EPIGENETIC REGULATION OF APOPTOSIS IN PROSTATE CANCER

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 and suppresses gene expression by catalyzing histone H3 methylation on lysine 27. EZH2 is overexpressed in metastatic prostate cancer and has been shown to promote cell proliferation and metastasis. Here we show that EZH2 also suppresses prostate cancer apoptosis by coordinating the epigenetic silencing of two pro-apoptotic microRNAs, miR-205 and miR-31.

We previously reported that miR-205 is silenced in prostate cancer through promoter methylation. In this study, we found that EZH2 suppresses miR-31 expression by trimethylation of H3K27 on the miR-31 promoter. SiRNA knockdown of EZH2 increased miR-31 expression and decreased the anti-apoptotic protein E2F6 (a target of miR-31), resulting in the sensitization of prostate cancer cells to docetaxel-induced apoptosis and vice versa. We further demonstrated that miR-205 silencing is linked to miR-31 silencing through EZH2. Suppression of miR-205 caused an increase of EZH2 protein, which in turn inhibited miR-31 expression and vice versa. Thus, EZH2 integrates the epigenetic silencing of miR-205 and miR-31 to confer resistance to chemotherapy-induced apoptosis.

Besides the histone modification by histone methyltransferases (HMTs) such as EZH2, histone deacetylases (HDACs) offer another mechanism to epigenetically regulate gene expressions in cancer. The class I selective inhibitor of HDACs, mocetinostat, has promising antitumor activities in both preclinical studies and the clinical trials. To understand how mocetinostat induces apoptosis in prostate cancer cells, we examined the effects of mocetinostat on miR-31. We found that miR-31 was significantly upregulated by mocetinostat in prostate cancer cells. E2F6 was decreased by mocetinostat treatment. Mocetinostat also increased the expression of pro-apoptotic protein Bad and activated caspase-3 and caspase-9. SiRNA

knockdown of E2F6 sensitized cancer cells to mocetinostat-induced apoptosis. Importantly, we found the same results in the primary prostate cancer stem cells. Thus, activation of miR-31 and downregulation of E2F6 contribute to mocetinostat-induced apoptosis in prostate cancer.

In summary, the epigenetic silencing of miR-31 confers a resistance mechanism for chemotherapy-induced apoptosis in prostate cancer cells. Using mocetinostat to activate miR-31 expression is a novel strategy to overcome resistance to apoptosis and improve response to therapy.

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LIST OF ABBREVIATIONS

3’-UTR	3'–Untranslated Region
µg/ml	microgram per milliliters
µM	micro Moles
ACS	American Cancer Society
cDNA	complementary Deoxyribonucleic Acid
ChiP	Chromatin imuunoprecipitation
CpG islands	Cytosine-phosphodiester bond-Guanine islands
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescence Protein
H3K27Me3	Histone3 Lysine27 trimethyl
H3K9Me2	Histone3 Lysine9 dimethyl
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
miR, miRNA	micro Ribonucleic Acid
mRNA	messenger Ribonucleic Acid
nM	Nano Moles

NC	Negative Control
Nm	Nano meter
nM	Nano mole
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
qRT-PCR	quantitative Real Time PCR
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis
SiRNA	small interfering Ribonucleic Acid
UV	Ultraviolet

CHAPTER 1. BACK GROUND INFORMATION; OBJECTIVES AND SPECIFIC AIMS; MATERIALS AND METHODS

1.1. Back Ground Information

1.1.1. Prostate Cancer

Prostate cancer is a small walnut-shaped gland that is below the urinary bladder and in front of the rectum. Prostate cancer has the topmost incidence rate of new cancer cases in men in US. And it is the second leading cause of cancer related deaths in the developed world with increasing rates in developing world. In the United States in 2015, there were an estimated 220,800 new cases of prostate cancer and about 27,540 deaths due to prostate cancer in men in the US (American Cancer Society, 2013-2014). The lifetime risk of developing prostate cancer is 1 in 7 in men in the US (National Cancer Institute 2014). Prostate cancer is serious disease that may cause pain, difficulty in urinating, and problems during sexual intercourse, erectile dysfunction. However, most men diagnosed with prostate cancer do not die from it. In fact, more than 2.9 million American men are living with current or past diagnosis of prostate cancer.

Prostate cancer starts in the cells of prostate gland. Actually, several types of cells are found in the prostate, but almost all prostate cancers develop from the gland cells (American cancer society 2010). Most prostate cancer cells tend to grow slowly compared with most other cancers, though there are cases of aggressive prostate cancers. The cells may begin to change 10, 20, or even 30 years before a tumor is big enough to cause obvious symptoms. Eventually, the cancer cells may metastasize from the prostate to other parts of the body, particularly the bones and lymph nodes (National cancer institute). As cancer progresses, the tumor at the inner part of

the prostate (around the urethra) keeps growing; the prostate tissue can squeeze the urethra, leading to problems in passing urine (Figure 1).

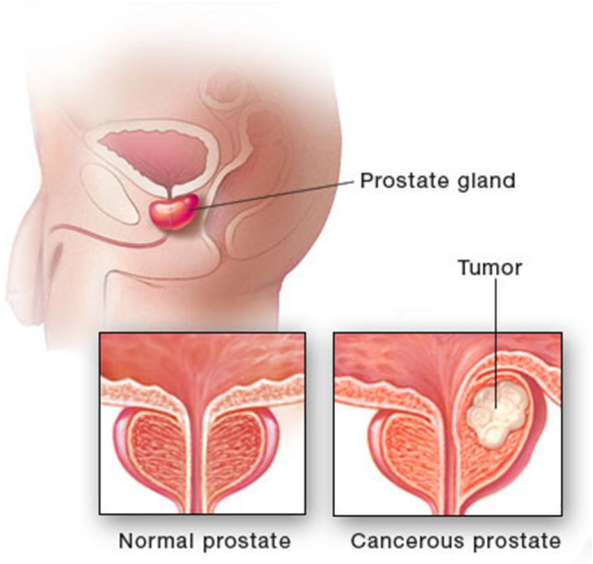

Figure 1. A normal prostate gland and a prostate with a tumor. (This figure is from Mayo foundation for medical education and research.)

1.1.1.1. Prostate cancer risk factors

A complete understanding of the causes of prostate cancer remains elusive (Hsing AW, Chokkalingam AP 2006). However the most probable cause is DNA mutations in cells. The DNA changes can pass down through families or occur due to environment or lifestyle. The demographic risk factors include age, genetics, and dietary and medication exposure.

Age: The incidence of prostate cancer is strongly related to age, young age people can develop prostate cancer, but the incidence rate increases exponentially with advancing age. More than 80% of prostate tumors are diagnosed in men over 65. Men under 65 years of age vs. 65 years and over, prostate cancer incidence rates were 56.8 vs. 974.7 per 100,000 person-year, respectively (Ries, L.A.G, M.P. Eisner 2003).

Genetics: Genetic background is associated with race, family, and specific gene variants that may contribute to prostate cancer risk. The risk of developing prostate cancer will be doubled in men who have a first-degree relative (father, brother, son) with prostate cancer, and five folded in men with two first-degree relatives (Steinberg GD, Carter BS 1990). In the United States, African American men are 60 percent (approximately) more likely to develop prostate cancer over their lifetime as compared to white or Hispanic men. African American men also have a higher motility rate (Hoffman RM, Gilliland FD 2001).

Dietary: A diet high in saturated fat, as well as obesity, increases the risk of prostate cancer (Alexander DD, Mink 2010). While there are several evidences provided to demonstrate the role for dietary fruits and vegetables in prostate cancer incidence, however more research is needed to determine if this is a causative relationship (Key TJ 2011).

1.1.1.2. Prostate cancer prevention

The known risk factors of prostate cancer, age, race, and family history, can barely be controlled. Because of the exact cause of prostate cancer is still unknown, at this time there is no proven prostate cancer prevention strategy. Given that, there is no effective method for preventing prostate cancer, making healthy choices may help to lower the risk of prostate cancer. Limiting the consumption of animal products that contain trans fats, saturated fats, and carbohydrates is thought to reduce the risk of prostate cancer, though the evidence is inconclusive (Masko EM, Allott EH 2012). And Omega-3 fatty acid (commonly exists in fatty fish and flaxseed) has been linked to a reduced risk of prostate cancer (Heinze VM, Actis AB 2012). Vitamin supplements have been studied and none of them are associated with lower risk of prostate cancer. Conversely, high intake of Calcium is correlated with advanced prostate cancer incidence (Datta M, Schwartz GG 2012). Besides the food and supplements, a medication

5-alpha-reductase inhibitor (Finasteride Proscar® and Dutasteride Avodart®) has shown a relative high degree of potential to reduce the overall risk of being diagnosed with prostate cancer. These drugs perform their role by impeding the formation of the most active form of testosterone that causes the prostate to grow. However, these drugs are still not approved by the FDA because of the insufficient data support to determine if they have an effect on the risk of death, and increased chance of more serious cases (Wilt TJ, MacDonald R 2008).

1.1.1.3. Early detection of prostate cancer

Due to the lack of reliable prevention strategies for prostate cancer, the early detection of prostate cancer is particularly important for the purpose of improving cure rate and reducing mortality. Screening is a common method that is utilized to detect a disease such as cancer in people who do not have obvious symptoms of that disease. For some types of cancer, screening can help to detect the warning signs of cancers at an early stage (American Cancer Satiety).

Prostate cancer screening is testing to find unsuspected and asymptoms cancers, and may follow up with more invasive tests such as a biopsy. The screening methods of prostate cancers include Prostate-Specific Antigen (PSA) blood test and Digital Rectal Exam (DRE) (Djulbegovic M, Beyth RJ 2010).

Prostate-Specific Antigen (PSA) blood test:

Prostate-Specific Antigen blood test is to measure the concentration of PSA in blood to look for signs of prostate cancer and help to diagnose prostate cancer in its early stages, when treatment is more effective. PSA is a glycoprotein enzyme with folate hydrolase activity that is produced by epithelial cells in prostate gland. And it helps liquefy semen in the seminal coagulum to allow sperm to swim freely (Aumüller G 1979; Balk SP, Ko YJ 2003). PSA exists in both cancerous (malignant) and noncancerous (benign) prostate tissue. Usually cancer cells

have a higher level of PSA than noncancerous cells. A small amount of PSA secreted by prostate gland normally can enter the bloodstream, and a level of less than 4 ng/ml (nanograms per milliliter) PSA blood concentration is regarded as normal. But the causes of elevating PSA levels in blood can also be a noncancerous condition such as prostatitis or an enlarged prostate. Therefore, PSA is not a unique indicator of prostate cancer and determining what a high PSA score means can be complicated (Velonas VM, Woo HH 2013).

Digital Rectal Exam (DRE):

A digital rectal exam is another screening test for prostate cancer early detection. As part of a complete physical examination, DRE is to check for growths in or enlargement of the prostate gland (WebMD.com). Since prostate is an internal organ that lies in front of the rectum, the exam is performed by inserting a lubricated, gloved finger into the rectum, and use another hand to press on the lower belly or pelvic area to feel for lumps, soft or hard spots, and other abnormalities. To minimize the risk of misdiagnosing prostate cancer, DRE should always be done with a PSA test (Fritz H. Schröder, Jonas Hugosson 2009).

Prostate biopsy and Gleason Score:

If the result from a PSA blood test rises to a level that prostate cancer is suspected to occur or the result from a DRE finds abnormality, prostate biopsy will be advisable for the patient to be performed. Prostate biopsy is a series of procedures that are performed to remove small hollow needle-core samples from a man's prostate gland. And the collected tissue samples are examined for the presence of cancer cells under microscope (Essink-Bot ML, de Koning HJ 1998-06-17).

Gleason score is used to help evaluate the microscopic features of biopsy cylindrical samples and incorporated into the strategy of prostate cancer staging that predicts prognosis and

helps guide therapy. A Gleason score is given to prostate cancer depended on its microscopic appearance. Lower scores are associated with small, closely packed glands. And as the score increases, cells exhibit a spread out pattern and start lose their glandular architecture ("Male Genital Pathology" 2009-05-13). Gleason scores range from 2 to 10, with 2 representing the most well differentiated tumors and 10 the least-differentiated tumors (Kumar V, Abbas AK 2004). Gleason score, PSA, and DRE work together to determine clinical risk, which then dictates treatment option (Epstein JI, Allsbrook WC Jr 2005; 29(9): 1228-42).

1.1.1.4. Prostate cancer treatment (American Cancer Society)

There are different types of treatment options for prostate cancer. And the choices doctors make depend on several factors, which include how widely it has spread and patients' overall health, as well as the benefits and the potential side effects of the treatment (Mayo clinic). Typically seven standard treatments are used: Watchful waiting or active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy, biologic therapy and bisphosphonate therapy. In addition to these, three new types of treatment are being tested in clinical trials: Cryosurgery, high-intensity focused ultrasound and proton beam radiation therapy.

1.1.2. Epigenetics

Epigenetics refers to changes in gene expression via covalent chemical modifications of DNA, associated proteins, or RNA, resulting in changes to the function and/or regulation of these molecules, without an alteration in their primary sequence (Vichithra R. B. Liyanage, Jessica S. Jarmasz 2013). The epigenetic changes comprise DNA methylation, histone modification, nucleosome remodeling and RNA-associated silencing (Costantine Albany, Ajjai S. Alva 2011). These changes lead to conformational transforms in the DNA double helix and chromatin packaging. And eventually, these changes impact the transcription of associated genes

by modifying the access of transcription factors to promoter regions upstream of coding sequences (D. Hanahan, R.A. Weinberg, 2011; A. S. Perry, R.W.G. Watson 2010). Epigenetic mechanisms are crucial for normal development and maintenance of tissue-specific gene expression patterns in mammals (Shikhar Sharma, Theresa K.Kelly 2010). Progressive epigenetic aberrations can exert profound outcomes such as gene function alteration and malignant cellular transformation. Global changes in the epigenetics contribute as a hallmark in initiation and progression of cancer (Shikhar Sharma, Theresa K. Kelly 2010).

1.1.2.1. DNA methylation

DNA methylation is a covalent biochemical modification process where a methyl group is enzymatically added to the carbon-5 position of the cytosine ring (P. M. Das, R. Singal 2004). And this modification can give rise to a spontaneous deamination of 5-methylcytosine to thymine, consequently cause conformational changes of chromatin and transcriptional suppression (A. P. Bird, 1986). (Figure 2) The sequence 5'-CG-3' (also called the CpG dinucleotide) is the most favorable substrate for DNMTs. In adult genome, 60-90% of CpG dinucleotides are methylated. The unmethylated CpG dinucleotides are usually present on the region of DNA in normal tissues, especially on promoter region, which are called CpG islands. CpG islands are regions with a high frequency of CpG sites, usually defined as a sequence with at least 200bp, and a CG content percentage larger than 50%, and with an observed to expected ratio of CpG that is more than 60% (D. Takai and P.A. Jones 2002). The DNA methylation is accomplished by three identified DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) that present a higher affinity to 5-methylcytosine than to cytosine, and lead to functional silencing of gene expression (M. Manoharan, K. Ramachandran 2007). (Figure 3)

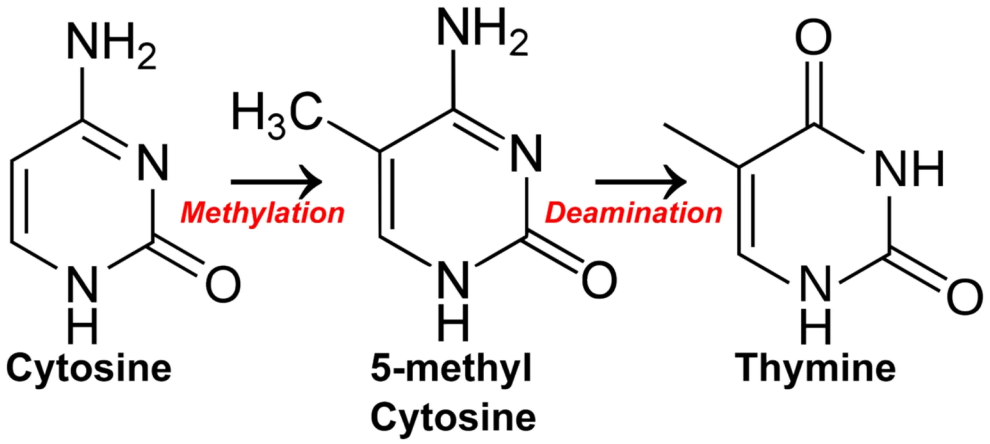

Figure 2. DNA methylation causes conformational changes of chromatin. DNA methylation adds a methyl group to the carbon-5 position of the cytosine ring by DNMT, then spontaneously deaminates to thymine in genomes. (This figure is from Scarano E., Laccarino M, et al., 1967.)

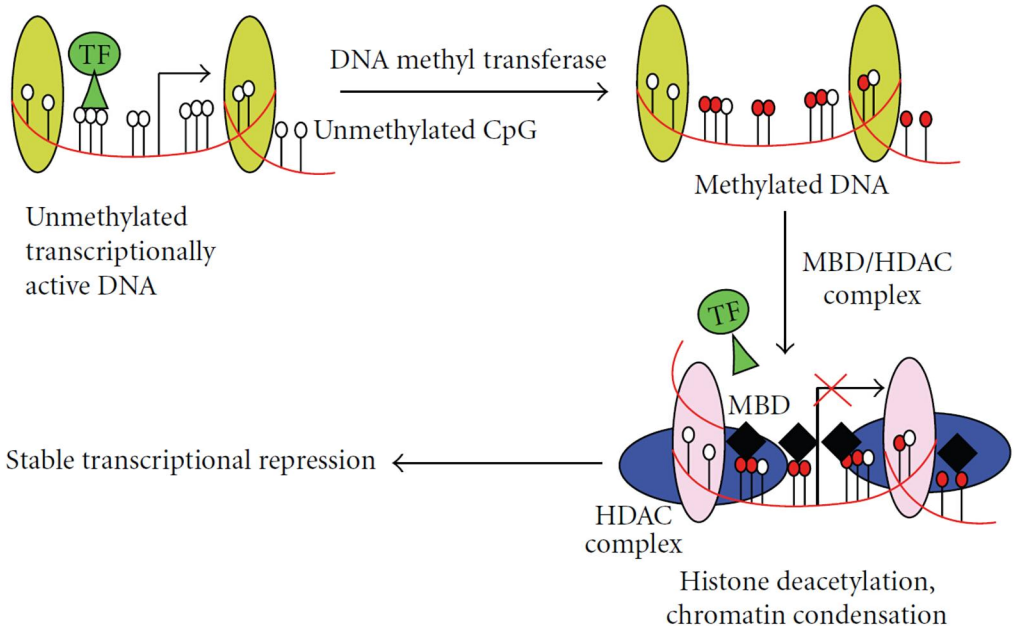

Figure 3. Epigenetics of gene expression silencing. (a) In unmethylated DNA (depicted by white hollow circles on left) transcription factors (TF) can easily access the gene promoter regions. In hypermethylated DNA (depicted in red filled-in circles on the right) access of TF to gene promoter regions is sterically hindered resulting in functional silencing of gene expression. (b) The methyl-CpG-binding domain protein (MPD)/histone deacetylase (HDAC) complexes can enzymatically cause histone deacetylation, which is associated with a condensed structure that inhibits normal gene transcription. (This figure is from M. Manoharan, K. Ramachandran, et al., 2007.)

1.1.2.2. DNA methylation in prostate cancer

To acquire and maintain the neoplastic phenotype of prostate cancer, the promoters are commonly hypermethylated. In prostate cancer, several classic tumor-suppressor gene functions are repressed by hypermethylation, which include hormone signaling, DNA repair, cell adhesion, cell-cycle control, and apoptosis (S. Yegnasubramanian, J. Kowalski 2004; C. Jeronimo, R. Henrique 2004; R. Maruyama, S. Toyooka 2002). (Table 1) Hypomethylation, a second methylation defect, is commonly observed in advanced stage prostate cancer and contributes to oncogenesis. A number of mechanisms of hypomethylation are applied for cancer occurrence, such as activation of oncogenes (c-MYC and H-RAS), activation of latent retrotransposons, and

Table 1. Hypermethylated genes in prostate cancer. (This table is from S. Yegnasubramanian, J. Kowalski 2004.)

DNA repair gene
GSTP1
MGMT
Tumor-suppressr genes
APC
RARβ
RASSF1
Hormone receptor genes
AR
ESR1,2
Cell adhesion genes
CDH1
CDH13
CDH4
Cell-cycle control genes
CCDN2
CDKN1B
SFN
Apoptotic genes
GADD45a
PYCARD
RPRM
GLIPR1

reduction of chromosome stability (W. G. Nelson, S. Yegnasubramanian, 2007; S. Yegnasubramanian, M. C. Haffner 2008).

1.1.2.3. Histone modification and chromatin remodeling

The nucleosome is the fundamental repeating units of eukaryotic chromatin, which consists of approximately 174bp of DNA tightly wrapped almost two times around the cylindrical histone octamer. Histone octamer composed of two copies of each of the four core histone proteins (Alberts Bruce 2002). Histone proteins are classified as either core histones (H2A, H2B, H3, and H4) or linker histones (H1 and H5) (Cox Michael, Nelson David R. 2005; Bhasin M, Reinherz EL, Reche PA 2006; Hartl Daniel L., Freifelder David 1988). Histones are not only enable packaging of the large genome into nucleus, but also realized as being dynamic regulators of gene activity that undergo many posttranslational chemical modifications, including methylation, acetylation, ubiquitination, and phosphorylation (M. Esteller 2007). The modifications commonly occurr on the N-terminal tails of core histones that are rich in positively charged amino acids, which protrude out of the nucleosome. (Table 2) Three prominent regulators of histone modification are histone deacetylases (HDACs), histone acetyltransferases (HATs), and histone methyltransferases (HMTs) (J. R. Dobosy and E. U. Selker 2001; M. H. Kuo and C. D. Allis 1998). Different modifications are likely to result in either gene activation or suppression that depends on the amino acid position and the number of added residues (C. Martin and Y. Zhang 2005; T. Kouzarides 2007). (Table 3) Although the biological significance of histone modifications is still elusive, these modifications are known to be involved in diverse biological processes including DNA repair, gene regulation, spermatogenesis (meiosis) and chromosome condensation (mitosis) (Ning Song, Jie Liu 2011).

Table 2. Types of histone modifications (This table is from Kouzarides T. 2007)

Amino Acid	Modification
Lysine	Methylation, Acetylation, Ubiquitination, Sumoylation, ADP-Ribosylation
Arginine	Methylation
Serine	Phosphorylation
Threonine	Phosphorylation

Table 3. "Histone codes", combination of histone modifications in transcription regulation. (This table is from Strahl BD, Allis CD 2000; Jenuwein T, Allis CD 2001.)

Type of modification	Histone							
	H3K4	H3K9	H3K14	H3K27	H3K79	H3K36	H4K20	H2BK5
Mono-methylation	activation	activation		activation	activation		activation	activation
di-methylaton		repression		repression	Activation			
tri-methylation	activation	repression		repression	Activation repression	activation		repression
acetlyation		activation	activation	activation				

1.1.2.4. Histone modification in prostate cancer

Increasing evidence indicates that histone modification has a crucial role in prostate tumorigenesis. The predictable clinical outcomes of different change levels in individual histone modifications can help to identify patients with adverse prognosis and high risk for recurrence (J. Ellinger, P. Kahl 2010; T.Bianco-Miotto; K. Chiam 2010). For instance, global methylation of H3K4 and acetylation of H3K18Ac can refer to the independent predictor of recurrence in

low-grade prostate cancer (D. B. Seligson, S. Horvath 2005; L. X. Zhou; T. Li 2010). Histone methyltransferase, EZH2 (Enhancer of zeste homolog 2) was shown to be amplified and overexpressed in a variety of cancers including prostate cancer (Chase A, Cross NC. 2011). High level expression of EZH2 has shown to be closely correlated with prostate cancer progression. Moreover, the increase of EZH2 expression is recognized as a predictor of a higher risk of recurrence after radical prostatectomy (Varambally S., Dhanasekaran SM 2002; Berezovska OP, Flinskii AB 2006). EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2). It suppresses gene expression by catalyzing the trimethylation of lysine 27 on histone 3 (H3K27) (Cao R., Wang L. 2008). (Figure 4) PRC2 can catalyze sequential methylation at H3K27, producing mono-, di-, and trimethylated H3K27 (H3K27me1, H3K27me2, and H3K27me3) (Simon JA, Lange CA. 2008). The PRC2 complex contains five subunits, including EZH2, EED, SUZ12, RbAp46/48, and AEBP2. The oncogenic activity of EZH2 has been extensively studied in prostate cancer and most results are focusing on its function in promoting proliferation and metastasis (Kleer CG, Cao Q. 2003; Bachmann IM, Halvorsen OJ 2006; Deb G., Thakur VS 2013; Velichutina I., Shaknovich R. 2010; Cao Q., Yu J. 2008). Recent studies have also demonstrated that EZH2 can suppress apoptosis (Q Zhang, SKR Padi 2014). However, the mechanisms of apoptosis suppression by EZH2 remain poorly understood.

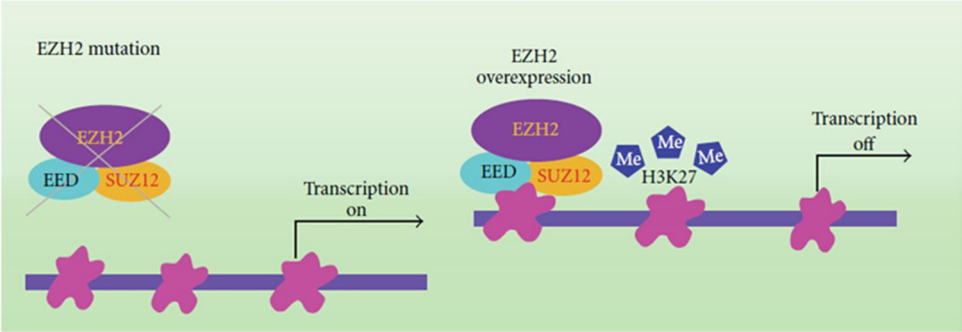

Figure 4. The mutation of EZH2 usually turns on gene transcription. (This figure is from Costantine Albany, Ajjai S. Alva 2011.)

1.1.2.5. Epigenetic therapy of cancer

Unlike the classic genetic alterations that encode information within the DNA sequence, epigenetic changes can store the information in multiple dimensions, such as in the form of DNA modifications, histone modifications, or RNA silencing. The multidimensional nature of epigenetics provides many and varied potentials for medical applications (Chahwan R, Wontakal SN, Roa S. 2011). Epigenetic therapy aims to use drugs or other epigenome-influencing techniques to reverse the abnormal epigenetic changes in cancer to treat medical conditions.

Table 4. FDA-Approved epigenetic therapies (This table is from Jane de Lartigue 2013.)

Agent	Class	Company	Approval Date	Approved Indication
Azacitidine (Vidaza)	DNMT inhibitor	Celgene Corportation	2004	FBA myelodysplastc syndrome subtypes
Decitabine (Dacogen)	DNMT inhibitor	Eisai	2006	Myelodysplastic syndrome
Vorinostat (Zolinza)	Pan-HDAC inhibitor	Merck	2006	Cutaneous T-cell lymphoma
Romidepsin (Isodax)	Class I HDAC inhibitor	Celgene Corportation	2009	Cutaneous T-cell lymphoma
Ruxolitinib (Jakafi)	JAK1/2 inhibitor	Incyte Pharmaceuticals	2011	Intermediate or highrisk myeofibrosis

Azacytidine and Deoxyazacytidine are discovered as two DNA methyltransferase inhibitors. They have already been approved by the Food and Drug Administration (FDA) to treat patients with myelodysplastic syndromes, a blood cancer produced by abnormal bone marrow stem cells (Jones PA, Baylin SB 2002). An inhibitor of histone deacetylase, Vorinostat (Suberoylanilide hydroxamic acid), has also been approved for the treatment of cutaneous T-cell lymphoma. (Table 4) In addition to DNA methylation and HDAC inhibitors, 3-Deazaneplanocin A (DZNep), which is a histone methyltransferase (HMT) inhibitor, has also been developed to target the degradation of histone methyltransferase EZH2 and then selectively inhibit trimethylation of H3K27me3 and H4K20me3 as well as reactive silenced genes in cancer cells

(Miranda TB, Cortez CC 2009). Other epigenetics drugs with diverse targets are in clinical trials or development as well.

1.1.3. Mocetinostat (MGCD): Class I Selective HDAC Inhibitor

Histone deacetylases (HDACs) are important epigenetic regulators of gene expression (Gray S.G. and T.J. Ekstrom 2001). These enzymes deacetylate lysines of core histone tail and result in transcription repression (Strahl B.D. C.D. Allis 2000). HDACs also deacetylate lysines of other proteins and regulate cellular functions ranging from proliferation, apoptosis, to metastasis and angiogenesis (Minucci S. and P.G. Pelicci 2006; Choudhary C., et al. 2009). There are eighteen mammalian HDACs and they are classified into four groups (Haberland M., R.L. Montgomery 2009): class I HDACs (HDAC1, -2, -3, and -8); class II HDACs (HDAC4, -5, -6, -7, -9, and -10); class III HDACs (sirt1-7); and class IV HDAC (HDAC11). The class I HDACs are frequently upregulated in various types of cancers compared to the corresponding normal tissues and their upregulation is associated with a poor prognosis (Weichert W., et al. 2008. 9(2): p.138-48; Weichert W., et al. 2008. 98(3):p.604-10; Weichert W., et al. 2008. 14(6):p.1669-77). HDAC inhibitors have been developed and extensively tested in phase I–III clinical trials. However, these new agents showed minimal clinical activity in patients with solid tumors while undesired side effects are also a problem (Slingerland M., H.J. Guchelaar 2014). One of the reasons for the poor performance of the early generation of HDAC inhibitors may be because they inhibit multiple classes of HDACs, which results in toxicity and limits the achievable therapeutic doses. Mocetinostat, also known as MGCD0103, is one of the new class I selective HDAC inhibitors (Fournel M., et al. 2008). Preclinical studies have demonstrated broad-spectrum antitumor activities of mocetinostat against various types of cultured cell lines and tumor xenografts in nude mice (Fournel M., et al. 2008; Bonfils C., et al. 2008). It has been

shown that mocetinostat inhibits colon cancer cell growth by upregulating WNT ligand DKK-1 expression (Sikandar S., et al. 2010). In B-cell chronic lymphocytic leukemia cells, mocetinostat has been shown to induce apoptosis through decreasing anti-apoptotic Mcl-1 protein and inducing Bax translocation to the mitochondria (El-Khoury V., et al. 2010). While mocetinostat has been shown to effectively kill prostate cancer cells (Fournel M., et al. 2008), the mechanisms of apoptosis induction remain poorly understood.

1.1.4. Noncoding MicroRNAs

MicroRNAs (microRNAs) are small non-coding RNAs with a length of about 22 nucleotides. Commonly, microRNAs genes are transcribed by RNA Polymerase II (Pol II) to form large ~80-nucleotide-long primary microRNAs with a hairpin structure. The primary microRNAs are processed by the ribonuclease Drosha and RNA binding protein Pasha to become 60-70 nucleotide-long precursor microRNAs. Following that, the enzyme Ran GTP/Exportin 5 exports the precursor microRNAs out of nucleus. In cytoplasm, the precursor microRNAs are further processed into 19-25 nucleotides long mature microRNAs by the RNase III protein Dicer. The mature microRNAs are incorporated into the RNA-induced silencing complex (RISC) to interact with target mRNAs and associate with the 3'-untranslated region (UTR). Eventually the interaction causes either degradation of mRNA or translational repression to regulate gene expression depending on the degree of complementarity (Bartel DP 2009; Lee y., et al 2002; Cai X., et al., 2004). (Figure 5)

MicroRNAs regulate very extensive biological functions and processes in plant and animals. In human, around 2000 microRNAs have been discovered, and individual microRNA targets about 100 to 200 messenger RNAs (Lim L., Lau N. 2005). Approximately 60% human protein-encoding genes are modulated by microRNAs at the level of transcription or translation

into proteins (Friedman R, Farh K 2009). Many microRNAs themselves are controlled by epigenetic changes including DNA methylation and histone modification or the combination of both (Bernal J, Duran C 2012). Dysregulation of various microRNAs has been shown to play critical roles in cancer biology, ranging from proliferation (Kota J, Chivukula RR 2009), differentiation (Chen CZ, Li L. 2004; Zhang J, Jima DD 2009), metabolism (Poy MN, Eliasson L 2004), metastasis (Ma L, Teruya-Feldstein J 2007), to apoptosis (Hermeking H. 2010). MicroRNAs have been reported to be potential targets for epigenetic therapy. By mimicking tumor suppressor microRNAs, treatment based microRNAs can be synthesized to selectively repress oncogenes in tumors and restore normal microRNA-mRNA regulation pathways. The challenge of this novel therapeutic strategy is to establish a safe and efficient deliver system to introduce synthetic microRNAs to tumor cells (Shikhar Sharma, Theresa K. Kelly 2010).

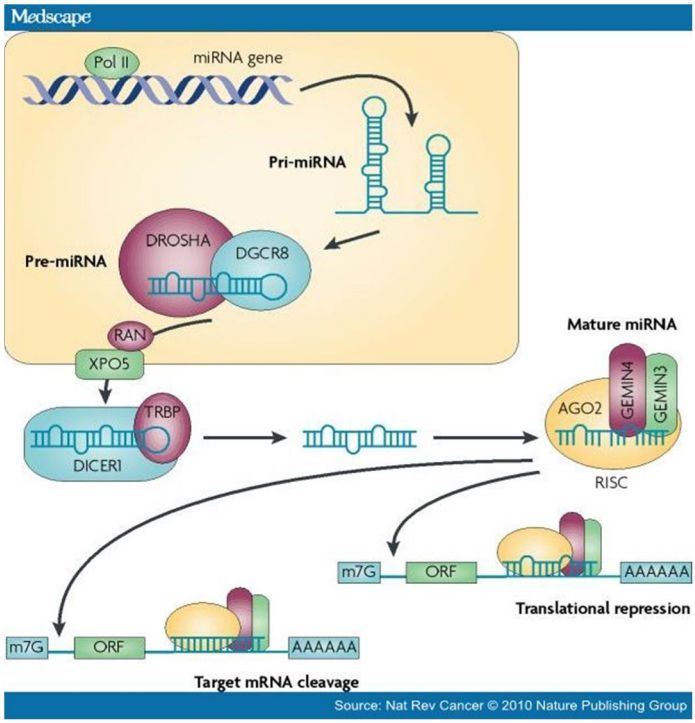

Figure 5. Biogenesis of microRNAs. (This figure is from Nat Rev Cancer 2010 Nature Publishing Group.)

1.2. Objective and Specific Aims

1.2.1. Objective of the Study (Project 1)

The main goal of this project was to investigate the novel epigenetic mechanisms of apoptosis regulation in prostate cancer.

Prostate cancer has the top incidence rate of new cancer cases, and it is the second leading cause of cancer-related death in the United States. The histone methyltransferase EZH2 has been reported to significantly overexpress in metastatic prostate cancer. High levels of EZH2 expression correlates with prostate cancer progression and a higher risk of recurrence after radical prostatectomy. Recent studies have also demonstrated that EZH2 can suppress apoptosis. However, the mechanisms of apoptosis suppression by EZH2 remain poorly understood. We have focused on the novel epigenetic mechanisms of apoptosis regulation in prostate cancer. MiR-31 is one of the most downregulated miRNAs in advanced prostate cancer cells. We identified that EZH2 is potentially responsible for the downregulation of miR-31. The antiapoptotic protein E2F6 (target of miR-31) downregulation is related to sensitize prostate cancer cells to docetaxel-induced apoptosis. Also, it has been recently reported that miR-205 can decrease the expression of EZH2 protein in prostate cancer. By silencing of miR-205, we have shown that the expression of EZH2 is upregulated, which in turn epigenetically represses miR-31 expression.

This study is important because the results will help us to understand the epigenetic mechanism of EZH2 regulation of apoptosis in prostate cancer.

Various small-molecule drugs have been developed to target EZH2, including EI1, GSK 126, DZNep and UNC1999. For instance, GSK 126 is a very potent EZH2 inhibitor (with a K_i of 0.5-3nM) and has high selectivity for EZH2 (more than 1000-fold higher activity than its activity

for 20 other human methyltranserases). And it is known that EZH2 inhibitors have significant anti-cancer properties by inducing apoptosis to suppress tumor growth and increase survival (McCabe MT, Ott HM 2012; Tan J Yang X 2007; Knutson SK, Wigle TJ 2012). However, the mechanisms of EZH2 in apoptosis regulation remain elusive.

This study has the potential to identify a new mechanism for EZH2 suppression of apoptosis in prostate cancer. Our study is innovative because the mechanism for miR-31downregulation in prostate cancer has never been investigated and the role of EZH2 in apoptosis regulation in prostate cancer is entirely novel. As a new epigenetic mechanism to regulate apoptosis, the axis of miR-205, miR-31 and EZH2 will provide a conceptual advance in cancer response to chemotherapy.

In this study, we hypothesize that EZH2 coordinates the silencing of the proapoptotic miR-205 and miR-31 to confer resistance to chemotherapy-induced apoptosis in prostate cancer.

1.2.2. Specific Aims (Project 1)

To test our hypothesis, the following specific aims were proposed:

i) To determine the mechanism of downregulation of miR-205 and miR-31 in prostate cancer in vitro.

The working hypothesis was that DNA methylation-mediated silencing of miR-205 can lead to histone methylation-mediated silencing of miR-31, with EZH2 as the coordinator of the two separate events. As a result, the expression of antiapoptotic protein E2F6 is increased and contributes to the development of apoptosis resistance. The experimental design for this part of the study was:

a) To determine the effect of EZH2 on miR-31expression in prostate cancer cells by Western blot and real-time PCR analysis.

b) To determine the mechanism by which miR-31 is downregulated by EZH2 with chromatin
immunoprecipitation assay.
c) To determine the effect on EZH2 on docetaxel-induced apoptosis in prostate cancer cells by cell death ELISA plus assay.
d) To determine the effect of E2F6 on chemotherapy-induced apoptosis in prostate cancer cells by cell death Elisa plus assay.
e) To determine the effect of miR-205 on EZH2 regulated miR-31 in prostate cancer cells.

ii) To determine the expression and regulation of miR-205, EZH2, and miR-31 in human prostate cancer specimens.

The working hypothesis was that miR-205 and miR-31 expression levels will be decreased in the cancer samples compared with the adjacent normal tissues. In the meantime, EZH2 expression will be upregulated in the cancer specimens. The experimental design for this part of the study was:

a) To determine the expression levels of miR-205 and correlation with EZH2 expression in human prostate cancer specimens by Real-Time PCR.
b) To determine the expression levels of miR-31 and correlation with EZH2 expression in human prostate cancer specimens by Real-Time PCR.

1.2.3. Objective of the Study (Project 2)

The main goal of this project was to investigate the mechanisms how mocetinostat induces apoptosis in prostate cancer.

Histone deacetylases (HDACs) are important epigenetic regulators of gene expression. Mocetinostat is a class I selective HDAC inhibitor and has promising antitumor activities in preclinical studies. Recent clinical trials have demonstrated excellent response of mocetinostat against myelodysplastic syndrome and relapsed Hodgkin's lymphoma. However, a phase II study also found that mocetinostat has limited efficacy as a single agent in relapsed and refractory chronic lymphocytic leukemia (CLL). Thus, it is important to understand the mechanism of mocetinostat-induced apoptosis in order to improve its efficacy in cancer therapy and overcome drug resistance.

This study has the potential to provide an insight mechanism of mocetinostat to exert antitumor activities by inducing apoptosis in prostate cancer. Our study is innovative because this is the first study of microRNAs in the antitumor action of mocetinostat. The axis of miR-31 and its target E2F6 is a novel mechanism to elucidate the apoptosis-inducing function of mocetinostat.

In this study, we hypothesize that activation of miR31 and downregulation of E2F6 contribute to mocetinostat-induced apoptosis in prostate cancer cells.

1.2.4. Specific Aims (Project 2)

To test our hypothesis, following specific aims were proposed:

i) To determine the role of mocetinostat on proapoptotic miR-31 expression in prostate cancer in vitro.

a) To determine if mocetinostat can induce apoptosis in prostate cancer by crystal violet assay.

b) To determine the effect of mocetinostat on miR-31 expression by selectively inhibiting the class I HDACs in prostate cancer using cell death Elisa plus assay.

c) To determine the expression status of activate caspases by western blot analysis.

d) To determine the effect of E2F6 on mocetinostat-induced apoptosis in prostate cancer cells by cell death Elisa plus assay.

ii) To determine the role of mocetinostat on proapoptotic miR-31 expression in primary prostate cancer stem cells.

a) To determine if mocetinostat can inducemiR-31 expression and activates apoptosis in primary prostate cancer stem cells.

1.3. Materials and Methods

1.3.1. Cell Lines Used and Transfection

The prostate cancer cell lines WPE1-NA22, PC-3, and DU-145 were obtained from American Type Culture Collection (Manassas, VA, USA). The WPE1-NA22 cells were cultured in Keratinocyte Serum Free Medium (K-SFM, Invitrogen), supplemented with bovine pituitary extract and human recombinant epidermal growth factor. PC-3 and DU-145 cells were cultured in RPMI-1640 medium (Hyclone, Thermo scientific) containing 10% fetal bovine serum (FBS). All cell lines were maintained in 5% CO2-humidified atmosphere at 37℃, and passaged using 0.05% trypsin-EDTA (Invitrogen). Human prostate cancer stem cells were purchased from CELPROGEN (San Pedro, CA) and cultured following the manufacturer's protocol. For transient transfection, plasmids were transfected into cells using LipofectamineTMPlus Reagent (Invitrogen) following the manufacturer's protocol. SiRNAs were transfected into cells using X-treme GENE siRNA transfection reagent (Roche) following the manufacturer's protocol.

1.3.2. Drugs and Chemicals

Docetaxel was purchased from Sigma. 3-Deazaneplanocin A (DZNep CAS 102052-95-9) was purchased from Cayman Chemical Company. Formaldehyde was purchased from Alfa

Aesar (Ward Hill, MA). Mocetinostat was purchased from Selleckchem (Houston, TX). Vorinostat was purchased from Biovision (Mountain View, CA).

1.3.3. Western Blot Analysis

Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). Complete protease inhibitor cocktail (Roche) was added to lysis buffer before use. Protein concentration was determined by Bio-Rad DC protein assay kit (Bio-Rad). Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The membranes were blocked with 5% non-fat milk in PBS overnight at 4℃ then incubated with primary antibody and subsequently with appropriate horse radish peroxidase-conjugated secondary antibody. The protein bands were developed with ECL reagents (Pierce) and exposure to X-ray films. Anti-β-tubulin and anti-E2F6 antibodies were purchased from Santa Cruz Biotechnology. Cleaved caspase-9, cleaved caspase-3, cleaved PARP, Bad, Bid, Bak, Puma, Bax, and EZH2 antibodies were purchased from Cell Signaling.

1.3.4. Detection of Apoptosis by Cell Death Assay Elisa^{PLUS}

The Cell Death Detection Elisa^{PLUS} kit (Roche) was used to detect apoptosis following the manufacturer's protocol. This assay determines apoptosis by measuring mono- and oligonucleosomes in the lysates of apoptotic cells. The cell lysates were placed into a streptavidin-coated microplate and incubated with a mixture of anti-histone-biotin and anti-DNA-peroxidase antibodies, and incubation buffer for two hours. The antibodies were bound to the histone- and DNA-component of the nucleosomes, and the immunocomplexes were fixed to the microplate by streptavidin-biotin interaction. The amount of peroxidase retained in the immunocomplex was photometrically determined with ABTS as the substrate. Absorbance was measured at 405 nm.

1.3.5. WST-1 Cell Proliferation Assay

WST-1 reagent (Roche, Indianapolis, IN) is a simple, colorimetric assay designed to measure the relative proliferation rates of cells in culture medium. The principle of WST-1 assay is based on the conversion of the tetrazolium salt, WST-1 (slightly red) into water soluble formazan dye (dark red) by mitochondrial dehydrogenase present only in viable cells. The activity of mitochondrial dehydrogenase increases proportionally to the number of viable cells, leading to an increase in the conversion of WST-1 to formazan dye. The resulting increased color intensity is in turn quantified by measuring the absorbance at 440 nm wavelength. Procedure in brief, after treatment with Docetaxel, prostate cancer cells were incubated with WST-1 for 4 hours at 37^{o} C in a humidified atmosphere maintained at 5 % CO_2 as recommended by the manufacturer. The absorbance of the formazan dye was measured colorimetrically.

1.3.6. Real-Time Polymerase Chain Reaction

Gene expression was measured by real-time PCR using TaqMan® assays (Cat # TM509 for miR205, TM1100 for miR-31, Hs00544833_m1 for EZH2) from Applied Biosystems (Foster city, CA). Total RNA was isolated using mirVana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions and quantified with a Nano-Drop spectrophotometer. 5μg of total RNA was used in the reverse transcription reaction. The cDNAs were used as templates to perform PCR on an Applied Biosystems 7500 Real-time PCR System following the manufacturer's protocol. Relative miRNA expression levels were calculated using 18S RNA as reference.

1.3.7. 5' RACE

The transcription start site of miR-31 pri-miRNA was identified by 5'RACE experiments with FirstChoice® RLM-RACE kit from Ambion, using total RNA isolated from WPE1-NA22

cells as template. cDNA was synthesized from total RNA (5 μg) with random primers. The 5' ends of the miRNAs were amplified with a gene-specific primer. Amplicons were reamplified successively with nested-gene-specific primers.

1.3.8. Transient Transfection of PC-3 Cells with pCEP4-EZH2 Plasmid

We created an expression vector to over express the full length *EZH2* gene in PC-3 cells. The *EZH2* cDNA was cloned into the pCEP4 vector (Figure 6) to express EZH2 as a Flag-tagged protein. Full length *EZH2* gene was obtained by polymerase chain reaction (PCR) using expressed-sequence tag (EST) clone as a template. Transient transfection was done in PC-3 cells with this plasmid using Lipofectamine ™ PLUS Reagent (Invitrogen) following manufacturer's protocol. 1.0 μg/ml of plasmid was used to form complexes with transfection reagent. EZH2 overexpression was confirmed by western blot using anti-Flag antibody.

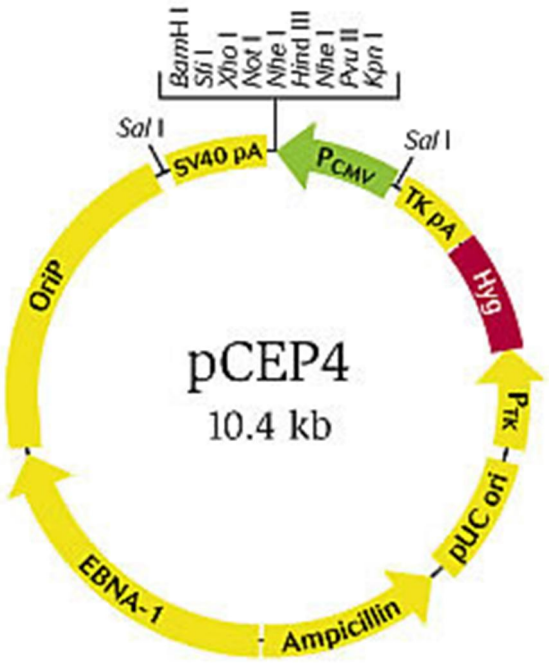

Figure 6. pCEP4 plasmid. (Invitrogen Catalog # V044-50).

1.3.9. Transient Transfection of PC-3 Cells and DU-145 Cells with Negative Control or EZH2 SiRNA

The Silencer® siRNAs for EZH2 (target sequence GACUCUGAAUGCAGUUGCU) and negative control (Catalog # AM4611) were purchased from Ambion. These siRNAs were transfected into PC-3 cells and DU-145 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianspolis, IN) following manufacturer's protocol. 100 nM negative control or EZH2 siRNA was used for transfection into PC-3/DU-145 cells and EZH2 knockdown was confirmed by western-blot. Effect of EZH2 knockdown on drug resistance was determined by Cell Death Elisa Assay.

1.3.10. Transient Transfection of PC-3 Cells with Negative Control or E2F6 SiRNAs

The Silencer® siRNAs for E2F6 (siRNA ID 4185) and negative control (Catalog # AM4611) were purchased from Ambion. These siRNAs were transfected into PC-3 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianspolis, IN) following manufacturer's protocol. 100 nM negative control or E2F6 siRNA was used for transfection into PC-3 cells and E2F6 knockdown was confirmed by western-blot. Effect of E2F6 knockdown on drug resistance was determined by Cell Death Elisa Assay.

1.3.11. Transient Transfection of PC-3 Cells with MiR-205 and MiR-NC Mimics

miRIDIAN® miR-205 mimic (Cat# C-300564-05) and negative control miRNA (Cat# CN-001000-01-05) were purchased from Dharmacon. miRIDIAN® Pre-miR™ miRNA Precursor Molecules are small, chemically modified, double-stranded RNA molecules designed to mimic endogenous mature miRNAs. These miRNA mimics were transfected into PC-3 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN) following

manufacturer's protocol. 100 nM negative control miRNA or miR-205 mimic was used for transfection into PC-3 cells and the effect of transfection was confirmed by western blot analysis.

1.3.12. Transient Transfection of PC-3 Cells with Anti-miRTM Inhibitor for MiR-205

Anti-miR^{TM} microRNA Inhibitor for miR-205 (ID AM11015) was purchased from Life Technologies. Anti-miR^{TM} microRNA Inhibitors are ideal for use as specific suppressors of microRNA activity. These miRNA inhibitors were transfected into PC-3 cells using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN) following the manufacturer's protocol. 100 nM Anti-miR^{TM} microRNA inhibitor specific to miR-205 or the negative control inhibitor was used for transfection. Total RNA and protein was isolated to analyze the miR-31 and EZH2 / E2F6 expression by RT-PCR and Western blot respectively.

1.3.13. Plasmid Construction

The full-length E2F6 cDNA was obtained by PCR using an EST clone as template and constructed into pcDNA3-HA vector.

1.3.14. Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit from Millipore (Billerica, MA, USA). Formaldehyde was added directly to the medium containing prostate cancer cells after desired DZNep treatment (5μM) or SiRNA knockdown of EZH2, to crosslink histones to DNA. After incubating cells with formaldehyde for 10 minutes at 37^0C and 5% CO_2, media was removed as much as possible. Cells were washed twice using ice cold PBS containing protease inhibitors. Cells were scrapped into a conical tube and centrifuged at 2000 rpm for 4 minutes at 4^0C. Cell pellet was resuspended in 200μl of SDS Lysis Buffer with protease inhibitors and incubated for 10 minutes on ice. (200μl of SDS Lysis Buffer is per 1 X 10^6 cells, the resuspended cell pellet should be divided into 200μl aliquots so that each 200μl

aliquot contains ~1 X 10^6 cells). Cell lysate was sonicated to shear DNA into fragments 200 and 1000 base pairs length, while keeping the samples on ice (Branson Digital Sonifier, 30 % power, 10 seconds pulse 3X, 1 minute interval between each pulse). After sonication, samples were centrifuged for 10 minutes at 13,000 rpm and 4^0C, cell supernatant was diluted 10 times in ChIP Dilution Buffer, adding protease inhibitors as above. This is done by adding 1800µl ChIP Dilution Buffer to the 200µl sonicated cell supernatant. 1% (20µL) of diluted supernatant is stored at -20^0C, this is considered as INPUT, acts as a loading control. To reduce nonspecific background, 80µl of Salmon Sperm DNA/Protein A Agarose-50% Slurry was added to 2ml diluted cell supernatant and incubated for I hour at 4ºC with agitation. Brief centrifugation (1000 rpm, 1 min at 4^0C) was performed to pellet the agarose and the supernatant fraction was collected. Immunoprecipitations were performed by incubating the supernatant fractions with anti- H3K27Me3 (Active Motif, Carlsbad, CA, 1:200 dilution) or control IgG antibodies for overnight at 4ºC with rotation. 60µl of Salmon Sperm DNA/Protein A Agarose Slurry was added to the immunoprecipitated complex and incubated for one hour at 4^0C with rotation to collect the antibody/histone complex. Protein A agarose /antibody/histone complexes were collected by brief centrifugation, these complexes were washed for 3 min on a rotating platform with 1 ml each of the following buffers listed in the order as:

1. Low Salt Immune Complex Wash Buffer, one wash
2. High Salt Immune Complex Wash Buffer, one wash
3. LiCl Immune Complex Wash Buffer, one wash
4. 1X Tris-EDTA Buffer, two washes

After each wash, complexes were centrifuged at 1000 rpm for 1 min at 4ºC. Histone complexes were eluted from the antibody by incubating with 250µL freshly prepared elution

buffer (1%SDS, 0.1M $NaHCO_3$) for 15 min at room temperature (RT). Supernatant fraction was collected after centrifugation at 13000 rpm for 5 min at RT. The elution process was repeated and eluates were combined (500µL). INPUTS as well as eluates were reverse crosslinked by treating with 20µL 5M NaCl at 65^0C for overnight. Finally, Polymerase Chain Reaction (PCR) was performed with primers designed from the sequence of the miR-31 promoter (5'-GCTATCTCAACCCACTCTCCGCCT-3' and 5'-GATTAGATGCTGATGTGAGTGCTG-3'), covering a ~200 bp fragment that is upstream of the transcription start site of the miR-31 gene.

1.3.15. Crystal Violet Staining

The cells were treated with various doses of mocetinostat or the same doses of vorinostat for 72h. The colonies were fixed with paraformadehayde and stained with 0.05% Crystal Violet.

1.3.16. Human Prostate Cancer Specimens

Frozen specimens of human prostate cancer tissue and paired normal prostate tissues were obtained from 8 patients at Mayo Clinic SPORE, and approved by the Mayo Clinic Institutional Review Board. These patients had biopsy-proven prostate cancer and were treated at the Mayo Clinic by radical retropubic prostatectomy between January 1995 and December 1998 without neoadjuvant therapy [43]. Samples were frozen in liquid nitrogen and stored at -80 ^{o} C until use. Total RNA was isolated from frozen samples, and quantified by real-time PCR.

1.3.17. Statistical Analysis

Differences between the mean values were analyzed for significance using the unpaired two-tailed Student's test for independent samples; $P \leq 0.05$ was considered to be statistically significant.

CHAPTER 2. POLYCOMB PROTEIN EZH2 SUPPRESSES APOPTOSIS BY COORDINATING THE SILENCING OF PRO-APOPTOTIC MIR-205 AND MIR-31

The working hypothesis for Project 1 was that EZH2 contributes to apoptosis resistance by integrating DNA methylation-mediated miR-205 silencing with histone methylation-mediated silencing of miR-31. The following experiments were performed to prove our hypothesis.

2.1. Results and Discussion

2.1.1. Determining the Effect of EZH2 on MiR-31 Expression

We reported previously that miR-31 expression was decreased in prostate cancer cells, resulting in resistance to apoptosis (Bhatnagar N, Li X, Padi SK, et al., 2010). It was recently shown that in adult T cell leukemia (Yamagishi M, Nakano K, et al., 2012), the polycomb repressive complex can be recruited to the miR-31 promoter (on chromosome 9q21) by transcription factor YY1. Subsequently, EZH2 increases trimethylated H3K27 and suppresses miR-31 expression (Yamagishi M, Nakano K, et al., 2012). To understand the mechanism of miR-31 silencing in prostate cancer, we examined if miR-31 is suppressed by EZH2. We found that siRNA knockdown of EZH2 restored miR-31 expression in PC-3 cells (Figure 1A and 1B)

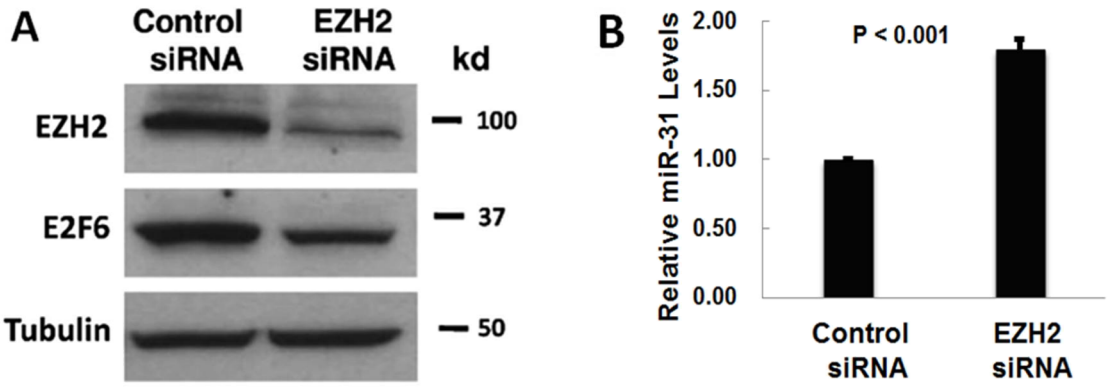

Figure 7. EZH2 suppresses miR-31 expression in PC-3 prostate cancer cell line. (A and B) PC-3 cells were transfected with the negative control or EZH2 targeting siRNAs for 48h. (A) Cell lysates were analyzed by western blot using the indicated antibodies. (B) Total RNA was isolated from the cells and real-time PCR analysis was performed as described in Materials and Methods. All experiments have been repeated three times, and data shown are mean values±S.D.

Similarly, depletion of EZH2 with DZNep (a known EZH2 inhibitor 27) increased miR-31 expression in PC-3 cells and decreased E2F6 (Figure 8A and 8B).

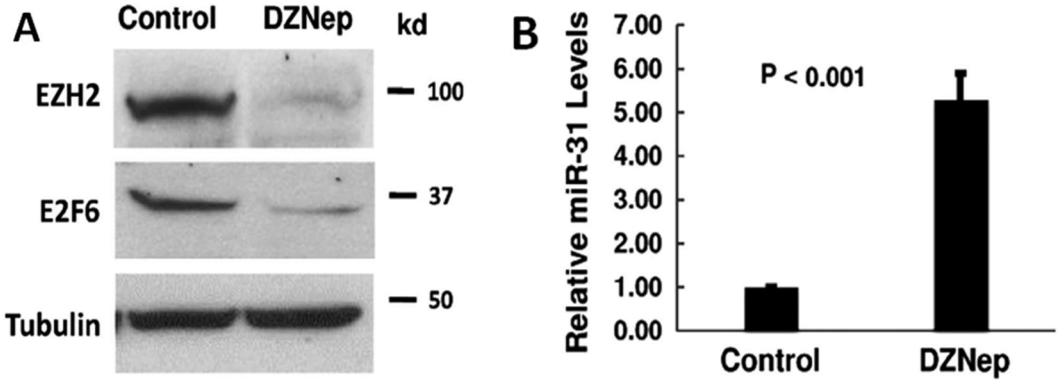

Figure 8. DZNep treatment suppresses miR-31 expression. (A) PC-3 cells were treated with 5 μM of DZNep for 24 h. Cell lysates were analyzed by western blot using the indicated antibodies. (B) Total RNA was isolated from the cells and real-time PCR analysis was performed. All experiments have been repeated three times, and data shown are mean values±S.D.

In another prostate cancer cell line DU-145, we also observed that siRNA knockdown of EZH2 or DZNeP treatment increased miR-31 expression and decreased E2F6 (Figure 9A and 9B).

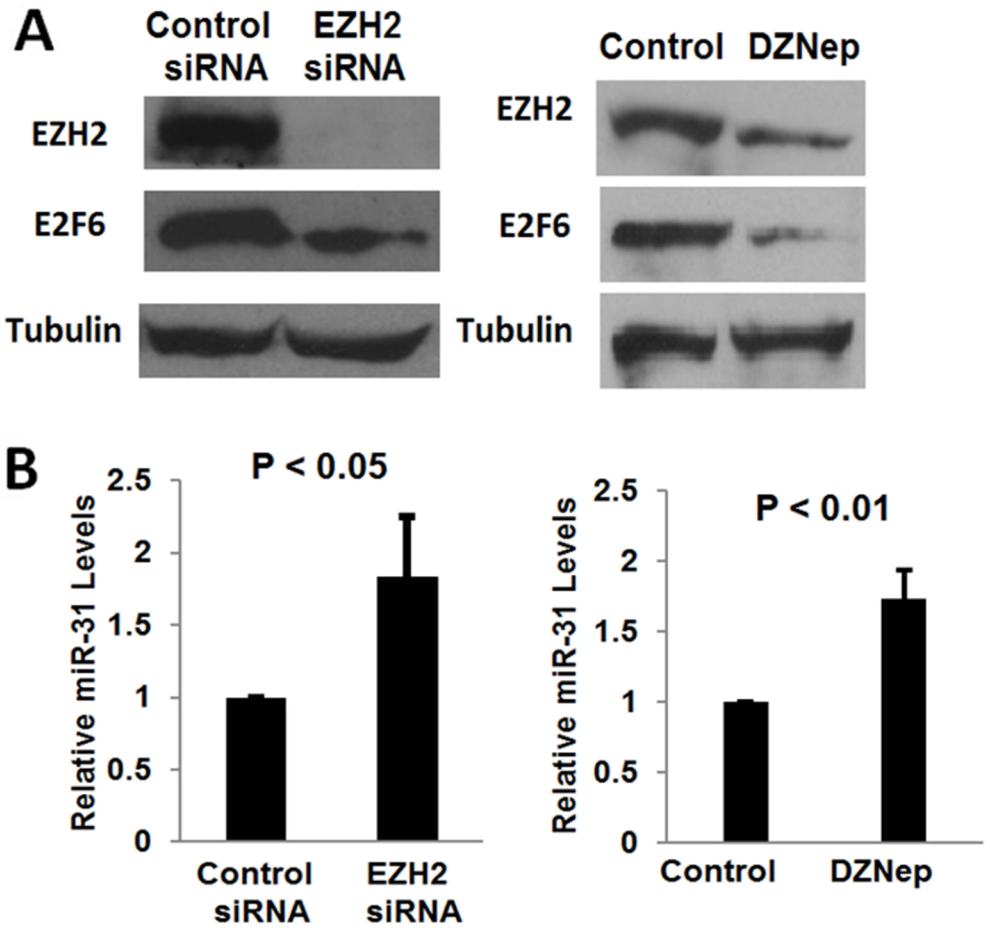

Figure 9. EZH2 suppresses miR-31 expression in DU-145 prostate cancer cell line. (A and B) Left, DU-145 cells were transfected with negative control or EZH2 targeting siRNAs for 48h. Right, DU-145 cells were treated with 5 μM of DZNep for 24 hours. (A) Cell lysates were analyzed by western blot using the indicated antibodies. (B) Total RNA was isolated from the cells and real-time PCR analysis was performed as described in Materials and Methods. All experiments have been repeated three times, and data shown are mean values±S.D.

2.1.2. EZH2 Regulates Histone Methylation on the MiR-31 Promoter

We performed 5' RACE experiments to identify the transcription start site and the promoter for the miR-31 gene. The gene encoding miR-31 is located on chromosome 9p21. The transcription start site for miR-31 was identified (Figure 10A). To determine if EZH2 regulates histone H3K27 methylation, we performed chromosome immunoprecipitation (ChIP) assay near the transcription start site (~360 bp upstream) on the miR-31 promoter. Chromatin immunoprecipitation assay was performed using the Chromatin Immunoprecipitation (ChiP)

Assay Kit (Upstate, Temecula, CA, USA) by following the manufacturer's protocol. Immunoprecipitations were performed using anti-dimethyl-histone H3 lysine 27 (H3K27Me3), and control IgG antibodies. Using ChIP assay followed by a PCR analysis, we measured the H3K27Me3 levels on the promoter region of miR-31 in EZH2 knockdown or EZH2 depleted PC-3 prostate cancer cells. As shown in Figure 10 (B and C), siRNA knockdown of EZH2 or DZNep treatment decreased the levels of H3K27 trimethylation on promoter of miR-31.

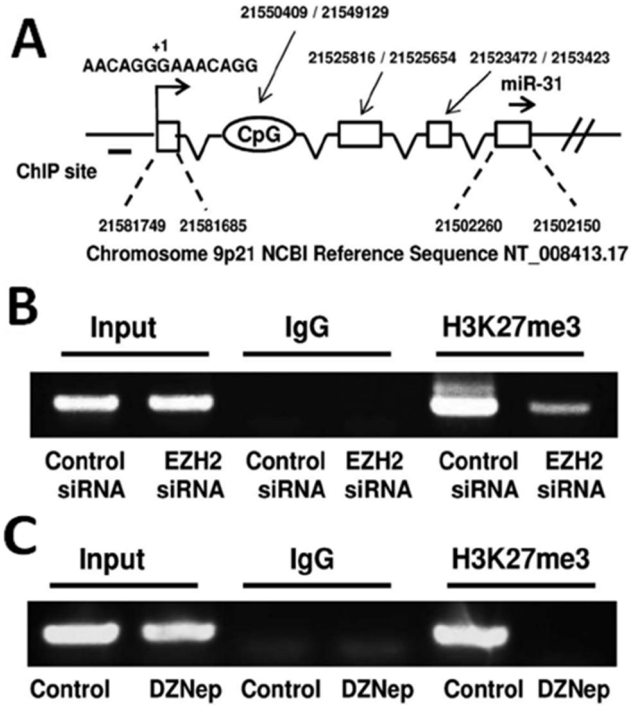

Figure 10. EZH2 regulates H3K27 methylation on the miR-31 promoter. (A) The putative transcription start site (indicated by +1) and genomic sequence of the miR-31 gene is shown. The mature miR-31 sequence is indicated by "→". The DNA fragment covered by ChIP assay is indicated by "–". The locations of the first four exons on the chromosomes were indicated. (B) PC-3 cells were transfected with the negative control or EZH2 targeting siRNAs for 48h. ChIP assay was performed as described in Materials and Methods, using primers specific for the miR-31 promoter and the indicated antibodies. (C) PC-3 cells were treated with 5 M of DZNep for 24 hours. ChIP assay was performed as in (B).

ChIP assay at 1250 bp upstream from the transcription start site did not detect H3K27 methylation, indicating that methylation occurs specifically at the transcription start site. As

shown in Figure 11, siRNA knockdown of EZH2 decreased the binding of EZH2 to the miR-31 promoter, while the levels of histone H3 on the promoter were not changed by EZH2 knockdown.

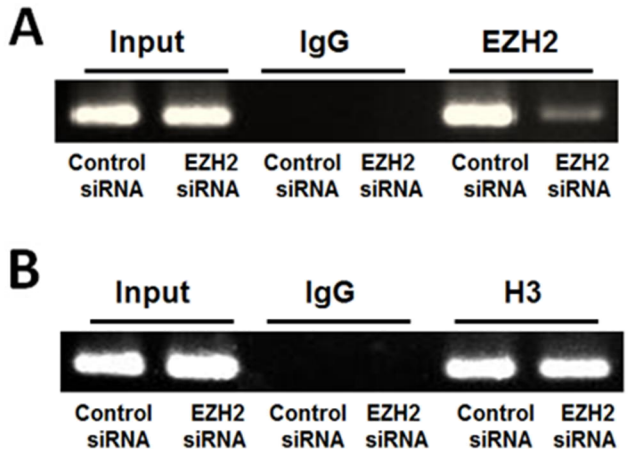

Figure 11. EZH2 regulates H3K27 methylation specifically at the transcription start site. PC-3 cells were transfected with the negative control or EZH2 targeting siRNAs for 48h. ChIP assay was performed as described in Materials and Methods, using primers specific for the miR-31 promoter and the indicated antibodies. The experiments have been repeated three times.

2.1.3. Downregulation of EZH2 Increases Docetaxel-induced Apoptosis in Prostate Cancer Cells

As miR-31 regulates apoptosis by targeting the anti-apoptotic protein E2F6 (Bhatnagar N, Li X, et al., 2010), we hypothesized that by silencing miR-31, EZH2 may regulate apoptosis in prostate cancer cells. We transfected PC-3 cells with the negative control or EZH2 targeting siRNAs and treated the cells with docetaxel, a drug used clinically to treat prostate cancer. EZH2 knockdown increased miR-31 expression with or without Docetaxel treatment in PC-3 and DU-145 prostate cancer cell lines (Figure 12).

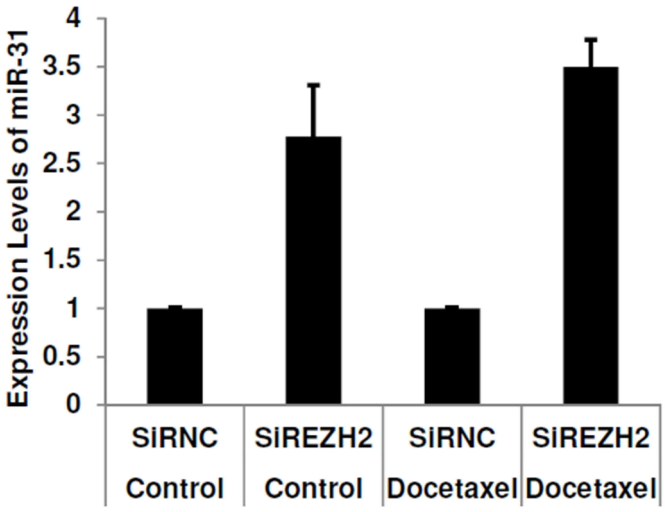

Figure 12. EZH2 regulates apoptosis in PC-3 prostate cancer cells. PC-3 cells were transfected with the negative control or EZH2 targeting siRNAs. At 24h after siRNA transfection, cells were treated with 10 nM docetaxel for additional 24h. Total RNA was isolated from the cells and real-time PCR analysis was performed as described in Materials and Methods. The experiments have been repeated three times, data shown are mean values + SD.

Docetaxel was able to induce higher level of apoptosis in cells that were transfected with EZH2 siRNA, compared to cells that were transfected with the negative control siRNA (Figure 13A,B). Higher levels of active (cleaved) caspase-3 and caspase-9, as well as PARP cleavage, were observed in cells after EZH2 siRNA transfection and docetaxel treatment (Figure 13C).

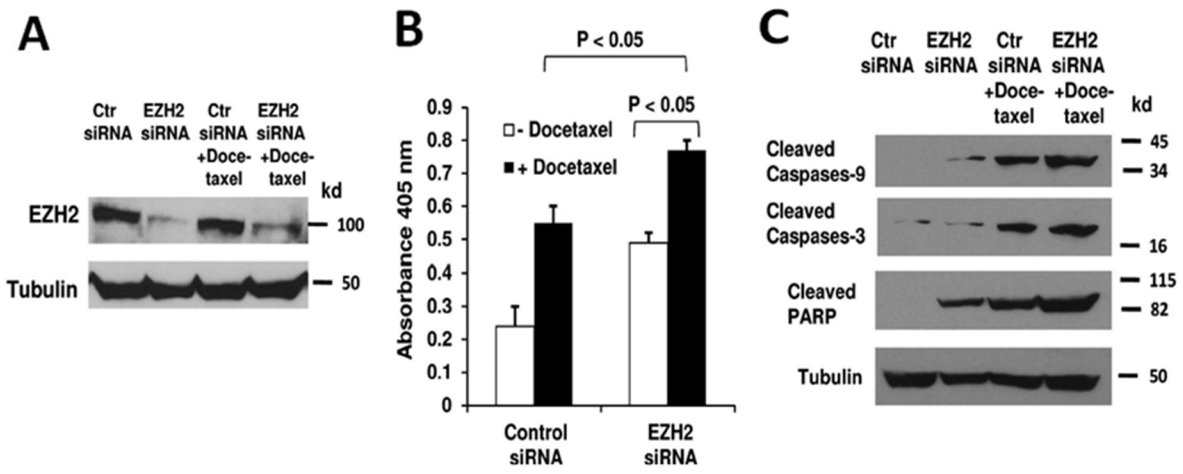

Figure 13. EZH2 knockdown increases docetaxel-induced apoptosis in PC-3 prostate cancer cells. PC-3 cells were transfected with the negative control or EZH2 targeting siRNAs. At 24 h after siRNA transfection, cells were treated with 10 nM docetaxel for additional 24 h. (A) Cell lysates were analyzed by western blot using the indicated antibodies. (B) Apoptosis was measured by Cell Death Detection ElisaPLUS analysis as described in Materials and Methods. (C) Western blotting was performed with the antibodies indicated. The experiments have been repeated three times, and data shown are mean values±S.D.

In DU-145 cells, we also observed that siRNA knockdown of EZH2 increased miR-31 expression (Figure 14A), docetaxel-induced apoptosis, as well as active caspase-3/caspase-9, and PARP cleavage (Figure 14B and 14C).

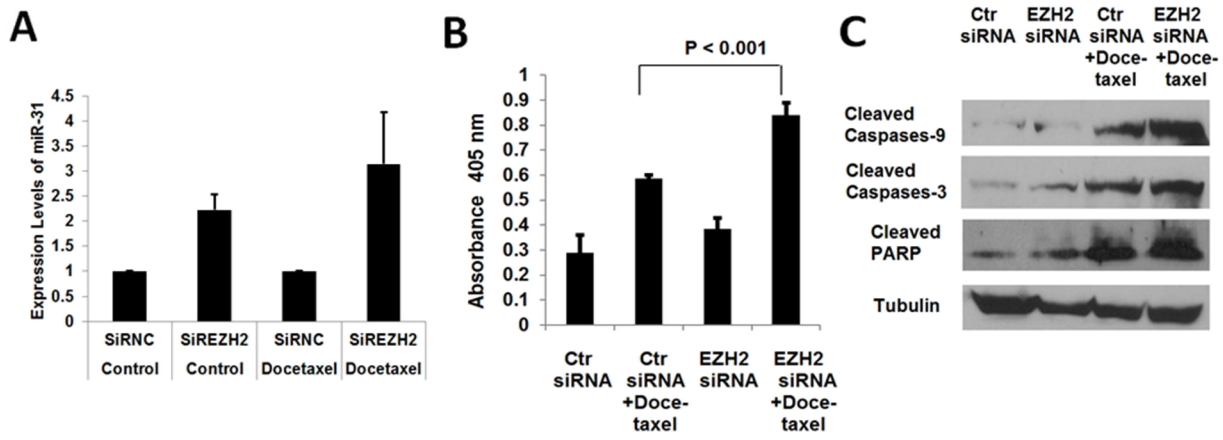

Figure 14. EZH2 knockdown increases docetaxel-induced apoptosis in DU-145 prostate cancer cells. (A) DU-145 cells were transfected with the negative control or EZH2 targeting siRNAs. At 24h after siRNA transfection, cells were treated with 10 nM docetaxel for additional 24h. Total RNA was isolated from the cells and real-time PCR analysis was performed as described in Materials and Methods. (B) Apoptosis was measured by Cell Death Detection ElisaPLUS analysis as described in Materials and Methods. (C) Western blotting was performed with the indicated antibodies. The experiments have been repeated three times, data shown are mean values + SD.

2.1.4. Overexpression of EZH2 Confers Resistance to Docetaxel-induced Apoptosis

We expressed EZH2 exogenously to determine if EZH2 can contribute to apoptosis resistance in prostate cancer cells. We transfected PC-3 cells with pCEP4-EZH2 expression vector to overexpress Flag-tagged EZH2 protein. Overexpression of EZH2 was confirmed by western blotting (Figure 15A). As expected, overexpression of EZH2 suppressed miR-31 (Figure 15B), and increased E2F6 protein (Figure 15A). When treated with docetaxel, cells expressing EZH2 were significantly more resistant to drug-induced apoptosis, comparing to empty vector-transfected cells (Figure 15C).

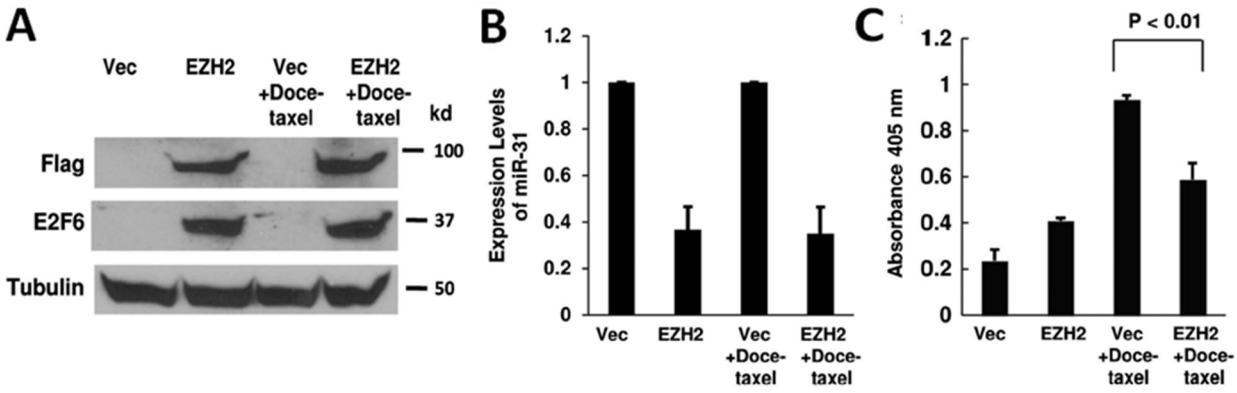

Figure 15. EZH2 confers resistance to docetaxel-induced apoptosis. PC-3 cells were transfected with the empty expression vector or pCEP4-EZH2. At 24h after transfection, cells were treated with 10 nM of docetaxel for additional 24h. (A) EZH2 and E2F6 expression was determined by western blot. (B) Total RNA was isolated from the cells and real-time PCR analysis was performed. (C) Apoptosis was measured by Cell Death Detection ElisaPLUS analysis. The experiments have been repeated three times, data shown are mean values + SD.

2.1.5. SiRNA Knockdown of E2F6 Sensitizes Prostate Cancer Cells to Docetaxel-induced Apoptosis

We have previously shown that miR-31 targets E2F6 (Bhatnagar N, Li X, et al., 2010). E2F6 is an anti-apoptotic protein that inhibits UV- and hypoxia-induced apoptosis (Yang WW, Wang ZH, et al., 2007; Yang WW, Shu B, et al., 2008). To determine the effects of E2F6 on chemotherapy-induced apoptosis in prostate cancer cells, we used siRNA to specifically knockdown E2F6 in PC-3 cells. As shown in Figure 16A, transfection of E2F6 targeting siRNA was able to decrease levels of E2F6 protein. SiRNA knockdown of E2F6 sensitized PC-3 cells to apoptosis induced by docetaxel (Figure 16B).

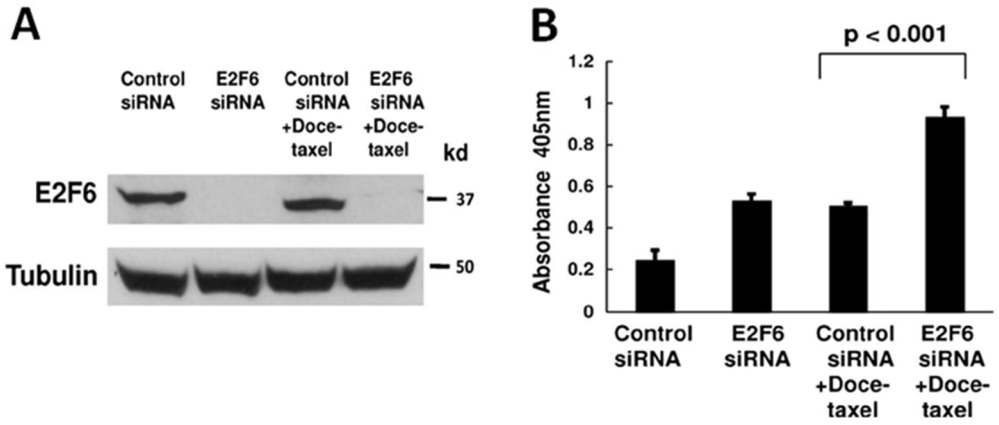

Figure 16. SiRNA knockdown of E2F6 sensitizes PC-3 cells to docetaxel-induced apoptosis. PC-3 cells were transfected with the negative control or E2F6 targeting siRNAs. At 24h after siRNA transfection, cells were treated with 10 nM docetaxel for additional 24h. (A) Cell lystates were analyzed by western blotting with the indicated antibodies. (B) Apoptosis was measured by Cell Death Detection ElisaPLUS analysis. The experiments have been repeated three times, data shown are mean values + SD.

In DU-145 cells, we also observed that siRNA knockdown of E2F6 increased docetaxel-induced apoptosis (Figure 17).

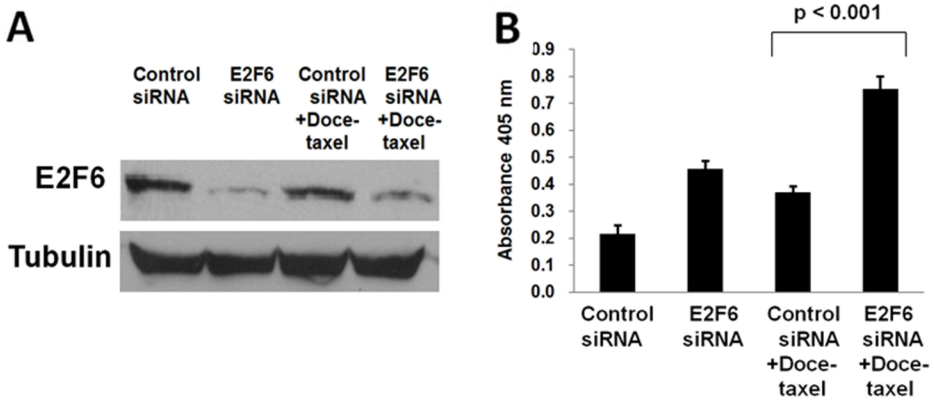

Figure 17. SiRNA knockdown of E2F6 sensitizes DU-145 cells to docetaxel-induced apoptosis. DU-145 cells were transfected with the negative control or E2F6 targeting siRNAs. At 24h after siRNA transfection, cells were treated with 10 nM docetaxel for additional 24h. (A) Cell lystates were analyzed by western blotting with the indicated antibodies. (B) Apoptosis was measured by Cell Death Detection ElisaPLUS analysis. The experiments have been repeated three times, data shown are mean values + SD.

In both PC-3 and DU-145 cells, miR-31 levels were not affected by E2F6 knockdown or docetaxel treatment (Figure 18).

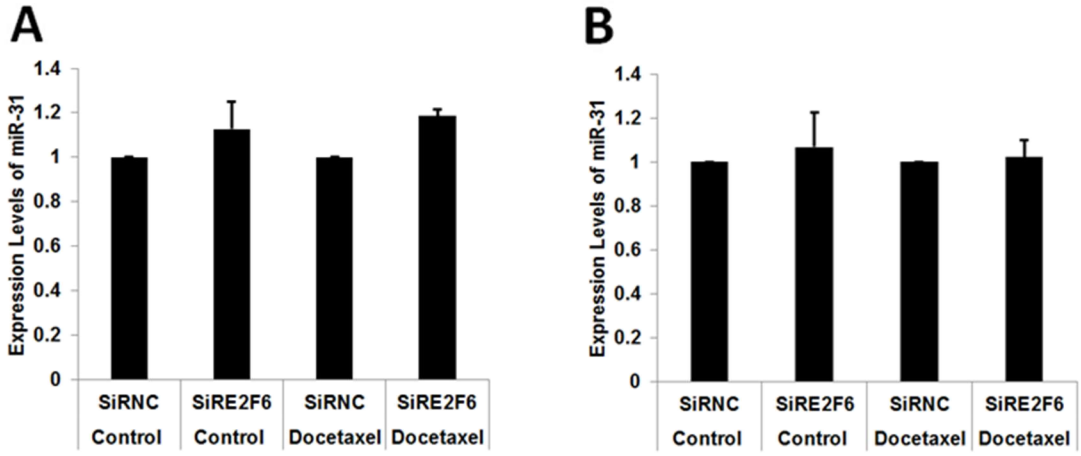

Figure 18. Real-Time PCR results confirming miR-31 expression levels after E2F6 knockdown and docetaxel treatment. PC-3 cells (A) and DU-145 cells (B) were transfected with the negative control or E2F6 targeting siRNAs. At 24h after siRNA transfection, cells were treated with 10 nM docetaxel for additional 24h. Total RNA was isolated from the cells and real-time PCR analysis was performed as described in Materials and Methods. The experiments have been repeated three times, data shown are mean values + SD.

2.1.6. MiR-205 Regulates MiR-31 through EZH2

We have reported that miR-205 is silenced in prostate cancer by promoter hypermethylation (Bhatnagar N, Li X, et al., 2010). Interestingly, miR-205 has been shown to decrease EZH2 protein in prostate cancer cells (Gandellini P, Folini M, et al., 2009). We hypothesized that the epigenetic silencing of miR-205 (through promoter methylation) will lead to increased expression of EZH2, which in turn epigenetically represses miR-31 expression through histone methylation. To test our hypothesis, we examined the effects of miR-205 on miR-31 expression. As shown in Figure 19A, blocking miR-205 with an anti-miR inhibitor in WPE1-NA22 cells (a cell line that expresses high levels of endogenous miR-31 (Bhatnagar N, Li X, et al., 2010)) decreased miR-31 expression, and increased EZH2 and E2F6 proteins. In contrast, overexpression of miR-205 in PC-3 cells caused a decrease of EZH2 and an increase of miR-31,

which in turn decreased E2F6 (Figure 19B). (The experiences were performed by Sathish Kumar Padi)

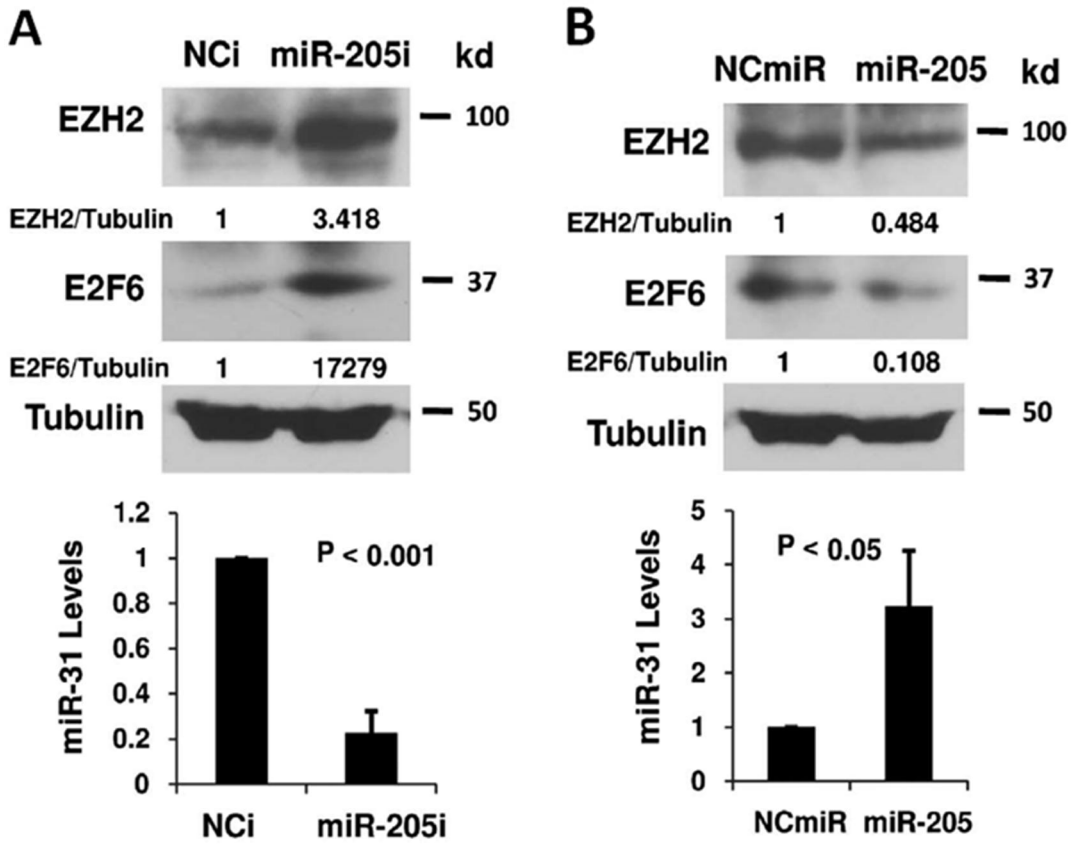

Figure 19. EZH2 integrates miR-205 silencing and miR-31 suppression. (A). WPE1-NA22 cells were transfected with negative control or anti-miR-205 inhibitor. After 48 h, total RNA and protein were collected and miR-31 expression was analyzed by real-time PCR and western blots were carried out using indicated antibodies. (B). PC-3 cells were transfected with the negative control miR or miR-205 for 48 h, and real-time PCR and western blots were performed as in (A). The experiments have been repeated three times, and data shown are mean values±S.D.

2.1.7. MiR-205, EZH2, and MiR-31 Expression in Human Prostate Cancer Specimens

We analyzed miR-205, EZH2, and miR-31 expression in eight pairs of human prostate cancer specimens and the adjacent non-malignant tissues using real-time PCR. We found that miR-205 and miR-31 expression levels were decreased in the cancer samples comparing to the normal tissues (Figure 20). In the meantime, EZH2 expression was increased in the cancer specimens. Interestingly, the expression levels of miR-205, EZH2, and miR-31 correlate well

among the individual patients (Pearson's correlation coefficient test: R= -0.7911 between the levels of EZH2 and miR-31; R= -0.6236 between EZH2 and miR-205). For example, low levels of miR-205 in patients PR2647 and PR1107 match the high levels of EZH2 in the same patients, which in turn lead to the very low levels of miR-31 in these patients. This observation supports our hypothesis that EZH2 may coordinate the silencing of miR-205 and miR-31 in prostate cancer.

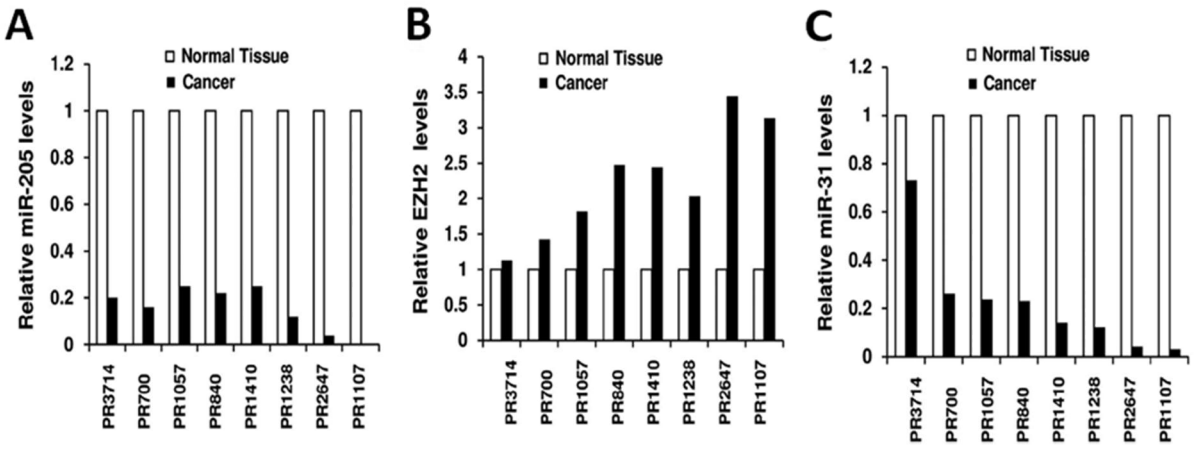

Figure 20. MiR-205, EZH2, and miR-31 expression in human prostate cancer specimens. Total RNAs were isolated from eight pairs of prostate cancer specimens and the adjacent normal prostate tissues. (Each pair of samples were from the same patient indicated by the patient ID on the X axis.) MiRNA and EZH2 expression were determined by real-time PCR. The ratios of miR-205 (A), EZH2 (B), and miR-31 (C) levels in the tumor samples versus that in the matched adjacent non-tumor samples were shown (the expression levels in each normal tissue were designated as 1)

2.2. Discussion

Overexpression of EZH2 is found in various types of solid tumors such as melanoma (Asangani IA, Harms PW, et al., 2012), breast cancer (Kleer CG, Cao Q, et al., 2003), cervical cancer (Holland D, Hoppe-Seyler K, et al., 2008), gastric cancer (Matsukawa Y, Semba S, et al., 2006), and prostate cancer (Varambally S, Dhanasekaran SM, et al., 2002). High levels of EZH2 are linked to tumor growth, metastasis, and poor prognosis for cancer patients. EZH2 suppresses apoptosis in a variety of cancers, including gastric cancer (Xie L, Zhang Z, et al., 2014), bladder cancer (Wang Y, Xiang W, et al., 2014), leukemia (Zhou J, Bi C, et al., 2011), and prostate

cancer (Li K, Liu C, et al., 2013). However, the mechanisms of apoptosis suppression by EZH2 remain poorly understood. It has been reported that miR-205 suppresses EZH2 expression (Gandellini P, Folini M, et al., 2009) in prostate cancer cells and miR-205 promotes apoptosis (Bhatnagar N, Li X, et al., 2010; Verdoodt B, Neid M, et al., 2013). In this study, we identified a novel mechanism of EZH2 regulation of apoptosis in prostate cancer. We propose that EZH2 coordinates the silencing of the pro-apoptotic miR-205 and miR-31. Thus, DNA methylation-mediated silencing of miR-205 (Bhatnagar N, Li X, et al., 2010) can lead to histone methylation-mediated silencing of miR-31, with EZH2 as the coordinator of the two separate events. As a result, the expression of anti-apoptotic protein E2F6 is increased and contributes to the development of apoptosis resistance. It has been reported that the genomic loss of miR-101 can result in the up-regulation of EZH2 in prostate cancer (Varambally S, Cao Q, et al., 2008) and miR-101 promote apoptosis in cancer cells (Wang Y, Xiang W, et al., 2014; Xu Y, An Y, et al., 2013). EZH2 may also contribute to apoptosis resistance by linking the genomic loss of miR-101 to miR-31 silencing.

At present, we do not know the mechanism of how miR-205 inhibits EZH2. There is no target sequence of miR-205 within the 3' UTR of EZH2 gene (www.targetscan.org). Thus, miR-205 may suppress EZH2 indirectly through its action on molecules that control EZH2 expression. For example, the oncogene Myc can regulate EZH2 at both transcriptional and post-transcriptional levels (Koh CM, Iwata T, et al., 2011). Furthermore, overexpression of ERG (as a result of fusion between the TMPRESS2 and ERG from chromosome 21 translocation) can directly activate EZH2 (Yu J, Mani RS, et al., 2010). Epigenetic silencing of miR-205 may result in the increase of these EZH2 activators, which in turn increases EZH2 expression.

While a previous study has reported promoter methylation of the miR-31 gene (Lin PC, Chiu YL, et al., 2013), the methylation analysis was done in the region of the CpG island that is located in the intron region between exons 1 and 2 (Figure 2A). The CpG island is about 32 kb away from the transcription start site that we identified with 5'RACE. Our ChIP assay was performed in the revised promoter region that has no CpG island (Figure 2), indicating that EZH2 may suppress miR-31 expression independent of DNA methylation. Previously, it has been shown that histone H3K27 trimethylation can silence the expression of miR-22 independent of promoter methylation (Lin PC, Chiu YL, et al., 2009).

CHAPTER 3. HDAC INHIBITOR MOCETINOSTAT INDUCES APOPTOSIS BY INCREASING MIR-31 EXPRESSION AND SUPPRESSION OF E2F6

The working hypothesis for Project 2 was that mocetinostat might be able to activate miR-31 and downregulation of E2F6, and consequently induce apoptosis in prostate cancer. The following experiments were performed to prove our hypothesis.

3.1. Results and Discussion

3.1.1. Mocetinostat Induces Apoptosis in Prostate Cancer Cells

We determined if mocetinostat can induce apoptosis in prostate cancer cells. As shown in Figure 21A, mocetinostat induced significant levels of apoptosis in DU-145 cells in a dose-dependent manner. Induction of apoptosis was determined by the cell death ELISA assay measuring mono- and oligonucleosomes in the lysates of apoptotic cells. Similarly, mocetinostat also induced significant levels of apoptosis in PC-3 cells (Figure 21B). We compared the antitumor activities of mocetinostat and vorinostat (suberanilohydroxamic acid, SAHA) (P.A. Marks 2007), a FDA-approved non-selective HDAC inhibitor. Mocetinostat was significantly more potent than vorinostat in prostate cancer suppression (Figure 21C).

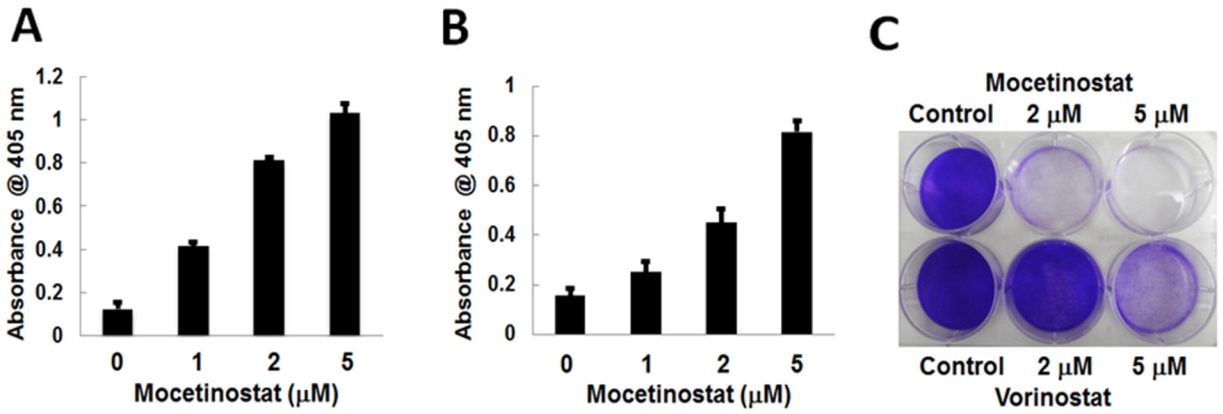

Figure 21. Mocetinostat induces apoptosis in prostate cancer cells. DU-145 cells. (A) and PC-3 cells (B) were treated with various doses of mocetinostat for 24 hours and apoptosis was analyzed using the Cell Death Detection ElisaPLUS kit as described in Materials and Methods. (C) DU-145 cells were treated with various doses of mocetinostat or vorinostat for 72 hours. The colonies were fixed with paraformaldehyde and stained with 0.05% Crystal Violet. All experiments have been repeated three times, data shown are mean values + SD.2.9.

3.1.2. Mocetinostat Induces MiR-31 Expression and Downregulates E2F6

We recently demonstrated that the downregulation of miR-31 contributes to apoptosis resistance in prostate cancer cells (N. Bhatnagar, X. Li, et al., 2010). Since miR-31 is repressed by epigenetic mechanisms in prostate cancer (P.C. Lin, Y.L. Chiu, et al., 2013), we hypothesized that mocetinostat may activate miR-31 expression. The effects of mocetinostat on miR-31 expression were determined by real-time PCR. As shown in Figure 22A, mocetinostat significantly induced miR-31 expression. We have previously shown that miR-31 targets E2F6 (N. Bhatnagar, X. Li, et al., 2010), which is a potent anti-apoptotic protein that can inhibit UV- and hypoxia-induced apoptosis (W.W. Yang, Z.H. Wang, et al., 2007; W.W. Yang, B. Shu, et al., 2008). As a result of miR-31 induction, mocetinostat significantly decreased E2F6 protein in DU-145 cells (Figure 22B).

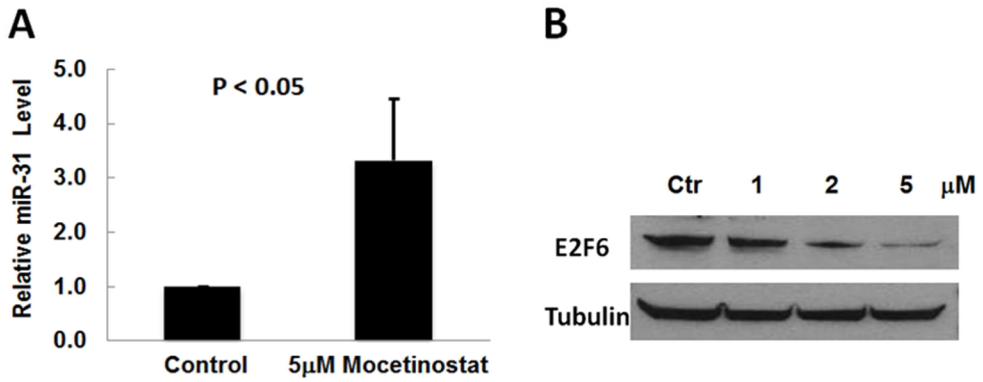

Figure 22. Mocetinostat activates miR-31 expression in prostate cancer cells. (A) DU-145 cells were treated with 5 μM mocetinostat for 24h. Total RNA was isolated from untreated (control) and mocetinostat-treated cells and real-time PCR analysis was performed as described in Materials and Methods. (B) DU-145 cells were treated with various doses of mocetinostat for 24h. Western blotting was performed with anti-E2F6 and anti-tubulin antibodies. The experiments have been repeated three times, data shown are mean values + SD.

To further understand the mechanism of mocetinostat-induced apoptosis, we determined the effects of mocetinostat on the expression of pro-apoptotic members of Bcl-2 family proteins (N.N. Danial, S.J. Korsmeyer, et al,. 2007). Interestingly, Bad was significantly increased by mocetinostat treatment while the expression levels of other proteins were either reduced (Puma, Bid, and Bax) or unchanged (Bak) (Figure 23A). Since Bad is a key pro-apoptotic protein that triggers the intrinsic pathway of apoptosis (P. Jiang, W. Du,et al., 2007; N.N. Danial 2008), we examined the effects of mocetinostat on the caspases. As shown in Figure 23B, mocetinostat treatment significantly increased the levels of activated (cleaved) caspase-9 and caspase-3. The cleaved products of PARP, substrates of the caspases, were also increased by mocetinostat.

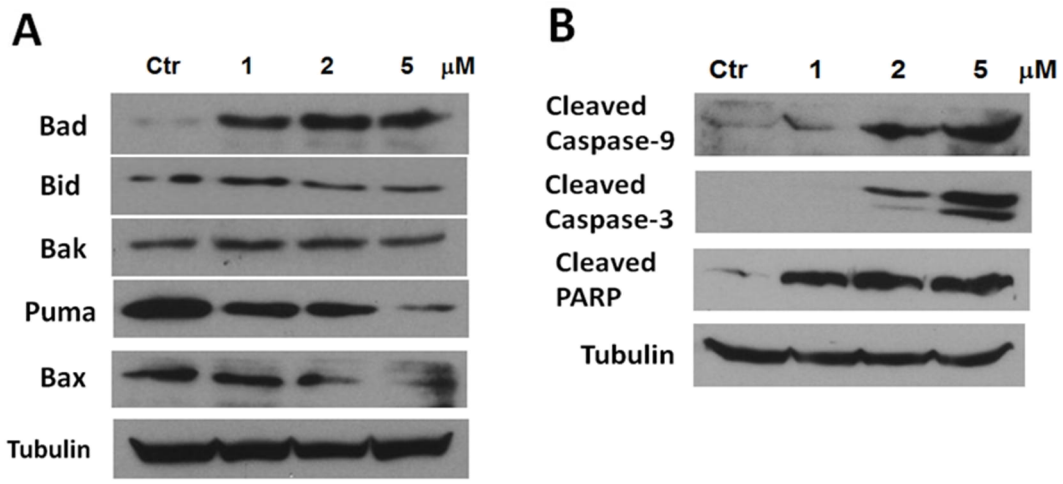

Figure 23. The expression levels of pro-apoptotic members of the Bcl-2 family proteins and caspases proteins after treatment of mocetinostat. (A and B) DU-145 cells were treated with various doses of mocetinostat for 24h. Western blotting was performed with the indicated antibodies.

3.1.3. E2F6 Regulates Mocetinostat-induced Apoptosis in Prostate Cancer Cells

To determine the role of E2F6 in mocetinostat-induced apoptosis, we used siRNA to knockdown E2F6 in DU-145 cells. As shown in Figure 24A, E2F6 protein expression was significantly reduced by siRNA treatment. Knockdown of E2F6 sensitized DU-145 cells to apoptosis induced by mocetinostat (Figure 24B). We also expressed E2F6 exogenously to determine if E2F6 can contribute to apoptosis resistance in prostate cancer cells. Overexpression of E2F6 was confirmed by western blotting (Figure 24C). When treated with mocetinostat, DU-145 cells overexpressing E2F6 were significantly more resistant to drug-induced apoptosis, compared to empty vector-transfected cells (Figure 24D).

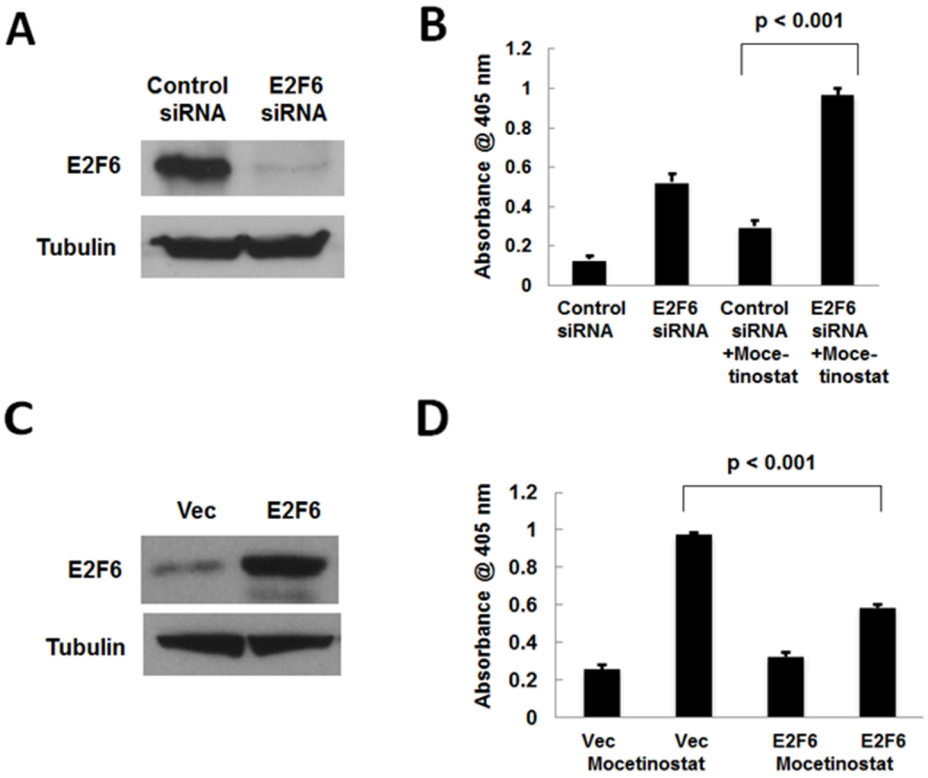

Figure 24. E2F6 regulates mocetinostat-induced apoptosis. (A) DU-145 cells were transfected with the negative control or E2F6 targeting siRNAs. At 48h after siRNA transfection, cell lystates were analyzed by western blotting with the indicated antibodies. (B) DU-145 cells were transfected with the negative control or E2F6 siRNAs. At 24h after siRNA transfection, cells were treated with 1 μM mocetinostat for additional 24h. Apoptosis was measured by Cell Death Detection ElisaPLUS analysis as described in Materials and Methods. (C) DU-145 cells were transfected with the empty expression vector or pcDNA3-HA-E2F6. At 24h after transfection, E2F6 overexpression was confirmed by western blot. (D) DU-145 cells were transfected with the empty expression vector or pcDNA3-HA-E2F6. At 24h after transfection, cells were treated with 5 μM of mocetinostat for additional 24h and apoptosis was measured by Cell Death Detection ElisaPLUS analysis as described in Materials and Methods. The experiments have been repeated three times, data shown are mean values + SD.

3.1.4. Mocetinostat Induces MiR-31 Expression and Activates Apoptosis in Primary Prostate Cancer Stem Cells

It is postulated that cancer stem cells mediate tumor formation, metastasis, and resistance to chemotherapy (B. Sharpe, M. Beresford, et al,. 2013). We tested if mocetinostat is effective against prostate cancer stem cells. As shown in Figure 25A, mocetinostat induced significant levels of apoptosis in patient-derived primary prostate cancer stem cells. Furthermore,

mocetinostat increased miR-31 expression (Figure 25B), and decreased E2F6 protein (Figure 25C) in prostate cancer stem cells. The level of pro-apoptotic protein Bad was increased by mocetinostat (Figure 25C).

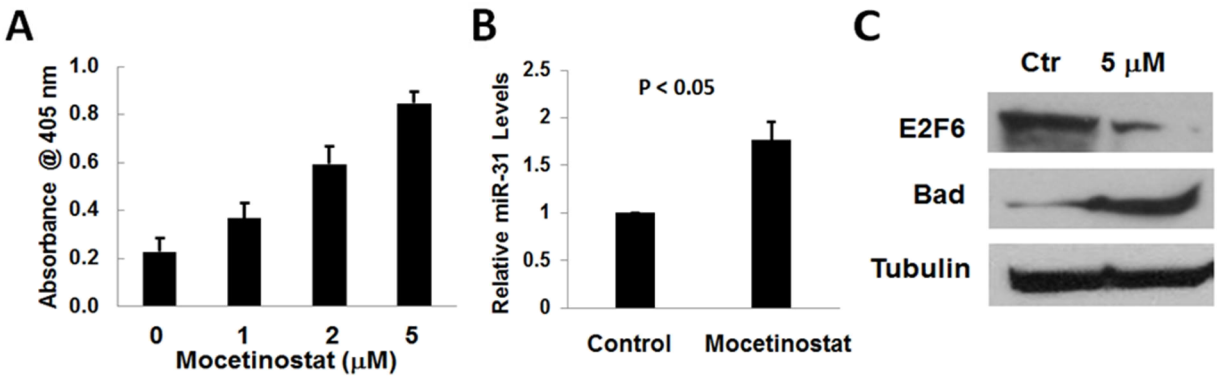

Figure 25. Mocetinostat induces apoptosis in the primary prostate cancer stem cells. (A) Prostate cancer stem cells were treated with various doses of mocetinostat for 24 hours and apoptosis was analyzed using the Cell Death Detection ElisaPLUS kit. (B) Prostate cancer stem cells were treated with 5 μM mocetinostat for 24h. Total RNA was isolated and real-time PCR analysis was performed as described in Materials and Methods. (C) Prostate cancer stem cells were treated with 5 uM of mocetinostat for 24h. Western blotting was performed with the indicated antibodies. The experiments have been repeated three times, data shown are mean values + SD.

3.2. Discussion

Mocetinostat is a class I selective HDAC inhibitor and has promising antitumor activities in preclinical studies (M. Fournel, C. Bonfils, et al., 2008; C. Bonfils, A. Kalita, et al., 2008). More importantly, recent clinical trials have demonstrated excellent response of mocetinostat against myelodysplastic syndrome and relapsed Hodgkin's lymphoma, with acceptable safety profiles (S.M. Luger, C.L. O'Connell, et al., 2013; A. Younes, Y. Oki, et al., 2011). However, a phase II study also found that mocetinostat has limited efficacy as a single agent in relapsed and refractory chronic lymphocytic leukemia (CLL) (K.A. Blum, A. Advani, et al., 2009). Thus, it is important to understand the mechanism of mocetinostat-induced apoptosis in order to improve its efficacy in cancer therapy and overcome drug resistance. Our findings in this report serve this purpose by helping to understand the molecular basis of mocetinostat-induced apoptosis. We

have previously shown that in prostate cancer cell lines derived from advanced metastatic cancers, miR-31 is downregulated to contribute to the resistance to chemotherapy-induced apoptosis (N. Bhatnagar, X. Li, et al., 2010). Here, we have demonstrated that mocetinostat activates the expression of miR-31 which in turn decreases expression of the anti-apoptotic protein E2F6. We also found that mocetinostat increases the expression of pro-apoptotic protein Bad. Our data have established a mechanistic model to explain how mocetinostat induces apoptosis in prostate cancer (Figure 4D). By inducing miR-31 to decrease E2F6 while in the meantime increasing Bad, mocetinostat tipped the balance between the anti-apoptotic and pro-apoptotic proteins (S.N. Willis, J.M. Adams, et al., 2005), resulting in increased apoptosis.

Evidence is emerging that the prostate cancer stem cells may play important roles in resistance to castration (M. Germann, A. Wetterwald, et al., 2012) and chemotherapy (B. Sharpe, M. Beresford, et al., 2013). Recently, it has been shown that mocetinostat induces cell cycle arrest and apoptosis in colon cancer initiating cells (S. Sikandar, D. Dizon, X. Shen, et al., 2010). Our data suggest that mocetinostat can efficiently induce apoptosis in the primary prostate cancer stem cells, through a mechanism involving miR-31 and E2F6 (Figure 4). Thus, mocetinostat is an effective drug for eliminating the prostate cancer stem cells.

Azacitidine can activate the expression of epigenetically silenced genes by inhibiting DNA promoter methylation (K. O'Dwyer, P. Maslak, et al., 2008). Moreover, recent clinical trials have demonstrated excellent results when azacitidine and mocetinostat are used together in cancer therapy (S.M. Luger, C.L. O'Connell, et al., 2013). It will be interesting to test if azacitidine can act with mocetinostat synergistically to activate miR-31 and induce apoptosis in prostate cancer cells, since the two drugs target different mechanisms of miR-31 silencing.

CHAPTER 4. SUMMARY AND CONCLUSIONS; CLINICAL IMPLICATIONS AND FUTURE DIRECTIONS

4.1. Summary and Conclusions

4.1.1. Polycomb Protein EZH2 Suppresses Apoptosis by Silencing the Proapoptotic MiR-31

Dysregulation of various miRNAs has been shown to have critical roles in cancer biology, ranging from proliferation, differentiation, metabolism, metastasis, to apoptosis (N. Bhatnagar, X. Li, et al., 2010). Our previous studies have shown that miR-205 and miR-31 are the two of the most significantly downregulated microRNAs in prostate cancer. And miR-205 undergoes downregulation by DNA methylation-mediated silencing on the promoter region. However the mechanism for downregulation of miR-31 in prostate cancer is still elusive. In the light of the recent demonstration that the polycomb repressor complex 2 (PRC2) can be recruited to the miR-31 promoter region by transcription factor YY1 in adult T-cell leukemia. Moreover, the polycomb protein EZH2 of the PCR2 increases trimethylated H2K27 to suppress miR-31 expression (Makoto Yamagishi, Kazumi Nakano 2012). EZH2 has also been found to be extensively overexpressed in metastatic prostate cancer (Varambally S, Dhanasekaran SM, 2002). Therefore, we proposed the hypothesis that miR-31 is epigenetically suppressed by polycomb protein EZH2 in prostate cancer. EZH2 suppresses apoptosis in a variety of cancers, including gastric cancer, bladder cancer, leukemia, and prostate cancer. However, the mechanisms of apoptosis suppression by EZH2 are still poorly understood. Our previous studies have identified an anti-apoptotic protein E2F6 as the target of miR-31; and downregulation of miR-31 can confer resistance to chemotherapy induced apoptosis. Based on these findings, we hypothesized that

EZH2 can suppresses apoptosis though decreasing miR-31 expression, and consequently cause upregulation of anti-apoptotic protein E2F6 expression. Interestingly, miR-205 has been shown to decrease EZH2 protein in prostate cancer cells. We hypothesized that the epigenetic silencing of miR-205 (through promoter methylation) will lead to increased expression of EZH2 which in turn epigenetically represses miR-31 expression through histone methylation.

Our results showed that EZH2 suppresses miR-31 expression by trimethylation of Histone 3 lysine 27 on the promoter region. SiRNA knockdown of EZH2 increased miR-31 expression, and consequently decreased E2F6 expression in PC-3 cells, resulting in the sensitization of prostate cancer cells to docetaxel-induced apoptosis. Conversely, overexpression of EZH2 blocked docetaxel-induced apoptosis. We further demonstrated that miR-205 silencing is linked to miR-31 silencing through EZH2. Suppression of miR-205 with a miRNA inhibitor caused an increase of EZH2 protein, which in turn inhibited miR-31 expression. In the other hand, overexpression of miR-205 decreased EZH2 protein and increased miR-31expression. By comparing eight pairs of human prostate cancer specimens with the adjacent non-malignant tissues, we found that miR-205 and miR-31 expression levels were decreased in the cancer samples. EZH2 expression was also increased in the cancer specimens, and had a negative correlation with miR-205 and miR-31 expression.

Finally, we have established novel epigenetic mechanisms to illustrate EZH2 coordination of miR-205 and miR-31 silencing in the development of apoptosis resistance (Figure 26). This study has also identified EZH2 and miR-31 as novel targets for the effective treatment of prostate cancer.

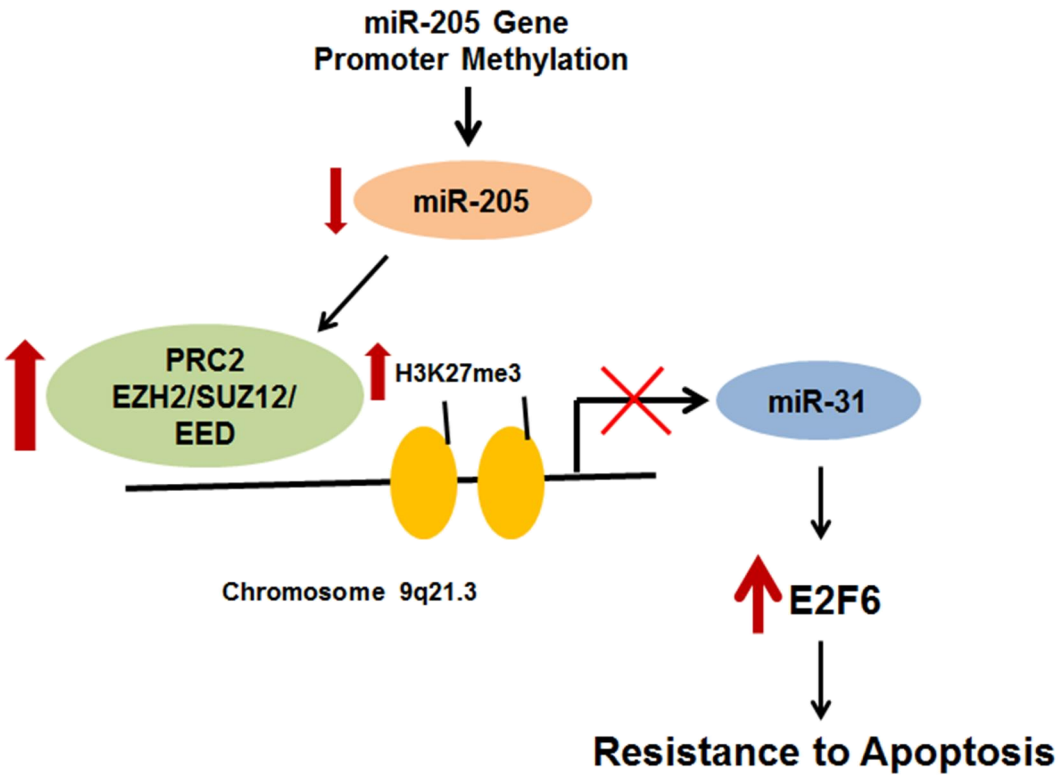

Figure 26. Schematic illustration of EZH2 coordination of miR-205 and miR-31 silencing in the development of apoptosis resistance. The promoter methylation-mediated silencing of miR-205 leads to the increase of EZH2 expression, which in turn suppresses miR-31 through H3K27 methylation on the miR-31 promoter. The reduction of miR-31 expression results in the increase of anti-apoptotic protein E2F6, which confers resistance to apoptosis.

4.1.2. HDAC Inhibitor Mocetinostat Induces Apoptosis by Increasing MiR-31 Expression and Suppression of E2F6

The mechanisms involved in epigenetic therapy typically cluster into three categories including DNA methylations, histone modifications, and regulatory RNAs (Razin Aharon 1998; Strahl, Brian D. 2000; Prasanth Kannanganattu V., David L. 2007). Mocetinostat, also known as MGCD103, is one of the class I selective histone deacetylases (HDACs) inhibitor that is developed for epigenetic therapy. Although mocetinostat has been shown to effectively kill prostate cancer cells, the mechanisms of apoptosis induction remain elusive. Recently, we have reported that miR-31 was significantly downregulated in prostate cancer cells and its downregulation resulted in

overexpression of anti-apoptotic protein E2F6 and resistance to apoptosis. Studies by Lin *et al* have shown that miR-31 is epigenetically silenced in prostate cancer by promoter hypermethylation and histone H3K27 trimethylation (Lin, P.C., e al. 2013). Since HDACs are often involved in DNA methylation-mediated gene silencing (McCabe M.T., J.C. Brandes 2009), we tested whether mocetinostat can activate miR-31 expression. Intriguingly, we found that mocetinostat significantly induced miR-31 expression in prostate cancer cells and decreased the expression of its target, the anti-apoptotic protein E2F6. The expressions of pro-apoptotic protein Bad, and activation of caspase-3 and caspase-9 is upregulated by mocetinostat as well. While siRNA knockdown of E2F6 sensitized cancer cells to mocetinostat-induced apoptosis, overexpression of E2F6 blocked mocetinostat-induced apoptosis. Importantly, we found that mocetinostat also increased miR-31 expression, decreased E2F6, upregulated Bad protein, and induced apoptosis in the primary prostate cancer stem cells.

Based on the results presented above, we have established a model to illustrate the role of miR-31in mocetinostat-induced apoptosis. Activation of miR-31 and downregulation of E2F6 contribute to mocetinostat-induced apoptosis in prostate cancer. These findings identified a novel mechanism that contributes to mocetinostat-induced apoptosis in prostate cancer.

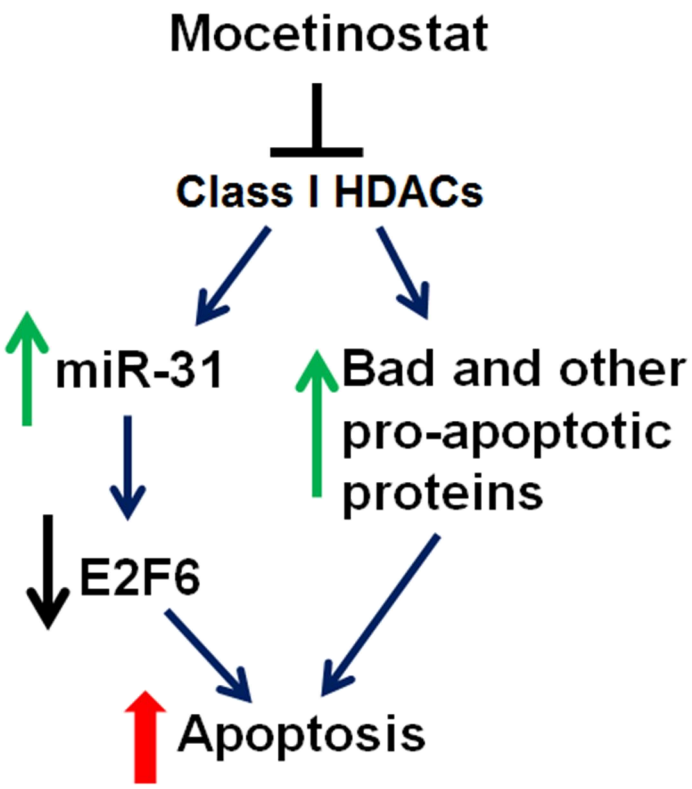

Figure 27. Schematic illustration of the role of miR-31 in mocetinostat-induced apoptosis. By inhibiting the class I HDACs, mocetinostat activates miR-31 expression and subsequently decreases E2F6. In the meantime, mocetinostat also increases the pro-apoptotic protein Bad. As a result, apoptosis is triggered in the prostate cancer cells.

4.2. Clinical Implications and Future Directions

Various small molecule drugs have been developed to target EZH2. For example, GSK126 is a very potent EZH2 inhibitor (with a Ki of 0.5–3 nM) and has high selectivity for EZH2 (more than 1000-fold higher activity than its activity for 20 other human methyltranserases). Importantly, GSK126 inhibits the growth of EZH2 mutant diffuse large B-cell lymphoma xenografts in mice (McCab MT, Ott HM 2012). Our findings indicate that EZH2 functions as a key coordinator in apoptosis suppression. Targeting EZH2 with drugs like GSK126 may not only induce apoptosis by itself but also sensitize cancer cells to other agents of chemotherapy.

In current clinical trials of mocetinostat and other HDAC inhibitors, HDAC activity inhibition and histone acetylation are used as biomarkers to measure pharmacodynamics responses. Our findings in this part of study have identified new parameters (miR-31, E2F6, Bad) that may be used to monitor tumor response to mocetinostat. These apoptosis regulators may be useful to predict how efficiently mocetinostat can kill the prostate cancer cells.

The future directions of this project include: (a) Synthesis of novel small molecule EZH2 inhibitors, (b) Systemic delivery of stable and synthetic miR-205 and miR-31.

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