Role of Epigenetics in Testicular Cancer Cell Drug Response

By

Clifford Yerby

Thesis
For the
Degree of Bachelor of Science
In
Biochemistry

College of Liberal Arts and Sciences
University of Illinois at Urbana

2021

Abstract

Testicular cancer is highly curable with the chemotherapeutic cisplatin. However, 15-20% of patients are resistant and succumb to their disease. Previously we showed that cisplatin refractory testicular cancer is highly sensitive to the DNA methyltransferase inhibitor, 5-aza deoxycytidine (5-aza). The mechanisms for cisplatin sensitivity and resistance in testicular cancer is unclear. If we can understand why testicular cancer is so curable, this knowledge could be applied to other cancer types. To better understand the mechanism of chemotherapy sensitivity and resistance in testicular cancer cells our lab generated two series of cell models, one resistant to cisplatin and the other resistant to 5-aza. We noted a reciprocal relationship between cisplatin and 5-aza resistance, with cisplatin resistance associated with increased sensitivity to 5-aza and 5-aza resistance associated with increased sensitivity to cisplatin. Transcriptomics revealed downregulation of the H3K27me3-mediated polycomb pathway in cisplatin resistant cells and upregulation of this pathway with 5-Aza resistance. To explore possible mechanisms for this reciprocal epigenetic modeling, the expression of the gene family responsible for histone lysine demethylation, the KDM family, was assessed by qPCR. Many KDM genes, including those responsible for H3K27me3 demethylation, were upregulated in cisplatin resistant cells and downregulated in 5-Aza resistant cells. Changes in KDM gene expression could explain, in part, globally altered levels of H3K27 methylation. We are performing genetic and pharmacologic studies to further validate a role for the H3K27me3 polycomb pathway in chemotherapeutic resistance, with the goal of devising novel therapies for testicular and potentially other cancers.

Acknowledgements

Emmanuel Bikorimana and Ratnakar Singh helped gather data from both cell viability assays and western blot assays. Michael J. Spinella assisted with revision of this thesis.

Table of Contents

Introduction

Testicular germ cell tumors (TGCTs) are the most common form of neoplasm found in men between 15 and 44 years old [1]. Since the mid-20th century, the occurrence of TGCTs in the US has been rising. TGCTs are among the most curable solid malignancies, even when the disease is highly metastatic [2]. The 5-year survival rate is above 80% using cisplatin-based combination chemotherapy [3]. About 20% of all patients are refractory to cisplatin treatment [4].

The mechanism to account for the cisplatin hypersensitivity and resistance of TGCTs is unknown, although it has been proposed that epigenetics may play a large role [5]. Compared to other tumors TGCTs have unique epigenetic markers, including patterns of DNA methylation as well as repressive and active histone modifications [6]. Cisplatin resistant cells overexpress DNMT3B, a key protein involved in de novo methylation of DNA [7]. An elevation in DNMT3B has been linked with elevated tumor invasion in TGCTs and a decrease in patient survival rate [7]. Interestingly, the Spinella lab has shown that TGTC cells are sensitive to very low dosages of the DNA methylation inhibitor 5-aza and that this is dependent on DNMT3B [8]. 5-azacytidine can be incorporated into DNA by mimicking cytosine and taking its place in DNA [9]. However, 5-aza is not able to be methylated, but inhibits the activity of DNA methyltransferases like DNMT3B. Besides the link to high levels of DNMT3B, the mechanism to account for why TGCT cells are so sensitive to very low doses of 5-aza in unknown. The potential of 5-aza treatment for cisplatin refractory testicular cancer patients is promising [10].

The Spinella lab, using RNA-seq, have shown a major up regulation of genes usually repressed by the polycomb pathway and H3K27 methylation in cisplatin resistant TGCTs [5]. Polycomb repressive complex 2 (PRC2) is a complex of proteins responsible for H3K27 methylation, one component is the H3K27 methyltransferase EZH2 [11]. Polycomb repressive complex 1 (PRC1) including the protein BMI1 is recruited to the site of H3K27me3 and further represses transcription by mediating H2AK119 ubiquitination [11]. H2AK119 ubiquitination and H3K27me3 levels were decreased in cisplatin resistant

TGCTs [5]. Interestingly pharmacological and genetic inhibition of EZH2 results in cisplatin resistance in TGCTs [5]. There are two proteins directly involved in the demethylation of H3K27 KDM6A and KDM6B [12].

In the current study we attempt to further our understanding of the mechanism to account for the hypersensitivity of TGCT cells to 5-aza by creating novel 5-aza resistant TGCTs. This study has established that cisplatin and 5-aza resistant cells show a reciprocal relationship in sensitivity to 5-aza and cisplatin, respectively. This led us to investigate the epigenetic difference between these two cell lines. We provide evidence that polycomb and DNA methylation are reciprocally co-regulated in cisplatin and 5-aza resistant cells with cisplatin resistant cells demonstrating decreased H3K27me3 and increased DNMT3B levels while 5-aza resistant cells demonstrate increased H3K27me3 and decreased levels of DNMT3B. We also demonstrate that alteration in H3K27me3 levels in the resistant cells could be explained in part by changes in the levels of polycomb components and H3K27me3 demethylases. Future work in this project would be to experimentally alter the expression or activity of DNMT3B and specific polycomb components to further define the crosstalk between these two pathways in the biology of TGCTs. A greater understanding of the epigenetics of TGCTs, both cisplatin sensitive and resistant, could lead to more effective and less harmful treatments for patients that do not respond to cisplatin.

Materials and Methods

Development of cisplatin and 5-aza resistant cell lines

Cells were cultured in DMEM (Gibco, Gaithersburg, MD, USA) with 10% FBS (Invitrogen, Carlsbad, CA, USA). 2102EP is a human testicular cancer derived EC cell line purchased from and verified by the American Type Culture Collection (Manassas, Virginia). The generation of cisplatin resistant cell

lines (B3, C1, C4) was previously reported by the Spinella lab [5]. To generate 5-Aza resistant cell lines parental 2102EP cells were exposed to increasing dosages of 5-aza-2'-deoxycytidine (5-Aza) (Sigma Aldrich, St. Louis, MO, USA) starting at 1 nM for three consecutive days and then allowed to recuperate for 1-2 weeks. The dosages increased step-wise from from 1 nM to 10n M over the span of 5 cycles. The 2102EP-derived cells were split into three groups AH1, AH2A, and AH2B. These groups received 5-Aza dosages gradually increasing to 20 nM, 100nM, and 50nM, respectively. Cells were cloned from each group using cloning cylinders. All clones retained resistant to 5-Aza for at least 4 months after being placed in 5-Aza free media. My role in deriving these cells was treating the cells as well as picking cells for cloning. 5-aza resistant clones, AH1A5, AH2A2 and AH2B9 were further characterized in this study.

Cell viability with cisplatin and 5-aza treatments

For cisplatin treatment cells were treated with indicated dosages of cisplatin for 6 h then assayed for survival 3 days later. For 5-aza treatment the cells were treated with the indicated dosages of 5-aza for 3 days, replenished daily. On the fourth day the cells were assayed for viability. The viability assays were performed using CellTiter-Glo (Promega, Madison, WI, USA) assay. The percent of viable cells, relative to the untreated cells, were calculated.

Western analysis

For western blot analysis cells were lysed with radioimmune precipitation (RIPA) buffer. A Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used to measure the protein concentration of each lysate and 30 ug of protein was used for Western analysis.

Samples were analyzed using SDA-PAGE transferred to nitrocellulose membranes which were then blocked using 5% non-fat dry milk. Membranes were then incubated with the indicated primary antibody overnight at 4 °C. DNMT3B (1:1000; 2851; Abcam Cambridge, MA, USA), DNMT1 (1:1000; 20701, Santa Cruz Biotechnology, Dallas, TX, USA), H3K27 me3(1:1000; 9733, Cell Signaling Technology, Danvers, MA, USA), Ubiquitin H2AK119, (1:1000; 3240, Cell Signaling Technology), BMI1 (1:1000; 6964, Cell Signaling Technology), actin (1:1000; MA1-744, Thermo Fisher Scientific, Waltham, MA, USA). Protein expression was detected using horseradish peroxidase-conjugated secondary antibody and chemiluminescence reagent (Thermo Fisher Scientific).

Real-time quantitative PCR

Cellular RNA was isolated using RNeasy Mini Kit (Qiagen). The concentration of RNA in each sample was measured using a NanoDrop machine. Complementary DNAs (cDNAs) were synthesized from 2 µg of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Bedford, MA, USA). Real-time quantitative PCR (RT-qPCR) assays were performed with *Power*SYBR Green PCR Master Mix (Applied Biosciences) and the QuantStudio 3 Real-time System (Thermo Fisher). The primer sequences are provided in **Table 1.**

Gene	Type	Sequence
Actin	Forward	5’-TTTGAGACCTTCAACACCCCAGCC-3’
	Reverse	5’-AATGTCACGCACGATTTCCCGC-3’
DNMT3B	Forward	5'-GAGTCCATTGCTGTTGGAACCG-3'
	Reverse	5'-ATGTCCCTCTTGTCGCCAACCT-3'
KDM 6A	Forward	5'-CACAGTACCAGGCCTCCTCATT-3'
	Reverse	5'-TCACTATCTGAGTGGTCTTTATGATGACT-3'
KDM 6B	Forward	5'-CGGAGACACGGGTGATGATT-3'
	Reverse	5'-CAGTCCTTTCACAGCCAATTCC-3'
EZH2	Forward	5'-GTGGAGAGATTATTTCTCAAGATG-3'
	Reverse	5'-CCGATCCAATCTGTTCTGGT-3'
BMI1	Forward	5’-CCAGGGCTTTTCAAAAATGA-3’
	Reverse	5’-CCGATCCAATCTGTTCTGGT-3’
JARID2	Forward	5’-TGTTCACAACGGGCATGTTT-3’
	Reverse	5’-TTGTGTTTTGAACAGGTTCCTTCT-3’

Table 1. Sequence of primers using for RT-qPCR

Results and Discussion:

Derivation of 5-Aza resistant 2102EP TGCT cells

Past experiments of the Spinella lab have shown that TGCT cells that express high levels of DNMT3B, are hypersensitive to very low dosages of 5-aza and quadectabine[8]. The mechanism of this 5-aza hypersensitivity is unknown, which prompted us to develop 5-Aza resistant cells. 2102EP cells were treated with gradually increasing doses of 5-Aza, from 1nM to 10nM, and allowed to recover between treatments. The cells were split into three groups and the dosages were gradually increased again. The cycle for how these cells were developed is described in Materials and Methods. **Figure 1** confirms that these newly generated cells were resistant to 5-Aza relative to the parental cell line.

Cisplatin resistant cells were previously developed and characterized by the Spinella lab [5]. The Spinella lab performed RNA-seq analysis on both 5-aza and cisplatin resistant cell lines. RNA-seq data indicated increased expression of polycomb target genes in cisplatin resistant cells compared to parental cells. However, 5-aza resistant cells showed decreased expression of polycomb target genes compared

to parental cells. The Spinella lab established that the repression of the polycomb pathway is an important mechanism of cisplatin resistance in the cisplatin resistant cell lines [5] . This prompted us to investigate the cisplatin sensitivity of the 5-aza resistant cells and to begin to explore the role of polycomb in 5-aza responses.

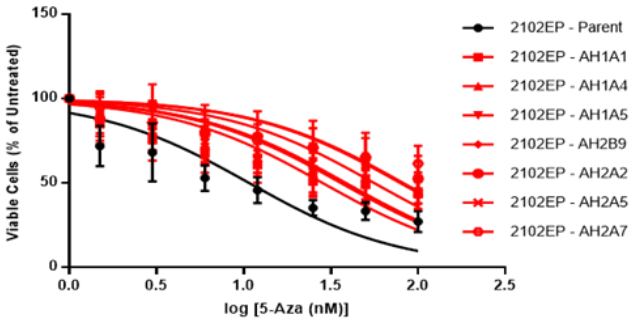

Figure 1. Confirmation of 5-Aza resistance in 5-Aza resistant cell lines. 5-Aza selected cell lines are consistantly resistant to 5-Aza treatment. Cell viablity was measured using CellTiter-Glo in parental and 5-Aza resistant cell lines with indicated dosages of 5-Aza. The precent of viable cells is releative to the untreated cells of each cell line. Bars are the average of biological triplicates. The error bars are S.D. The results in this figure were generated by Emmanual Bikorimana but I have periodically confirmed resistance in other experiments. Representative of three independent experiments.

There is a recipriocal drug resistance relationship between 5-Aza and cisplatin-resistant cell lines derived from 2102EP

To explore the role of the opposing expression of the polycomb target genes in 5-aza and cisplatin resistant cell lines a cell viability experiment was conducted to determine sensitivity to cisplatin and 5-aza. Seven distinct cell lines were tested **(Figure 2A)**. Those cell lines being 2102EP parent cells , 2102EP cells resistant to 5-Aza (AH1A5, AH2B9, AH2A2), and 2102EP cells resistant to cisplatin (B3, C1,

C4). Compared to the 2102EP parent cells, 5-Aza resistant cell lines were more senstive to cisplatin. The resistance of the cisplatin resistent cell lines to cisplatin was also confirmed. To further this study the viability of these same cell lines to 5-Aza was tested **(Figure 2B)**. The results showed that cisplatin resistant cell lines are more sensitive to 5-Aza treatment. I participated in the derivation of these resistant cell lines which is further detailed in the Materials and Methods section.

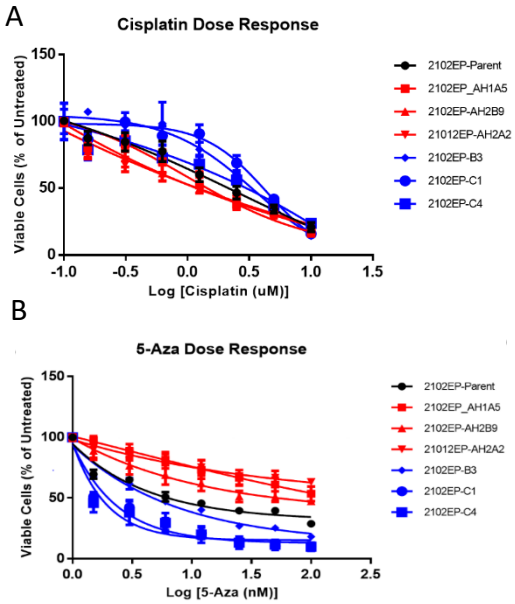

Figure 2. 5-Aza and cisplatin resistant cell lines show opposite drug response. A, Cell viability was measured using CellTiter-Glo in parental, 5-Aza resistant, and cisplatin resistant cell lines with indicated dosages of cisplatin. The percent of viabile cells was measured relative to the untreated cells in each cell line. **B,** Cell viablity was measured using CellTiter-Glo in parental, 5-Aza resistant, and cisplatin resistant cell lines with indicated dosages of 5-Aza. The precent of viable cells is releative to the untreated cells of each cell line. Bars are average of biological triplicates. The error bars are S.D. The results in this figure were generated by Emmanual Bikorimana but I have conducted similar experiments. Representative of 3 independent experiments.

5-Aza and cisplatin resistant cells have reciprocal expression of DNMT3B, H3K27me3, and BMI1

It is well established that DNMT3B overexpression in TGCT cells leads to 5-aza sensitivity [8]. Using RNA-seq we showed that the polycomb pathway is reciprocally regulated in 5-aza and cisplatin resistant cell lines. To confirm the RNA-seq results, Western Blot assays were conducted **(Figure 3)**. Eight different cell lines; two parent cell lines, three 5-Aza resistant cell lines (AH1A5, AH2B9, AH2A2), and three cisplatin resistant cell lines (B3, C1, C4) all derived from 2102EP cells were employed. The results show DNMT3B is overexpressed in cisplatin resistant cell lines and has decreased expression in 5-Aza resistant cell lines. However, not all DNA methyltransferases are expressed reciprocally, DNMT1 expression is largely unchanged between the cell lines. BMI1 is a polycomb protein that is expressed highly in the 5-aza resistant cell line AH1A5, but not in the other 5-Aza resistant cell lines. The cisplatin resistant cell lines have lower BMI1 expression. In addition, there was a striking opposite expression pattern of H3K27me3 in 5-Aza and cisplatin resistant cell lines relative to parental cells, which was consistent with the alterations in expression of polycomb target genes as seen in RNA-seq data.

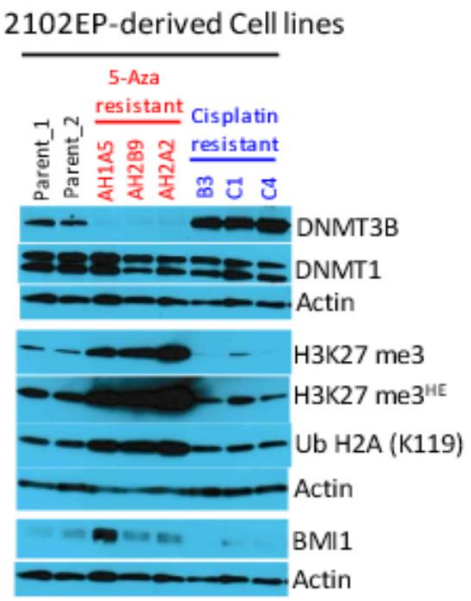

Figure 3. DNMT3B, H3K27me3, and BMI1 are reciprocally expressed in 5-Aza and cisplatin resistant cells. Western blot showing reciprocal expression of DNMT3B and H3K27me3 in cisplatin and 5-aza resistant cells. Representative blot of three separate experiments. This blot was performed by Ratnakar Singh but I have conducted similar experiments. Representative of three independent experiments.

Reciprocal expression of DNA methyltransferases, histone demethylases, and PRC2 components in 5-Aza and cisplatin resistant cell lines

To begin investigating the mechanism that accounts for the reciprocal expression of DNMT3B and H3K27me3 in resistant cell lines we performed RT-PCR of several proteins that regulate expression of H3K27me3 (Figure 4) and the polycomb pathway. The lysine demethylases KDM6A and KDM6B are known to demethylate H3K27me3, they were chosen to further our understanding of H3K27me3 regulation in 5-aza resistant cells. Other genes involved in the polycomb repressive complex were chosen based on the RNA-seq data showing reciprocal expression of this pathway in 5-aza and cisplatin resistant cell lines.

EZH2 and JARID2 are part of PRC2 and EZH2 is the methyltransferase responsible for adding the H3K27me3 mark. This experiment was performed in seven cell lines namely 2102EP parent, three 5-Aza resistant cell lines (AH1A5, AH2B9, AH2A2), and three cisplatin resistant cell lines (B3, C1, C4). The results show that several genes have a reciprocal expression pattern between 5-Aza and cisplatin resistant cell lines. This is especially apparent in the expression of DNMT3B where 5-Aza resistant cell lines have 0.5 times less expression of DNMT3B compared to the parental cell line (Figure 4). In contrast cisplatin resistant cell lines overexpress DNMT3B 4-fold compared to the parental cell line. The opposite is true for expression of BMI1, 5-Aza resistant cells expressed BMI1 1.3-fold higher compared to the control. Cisplatin resistant cell lines express this gene 0.3-fold lower compared to parental cells (Figure 4). The expression of KDM6A and EZH2 was inconsistent between the cell lines with KDM6A being overexpressed in only one of the cisplatin resistant lines but unchanged in the other lines. EZH2 was slightly repressed in one cisplatin resistant line and slightly overexpressed in two of the 5-aza resistant lines (Figure 4). Interestingly, the H3K27me3 demethylase KDM6B was consistently overexpressed in the three cisplatin resistant lines which could explain why these lines have decreased H3K27me3 (see Figure 3). However, the expression of KDM6B was unchanged in the 5-aza resistant lines. In summary the RT-PCR data points to multiple mechanisms that may be involved in the regulation of H3K27me3 across the cell lines. Further experiments will be required to further understand the mechanism of reciprocal regulation of H3K27me3 in 5-aza and cisplatin resistant cells.

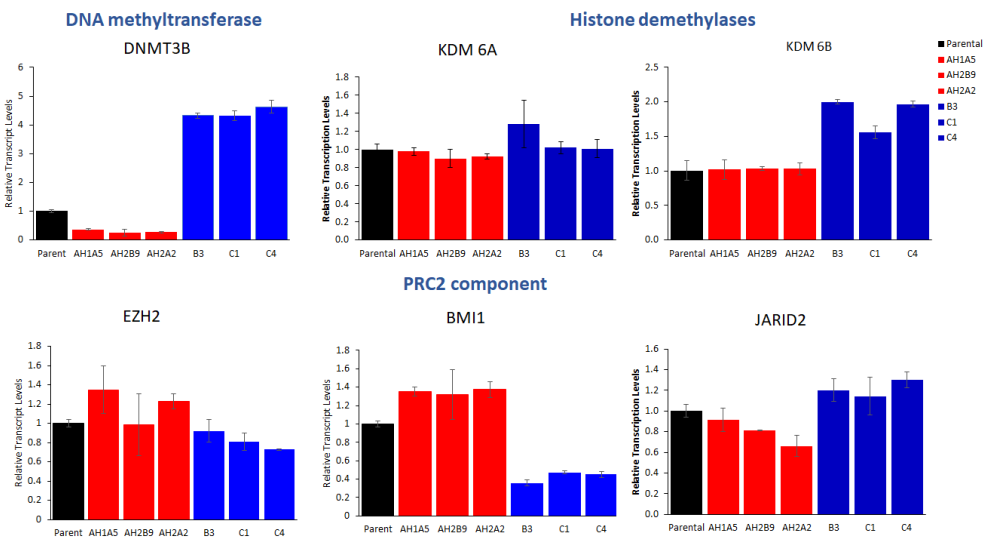

Figure 4. Cisplatin and 5-Aza resistance is associated with altered expression of genes involved in DNA methylation, histone H3K27 methylation and PRC2. Real-time PCR analysis of mRNA expression of DNMT3B, KDM6A, KDM6B, EZH2, BMI1, and JARID2 in parent, 5-aza resistant, and cisplatin resistant 2102 EP cells. Bars are the average of three biological replicates. Expression is relative to the parental cell line. The Error bars are S.D. Representative of two independent experiments.

Conclusion:

Arguably the biggest obstacle to curing advanced stage cancers is acquired chemotherapeutic resistance. TGCTs are one of few solid tumors that in advanced stages can be cured at a high rate with standard chemotherapy [2]. However, the mechanism behind sensitivity and resistance to cisplatin-based chemotherapy in TGCTs remains poorly understood. The Spinella lab has provided evidence that repression of the polycomb pathway associated with decreased H3K27 methylation and increased expression of polycomb target genes may be a major pathway responsible to acquired resistance in TGCT cells[5]. Further a clinically significant number of TGCT patients are resistant to cisplatin and die of progressive disease as there are no effective alternative therapies for these patients [3]. The Spinella lab

discovered that TGCT cells, even those resistant to cisplatin, are exquisitely sensitive to very low doses of the hypomethylating agents 5-aza and quadecitabine (prodrug of 5-Aza) an that this is associated with high levels of the DNA methyltransferase DNMT3B[8]. This has led to promising results in a phase I clinical trial combining guadecitabine and cisplatin in cisplatin refractory patients heavily pretreated with cisplatin.

In the current study we created novel 5-aza resistant TGCT cell lines to further our understanding the mechanism to account for the hypersensitivity of TGCT cells to 5-aza. The cisplatin and 5-aza resistant cells show a reciprocal relationship in sensitivity to 5-aza and cisplatin, respectively. We provide evidence that polycomb and DNA methylation are reciprocally co-regulated in cisplatin and 5-aza resistant cells with cisplatin resistant cells demonstrating decreased H3K27me3 and increased DNMT3B levels while 5-aza resistant cells demonstrate increased H3K27me3 and decreased levels of DNMT3B.

One of the most dramatic differences between 5-aza and cisplatin resistant cells was the expression pattern of DNMT3B while expression of DNMT1 is unchanged (Fig 3). These two proteins are both associated with DNA methylation, but they have distinct roles. DNMT1 is responsible for methylation of hemimethylated sites in newly replicated DNA strands, contributing to the stability of gene expression passed on from parental to daughter cells [13,14]. DNMT3B on the other hand is responsible for de novo methylation of CpG islands on autosomes and the X chromosome [14]. The methylation of cytosine on CpG islands blocks gene expression by preventing transcription factors from interacting with the promotor region [14]. DNMT3B was highly expressed in cisplatin resistant TGCTs which are highly sensitive to 5-aza. DNMT3B is known to form covalent adducts with 5-aza containing DNA that could lead to a DNA damage response, hence providing a mechanism for the increased 5-aza sensitivity of cisplatin resistant cells[8]. To accurately determine the role of increased DNMT3B in 5-aza

hypersensitivity will require further experiments including the overexpression and knockdown of DNMT3B in TGCTs.

The mechanism to account for the reciprocal regulation of DNMT3B and H3K27me3 is currently unclear and will require further study. One potential mechanism is that DNMT3B actively represses key components of the polycomb pathway by DNA methylation mediated gene promoter silencing in cisplatin resistant cells. We performed initial RT-PCR experiments to assess this mechanism. The methylation of H3K27 is mediated by the PRC2 complex. The core components of PRC2 are EZH2, SUZ12, and EED [11]. JARID2 is also a component of PRC2 but is devoid of catalytic function. Depletion of JARID2 is associated with increased proliferation and cell accumulation in S phase [15]. EZH2, in contrast, has catalytic activity and directly mediates H3K27 methylation [11]. We found a modest upregulation of EZH2 expression in a subset of the 5-aza resistant cells and a modest downregulation in a subset of the cisplatin resistant cell lines consistent with the changes in H3K27me3. Alterations if JARID2 expression did not correlate with expression changes in H3K27me3. The most promising change found was in PRC1 component BMI1. PRC1 is responsible for recognizing methylated H3K27 and then mediating ubiquitination of H2AK199 [16,17]. This results in polycomb target gene repression which is consistent with RNA-seq data from previous studies [5]. Hence changes in BMI1 could explain the reciprocal regulation of Ub-H2AK119 and polycomb target gene seen in 5-aza and cisplatin resistant cells but not the expression pattern of H3K27me3. In contrast to EZH2 that mediates H3K27me3, there are two demethylases which remove this mark, KDM6A and KDM6B. We provide evidence for increased expression of KDM6B in cisplatin resistant cells which is consistent with decreased H3K27me3 levels in cisplatin resistant cells [12]. A further interrogation of the RNA-seq data may also provide other candidate polycomb components that could be mediating the reciprocal regulation of H3K27me3 in 5-aza and cisplatin resistant cells.

In summary we provide evidence that the distinct and exquisite sensitivity of TGCT cells to 5-aza and cisplatin may involve interconnected DNA methylation and polycomb pathway alterations that are reciprocally regulated. Future work in this project would be to experimentally alter the expression or activity of DNMT3B and specific polycomb components to further define the crosstalk between these two pathways in the biology of TGCTs. Our findings have important implications for TGCT patients that fail cisplatin-based therapy and could lead to the design of less toxic epigenetic based therapies for both cisplatin sensitive and resistant TGCTs.

Bibliography

1. Ghazarian, A. A., Kelly, S. P., Altekruse, S. F., Rosenberg, P. S., & McGlynn, K. A. (2017). Future of testicular germ cell tumor incidence in the United States: Forecast through 2026. *Cancer*, *123*(12), 2320–2328. https://doi.org/10.1002/cncr.30597
2. Koychev, D., Oechsle, K., Bokemeyer, C., & Honecker, F. (2011). Treatment of Patients with Relapsed and/or Cisplatin-refractory Metastatic Germ Cell Tumours: an update. *Internal Journal of Andnrology, 34*(4), 266-273. https://doi.org/10.1111/j.1365-2605.2011.01145.x
3. Oing, C., Alsdorf, W.H., von Amsberg, G. et al. (2017) Platinum-refractory germ cell tumors: an update on current treatment options and developments. *World J Urol 35*, 1167–1175. https://doi.org/10.1007/s00345-016-1898-z
4. Einhorn, L., Williams, S., Chamness, A., Brames, M., Perkins, S., & Abonour, R. (2007). High-Dose Chemotherapy and Stem-Cell Rescue for Metastatic Germ-Cell Tumors. *The New England Journal of Medicine, 357*, 340-348 DOI: 10.1056/NEJMoa067749
5. Singh, Ratnakar; Fazal, Zeeshan; Corbet, Andrea K.; Bikorimana, Emmanuel; Rodriguez, Jennifer C.; Khan, Ema M.; Shahid, Khadeeja; Freemantle, Sarah J.; Spinella, Michael J. (2019). "Epigenetic Remodeling through Downregulation of Polycomb Repressive Complex 2 Mediates Chemotherapy Resistance in Testicular Germ Cell Tumors" *Cancers 11*(6), 796. https://doi.org/10.3390/cancers11060796
6. Taylor-Weiner, A., Zack, T., O'Donnell, E., Guerriero, J. L., Bernard, B., Reddy, A., Han, G. C., AlDubayan, S., Amin-Mansour, A., Schumacher, S. E., Litchfield, K., Turnbull, C., Gabriel, S., Beroukhim, R., Getz, G., Carter, S. L., Hirsch, M. S., Letai, A., Sweeney, C., & Van Allen, E. M. (2016). Genomic evolution and chemoresistance in germ-cell tumours. *Nature*, *540*(7631), 114–118. https://doi.org/10.1038/nature20596
7. Sonnenburg, D., Spinella, M. J., & Albany, C. (2016). Epigenetic Targeting of Platinum Resistant Testicular Cancer. *Current cancer drug targets*, *16*(9), 789–795. https://doi.org/10.2174/1568009616666151222150359
8. Beyrouthy, M. J., Garner, K. M., Hever, M. P., Freemantle, S. J., Eastman, A., Dmitrovsky, E., & Spinella, M. J. (2009). High DNA methyltransferase 3B expression mediates 5-aza-deoxycytidine hypersensitivity in testicular germ cell tumors. *Cancer research*, *69*(24), 9360–9366. https://doi.org/10.1158/0008-5472.CAN-09-1490
9. Christman, J. (2002) 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene 21*, 5483–5495. https://doi.org/10.1038/sj.onc.1205699
10. Albany, C., Fazal, Z., Singh, R., Bikorimana, E., Adra, N., Hanna, N. H., Einhorn, L. H., Perkins, S. M., Sandusky, G. E., Christensen, B. C., Keer, H., Fang, F., Nephew, K. P., & Spinella, M. J. (2021). A phase 1 study of combined guadecitabine and cisplatin in platinum refractory germ cell cancer. *Cancer medicine*, *10*(1), 156–163. https://doi.org/10.1002/cam4.3583
11. Kim, S. H., Yang, W. I., Min, Y. H., Ko, Y. H., & Yoon, S. O. (2016). The role of the polycomb repressive complex pathway in T and NK cell lymphoma: biological and prognostic implications. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, *37*(2), 2037–2047. https://doi.org/10.1007/s13277-015-3977-y

12. Labbé, R. M., Holowatyj, A., & Yang, Z. Q. (2013). Histone lysine demethylase (KDM) subfamily 4: structures, functions and therapeutic potential. *American journal of translational research*, *6*(1), 1–15.
13. Bulijasic, R., Bulijasic, M., Bojanac, A., Ulamec, M., Vlahovic, M., Jezek, D., Nulic-Jakus, F., & Sincic, N. (2018). Epigenetics and testicular germ cell tumors. *Gene, 661*, 22-33
14. Auclair, G., Guibert, S., Bender, A. *et al.* (2014) Ontogeny of CpG island methylation and specificity of DNMT3 methyltransferases during embryonic development in the mouse. *Genome Biol 15*, 545. https://doi.org/10.1186/s13059-014-0545-5
15. Adhikari, A., Mainali, P., & Davie, J. K. (2019). JARID2 and the PRC2 complex regulate the cell cycle in skeletal muscle. The Journal of biological chemistry, 294(51), 19451–19464. https://doi.org/10.1074/jbc.RA119.010060
16. Li, F., Han, H., Sun, Q., Liu, K., Lin, N., Xu, C., Zhao, Z., & Zhao, W. (2019). USP28 regulates deubiquitination of histone H2A and cell proliferation. *Experimental cell research*, *379*(1), 11–18. https://doi.org/10.1016/j.yexcr.2019.03.026
17. Wei, J., Zhai, L., Xu, J., & Wang, H. (2006). Role of Bmi1 in H2A Ubiquitylation and Hox Gene Silencing. *Journal of Biological Chemistry 281* (32), 22537-22544. https://doi.org/10.1074/jbc.M600826200