between the E006AA parental and E006AA ZIC2 -/- cells at day 5 because E006AA parental has a doubling time which influences the growth effect compared to the other mutant cell lines. The G12 ZIC2 -/- cell line, grew approximately 5 times slower than the E006AA WT. We noticed that the growth rates are reduced in the presence of CRISPR-Cas 9 114/118.

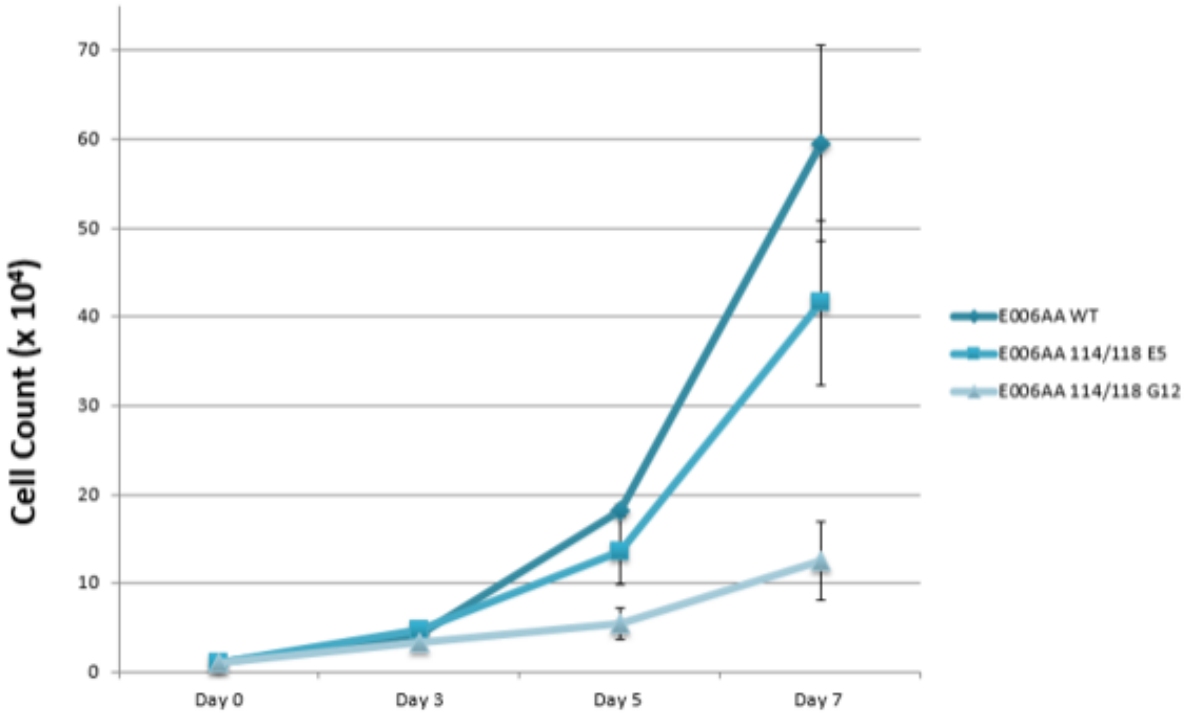


Figure 4. Growth curves for parental and CRISPR.Cas9 mutant cell types. Growth analysis of E006AA parental cell lines demonstrate significant growth differences with the knockdown cells. E006AA 114/118 CRISPR/CAS9 G12 AND E5 cells exhibit significant growth differences with the knockdown cells showing slower growth proliferation.

4.4 PCR of ZIC2 Loci in Edited Lines of E006AA-PR for Characterization of Genomic Alterations Introduced by CRISPR/Cas9

Plasmids are fragments of double-stranded DNA that typically carry genes and can replicate independently from chromosomal DNA. In a plasmid, we inserted genes that are either promoters or enhancer that help control gene expression. The sequence of the promoter region controls the binding of the RNA polymerase and transcription factors, so promoters play a large role in determining where and when ones gene of interest will be expressed.[33] Once you have constructed a plasmid, one can easily make an endless number of copies of the plasmid using bacteria, which can uptake plasmids and amplify them during cell division. Because bacteria are easy to grow in a lab, divide relatively quickly, and exhibit exponential growth rates, plasmids can be replicated easily and efficiently. An example plasmid map for targeted ZIC2 knockout using CRISPR/Cas9 systems is shown in figure 5.

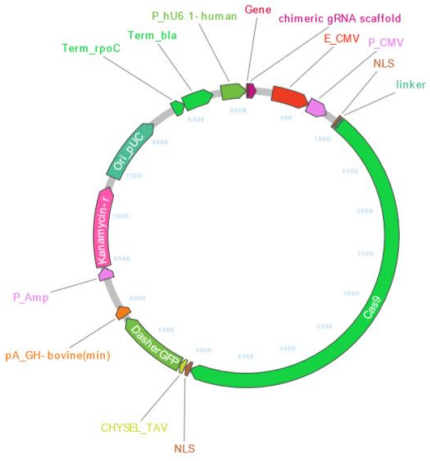


Figure 5. Cas9 Plasmid. DNA2.0's Cas9 vectors express the Cas9 nuclease and the RNA sequences that guide the nuclease to its genomic target. Cas9 expression is driven by a choice of promoters and can be monitored by linked expression of green or red fluorescent proteins.

We determined that ZIC2 contributes to aggressive prostate cancer cell growth using CRISPR-Cas9 gene editing of ZIC2 and expressed it to E006AA cell lines. CRISPR-Cas9 gene editing was successfully performed with the single guide RNA 114/118 inserted in a plasmid that spans coding nucleotides 161 to 180 in the first exon of the ZIC2. We evaluated the ZIC2 alterations at both the DNA sequence and protein expression levels. Regular human DNA should express itself at 659 base pairs. Transcriptome changes were revealed in E006AA-PR VS. E006AA G12, E5. E006AA 114/118. E006AA ZIC2-/- G12 cell line expressed a decrease in base pairs at the 500 markers of the ladder. This proved to be a 150 bp deletion expressing a mutation in ZIC2 gene using KO primers within the cells (Figure 4).

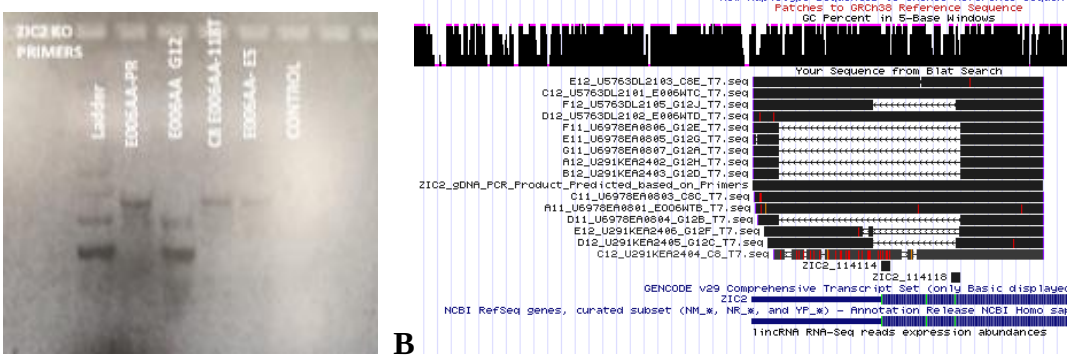


Figure 6. **CRISPR-Cas9 targeting ZIC2 sequencing.** (A) ZIC2 KO primers resulted in a frameshift mutation in the E006AA ZIC2-/- G12 cell line. (B) Sequences of E006 G12 114/118 showed results of a frameshift mutation at the 114 and 118 locations.

CHAPTER V

CONCLUSION

The experimental observations of ZIC2 in prostate cancer showed that ZIC2 is a gene that plays a role to activate and repress other genes. It is a transcription factor that is upregulated in aggressive prostate cancer. From our identification of ZIC2 expression in prostate cancer cell lines, we choose two ZIC2 modified E006AA cells for further study being that ZIC2 protein levels may express metastatic potential.

CRISPR/Cas9 mediated genome editing method inserts a guide RNA 114/118 into a ZIC2 gene with CRISPR/Cas9 mediated genome editing to produce a plasmid. The E006AA cell lines were then produced using a host cell line co-transfected with the plasmid produced by CRISPR/Cas9 mediated genome editing. The 114/118 guide RNA used was capable of guiding CRISPR/Cas9 to the double strand break of host gene.

To evaluate the loss of function of ZIC2 in prostate cancer cell lines, we used CRISPR-Cas9 gene editing of ZIC2 in the parental cell line of E006AA. E006AA ZIC2 -/- cells show altered expression of ZIC2 and significantly decreased proliferation rates when compared to the E006AA parental cells. We were able to determine the phenotype of the E006AA ZI2 modified cell lines by assessing a proliferation growth curve compared to the E006AA Parental cell line (figure 4). Ten thousand cells were the counting starting point of the parental and two ZIC2 -/- cell lines and observed over 3, 5

and 7 days. At day 5 the ZIC2 modified cells showed a five-fold significant decrease compared to the E006AA parental cell line.

Genomic alterations were introduced by CRISPR/Cas9 by PCR of ZIC2 in edited cell lines of E006AA-PR. We determined that regular human DNA expresses itself at 659 bp when loaded on an electrophoresis gel. The ZIC2-/- E5 cell lines expressed itself at 659 bp and the G12 cell line expressed its band at 500 bp (figure5). Sequencing identified the cloned genomic DNA PCR products to be expressed in Sanger sequencing frameshift mutations. Full Length PCR product from WT cell expressed 659 bp deletions. Deletion 1 allele detected by sequencing occurred at 473 bp while deletion 2 allele were detected by sequencing at 249 bp. We hypothesized that a homozygous null allele for the ZIC2 protein in the E006AA cell was created. This means that a frameshift mutation has occurred from Methionine 1,2, and 3 to Glycine.

In the present study, a site-specific CRISPR/Cas system was designed to decrease the growth of androgen-dependent prostate cancer. Stable E006AA cell lines harbouring Cas9 and guide RNAs (114/118) was constructed to knock out ZIC2, and the knockout activity and efficiency of CRISPR was investigated by immunoblot. In each cell line, we observed the E006AA ZIC2-/- G12 cell line expressed no bands compared to the E006AA parental cell line. The parental cell line showed a molecular weight difference at 65 kDa compared to the ZIC2 modified cell line of G12 and E5 that weighed below 55 kDa (figure 3). The results of the present study demonstrated that the treatment with CRISPR/Cas significantly inhibited the growth of E006AA cells.

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