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14. ABSTRACT
The purpose of this project is to validate the concept, provide data on mechanism, and uncover biomarkers of response to support future clinical trials targeting the polycomb pathway in testicular germ cell tumor (TGCT) patients. The major findings in the first two years have shown that targeting the polycomb pathway both pharmacologically and genetically alters cisplatin sensitivity of TGCTs in vitro and in vivo in mice. In terms of mechanism and biomarker identification we have performed a number of unbiased genome-wide studies including RNA-seq, H3K27me3 ChIP-seq and p53 ChIP-seq. **Update for year 2.** This past year we have performed xenografts with polycomb targeted drug GSKJ4 on a second TGCT cell line and have generated polycomb component KDM6A and KDM6B dual knockdown cells and tested their cisplatin sensitivity in vitro and in vivo (started just last week). We are at the stage of familiarity with the genetically engineered mouse model to start our first therapeutic trail with GSKJ4 plus cisplatin by next month and have conducted further RNA-seq studies on two cell lines treated with cisplatin, GSKJ4, and the combination. We have assessed whether polycomb targeting affects cisplatin sensitivity in somatic cancer cells and we have stained 50 primary TGCT samples with an antibody to the polycomb mark, H3K27me3. We have not encountered any insurmountable issues and are on track to complete all the tasks of the SOW. Our results strongly support that polycomb may be a valuable and useful therapeutic target to treat cisplatin refractory TGCTs. Further, the lab has been working closely with the leadership of the Malignant Germ Cell International Consortium (MaGIC) to conduct a second, large National Clinical Trial Network-wide trial of epidrug and DNA hypomethylating agent, ASTX727, in cisplatin refractory TGCT patients to be started approximately 1 year from now. We envision the GSKJ4 (which would be the second epidrug for TGCT patients) could be tested clinically via this same collaboration. Finally, we have applied this October for a R01 grant using preliminary data described here for continued support of the project. These have been exciting times for the lab, and we are appreciative of the DOD for their support in getting this project off the ground.

15. SUBJECT TERMS
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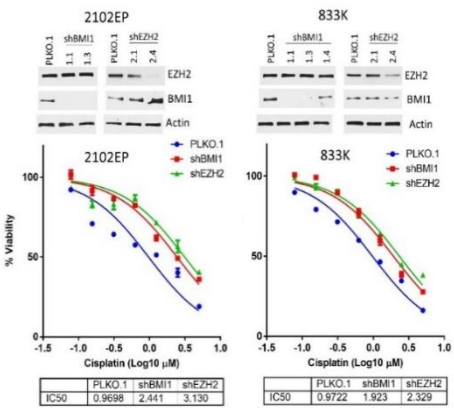
Introduction

There is a pressing clinical need to devise new strategies to treat refractory testicular germ cell tumors (TGCTs) and a rationale to devise targeted, cisplatin-sparing therapies. We employed unique cisplatin resistant cell models and de novo transcriptional profiling approaches that unexpectedly uncovered an important role for polycomb in TGCT sensitivity and resistance to cisplatin. Our hypothesis is that specific epigenetic states mediated by the polycomb pathway play a crucial role in the curability of testicular cancer and may sensitize other types of cancer to chemotherapy. The goal of the project is to further validate this epigenetic therapy in cell and animal models and to perform experiments that will provide markers of whether a patient's tumor will respond to polycomb targeting drugs and cisplatin.

Research Accomplishments (as organized by SOW Major Tasks).

1. Assess the degree that genetic/pharmacologic targeting of polycomb in TGCTs alters sensitivity to cisplatin in vitro.

We generated EZH2 and BMI1 shRNA knockdowns in NT2/D1, 833K and 2102EP cells and showed that they are resistant to cisplatin (Figure 1). We showed that GSK126 pretreatment of TGCT cells results in cisplatin resistance and that GSKJ4 treatment results in cisplatin sensitivity (Figure 2). We also generated KDM6A/6B dual knockdown TGCT cells and showed that they have increased sensitivity to cisplatin (Figure 3).



	PLKO.1	shBMI1	shEZH2
IC50	0.9698	2.441	3.130

	PLKO.1	shBMI1	shEZH2
IC50	0.9722	1.923	2.329

Fig. 1. Knockdown of EZH2 and BMI1 mediates cisplatin resistance in TGCT cells.

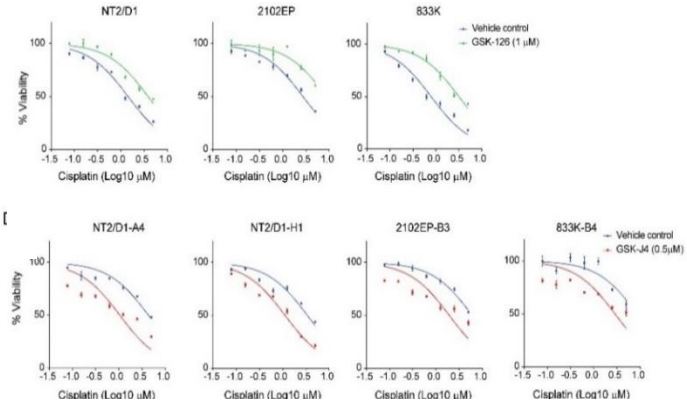


Fig. 2. Inhibition of H3K27 methylation mediates cisplatin resistance in TGCT cells, inhibition of H3K27 demethylation sensitizes TGTC cells to cisplatin. Cells pretreated with H3K27 methyltransferase inhibitor (GSK126 1 μM) or H3K27 demethylase inhibitor (GSK-4 (0.5 μM).

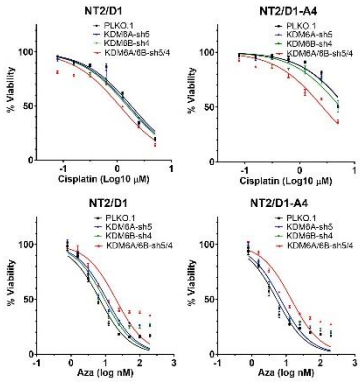


Fig. 3. KDM6A/B-KD mediates cisplatin sensitization and 5-aza resistance in TGCT cells.

2. Assess the degree that genetic and pharmacologic targeting of polycomb in somatic solid tumor cells alters sensitivity to cisplatin.

We have begun to assess they effects of polycomb targeting on other types of cancer cells. Thus far we have assessed two breast cancer cell lines and a colon cancer cell line (Figure 4). Polycomb targeting drugs GSKJ4 and GSK126 did not alter cisplatin sensitivity in these cells in contrast to what is seen with TGCT cells, suggesting that the key role of polycomb in mediating cisplatin sensitivity may be unique to TGCT cells. We are currently assessing additional somatic cancer cell lines.

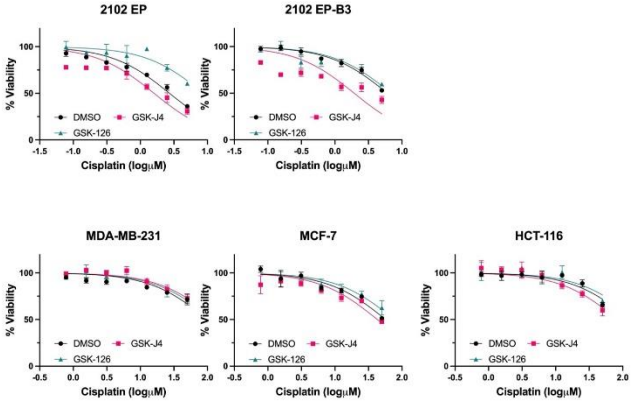


Fig. 4. Pharmacologic manipulation of polycomb in breast cancer cells (MDA-MB-231, MCF7) and colon cancer cells (HCT-116)

3. Assess the degree that genetic and pharmacologic targeting of polycomb in TGCT cells alters sensitivity to cisplatin in vivo.

We have assessed the in vivo effects of the polycomb targeting drug GSKJ4 in xenografts using two TGCT cell lines, the cisplatin resistant cell line 2102EP-C1 and the cisplatin sensitive cell line NT2/D1. For both cell lines, GSKJ4 mediated dramatic increased sensitivity to cisplatin (Figure 5). This indicates that GSKJ4 is a promising new epidrug for TGCTs. Two additional experiments are ongoing. In the first, we have made KDM6A/B 2102EP cells and are testing whether these cells will be hypersensitive to cisplatin in xenografts. In the second we are gearing up to treat the genetically engineered mouse model the forms spontaneous TGCTs with GSKJ4 and cisplatin. This mouse model gets very aggressive tumors that kills mice by 30 days post weening and cisplatin alone only marginally prolongs survival. If we can demonstrate that GSKJ4 dramatically prolongs survival in this model, it would be a very impactful result.

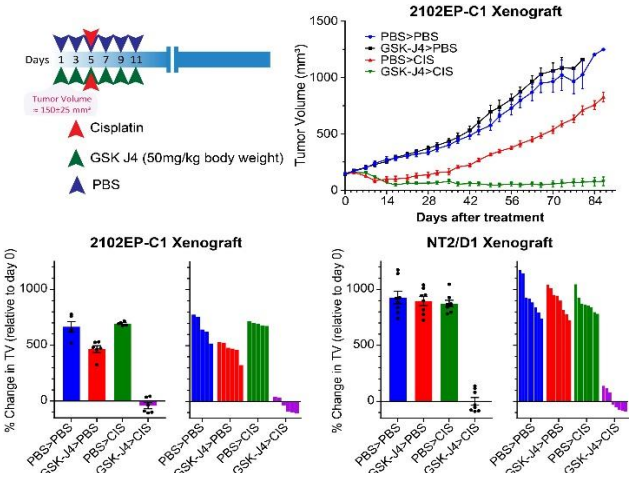


Fig. 5. GSKJ4 increases sensitivity of TGCT cells to cisplatin in vivo.

4. Determine whether polycomb alterations associated with cisplatin resistance extend to human TGCT samples.

We developed an immunohistochemical assay for H3K27me3 expression in testicular germ cell tumors (TGCTs). In Figure 6 we fixed embryonal carcinoma (EC) cells (2101EP) treated with vehicle or EZH2 inhibitor GSK126 and then formalin fixed and paraffin embedded the cells and prepared slides for staining with the H3K27me3 monoclonal rabbit antibody (Cell Signaling, cat#9733) at three different dilutions. Slides were stained on an automated workstation. We chose the 1:1000 dilution for clinical staining. Using this workflow, we ran a pilot study on 4 embryonal carcinoma (EC) primary samples, 1 sample from a cisplatin sensitive patient and 3 from cisplatin resistant patients (Figure 7). QuPath was used for automated WSI IHC quantification and analysis of 5 representative areas per slide. Each area had automated H score calculated based on percent positive cells and staining intensity. Interestingly the cisplatin sensitive patient had robust H3K27me3 staining (H-score 148.3), while two of the cisplatin resistant patient samples had very low staining (H-score, 1.15 and 24.18) while a third resistant sample had intermediate staining. This is in line with our hypothesis that decreased H3K27me3 expression and polycomb repression is associated with cisplatin resistance of TGCTs and exploitable by H3K27me3 demethylase inhibition therapy to resensitize patients to cisplatin. We are currently performing larger scale analysis on a series of 25 sensitive and 25 refractory TGCT specimens.

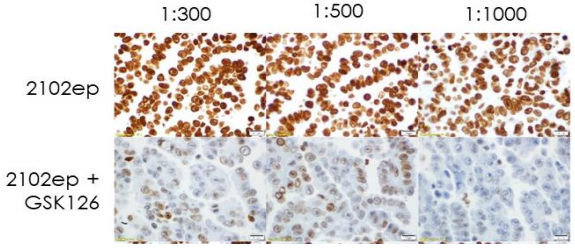


Figure 6. Optimization of H3K27me3 antibody #9733 on EC cells line positive and negative controls.

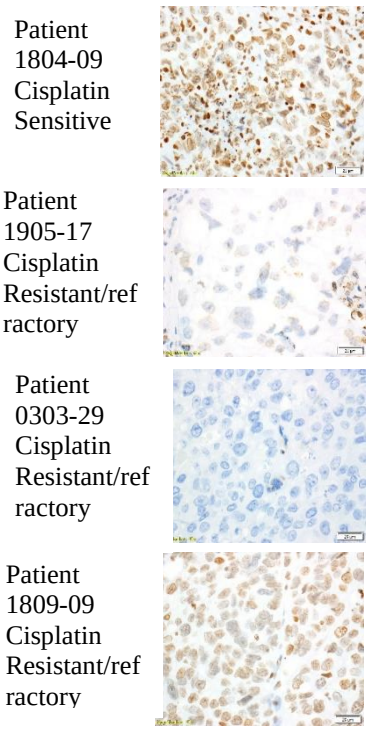


Figure 7. H3K27me3 staining of cisplatin sensitive and resistant/refractory TGCTs. 40x

5. Perform RNA-seq on TGCT cells targeting the polycomb pathway with pharmacologic and genetic approaches.

We preformed RNA-seq in biological triplicate in two cisplatin resistant cells lines 2102EP-C1 and NT2/D1-A4. Treatments were vehicle control, GSKJ4 alone, cisplatin alone, and GSKJ4 + cisplatin. In Figure 8 principal component analysis of cisplatin resistant NT2/D1-A4 cells reveals that biological triplicates of each treatment group cluster tightly. Figure 9 is an unsupervised hierarchal cluster analysis of genes changed 2 fold or greater with FDR of < 0.05 in NT2/D1-A4 cells, again revealing that treatment groups cluster together. Similar results were obtained with 2102EP-C1 cells. We are currently in the process of analyzing this data to determine gene changes that correlate with the ability of GSKJ4 to sensitive TGCT cells to cisplatin.

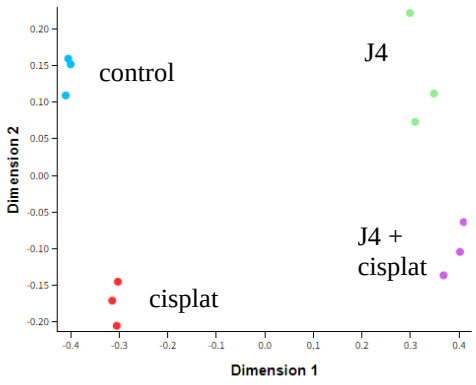


Figure 8. PCA Plot of NT2/D1-A4 cells.

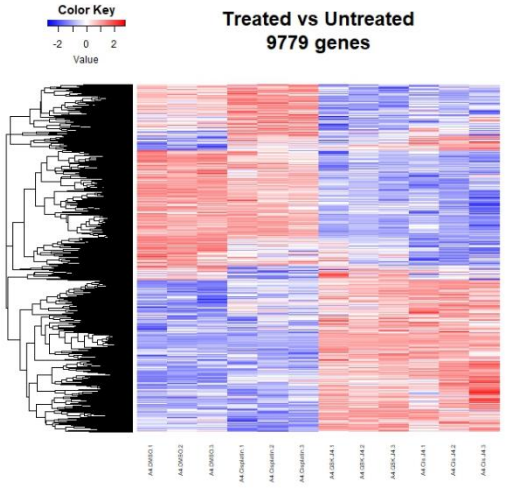


Figure 9. Cluster analysis of NT2/D1-A4

6. Perform H3K27me3-ChIP-seq and EZH2 ChIP-seq on cisplatin sensitive and resistant TGCT cells.

We have optimized H3K27me3 chip-seq and performed analysis of cisplatin sensitive vs resistant TGCT cells and showed that global H3K27me3 is decreased in cisplatin resistant cells (Figure 10). We plan to perform H3K27me3 ChIP-seq on GSK126 and GSKJ4 treated cells in year 3.

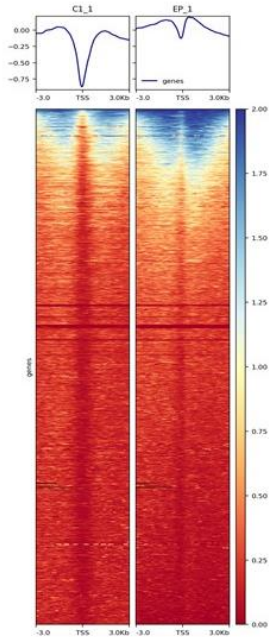


Figure 10. H3K27me-3 ChIP-seq of 2102EP and 2102EP-C1 cells.

7. Perform integrative analysis of RNA-seq and H3K27me3 ChIP-seq in cisplatin sensitive/resistant TGCT cells.

We have begun to corelate changes in global gene expression between cisplatin sensitive and resistant cells and changes in global H3K27me3 (Figure 11). These studies are ongoing. We have not yet used the NIH Roadmap database to compare our results with other cell types.

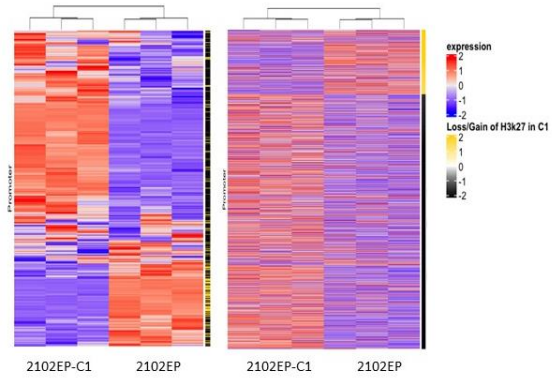


Figure 11. Integrative analysis of RNA-seq and H3K27me-3 ChIP-seq.

8. Validate select polycomb targets that correlate with cisplatin sensitivity.

We have not started these experiments yet. We need to complete the above experiments before deciding on which genes to focus on.

9. Assess chromatin accessibility and perform p53 ChIP-seq in cisplatin sensitive and resistant TGCT cells

We have optimized and performed p53 ChIP seq on cisplatin sensitive and resistant TGCT cells plus and minus cisplatin and are currently analyzing this data. We plan to do the ATAC-seq experiments in year 3.

10. Assessment of acute transcriptional response to cisplatin in cisplatin resistant and polycomb targeted cells.

We have performed these RNA-seq experiments and are currently analyzing the results.

11. Assessment of cisplatin-mediated DNA adducts, DNA damage and repair in cisplatin resistant and polycomb targeted cells.

We have not yet begun the DNA damage experiments but plan to do these in year 3.

Reportable Outcomes

We have applied for an R01 (earliest projected start date April 2024) with the data generated so far, in order to continue the project.

Conclusions

We have made excellent progress on most of the milestones and have generated genetically altered polycomb pathway TGCT cell lines and have tested cisplatin sensitivity in TGCT cells in which the polycomb pathway has been pharmacologically and genetically modified. We have also performed mouse xenograft studies with pharmacological modulation of the polycomb pathway. We have not encountered any insurmountable issues and are on track to complete all the tasks of the SOW. Our results strongly support that polycomb may be a valuable and useful therapeutic target to treat cisplatin refractory TGCTs. We have performed genomic studies to get at mechanism in the form of RNA-seq, H3K27me3 ChIP-seq and p53 ChIP-seq experiments. We have optimized and performed the H3K27me3 antibody staining of human TGCT samples. A major focus on year three will be to analyze and integrate all of this data and to submit at least two major papers on our findings. Further, the lab has been working closely with the leadership of the Malignant Germ Cell International Consortium (MaGIC) to conduct a second, large National Clinical Trial Network-wide trial of epidrug and DNA hypomethylating agent, ASTX727, in cisplatin refractory TGCT patients to be started approximately 1 year from now. We envision the GSKJ4 (which would be the second epidrug for TGCT patients) could be tested clinically via this same collaboration. Finally, we have applied this October for a R01 grant using preliminary data described here for continued support of the project. These have been exciting times for the lab, and we are appreciative of the DOD for their support in getting this project off the ground.

References

none

Appendices

none