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TITLE: THERAPEUTIC TARGETING OF CIMP+ COLORECTAL CANCERS

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14. ABSTRACT
Widespread CpG island hypermethylation is seen in 25% of colorectal tumors and establishes a distinct subset of colorectal cancer termed CIMP. It is generally characterized by silencing of tumor suppressor genes and is associated with a poor prognosis. Recent studies implicate both repressive DNA methylation and histone modification marks at the promoter elements of these tumor suppressor genes. In line with this, we discovered a therapeutic restoration of CIMP-specific tumor suppressor *CDKN2A* by inhibiting DNA methyltransferases and histone deacetylases. Unfortunately, we were not able to identify other epigenetic modifiers working synergistically with DNA methyltransferases in silencing CIMP-specific tumor suppressor *CDKN2A*. Furthermore, overexpression of *CDKN2A* in CIMP-positive cells did not have any effect in disease amelioration. As we were not able to meet our proposed objectives, we have proposed changes in the direction of our project by submitting a revised SOW. These changes will be explored in detail in the Year 2 of this project.

15. SUBJECT TERMS

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1. INTRODUCTION:

Widespread CpG island hypermethylation is seen in 25% of colorectal tumors and establishes a distinct subset of colorectal cancer termed CIMP (CpG Island Methylator Phenotype), generally characterized by silencing of tumor suppressor genes and is associated with a poor prognosis. Classically, the silencing of tumor suppressor genes is attributed to the presence of repressive DNA methylation and the absence histone acetylation marks at their promoter elements. In line with this prevailing dogma, our data show restored *CDKN2A* expression, one of the most commonly silenced tumor suppressor genes in CIMP-positive colorectal cancer, upon inhibition of DNA methylation activity. The restoration of CIMP-specific *CDKN2A* was further enhanced when DNA methylation and histone deacetylation activity were simultaneously inhibited. The overarching goal of the research in this first 12-month milestone is to identify novel epigenetic modifiers, other than the classical histone deacetylases, that work synergistically with DNA methylation event in inhibiting CIMP-specific tumor suppressor genes.

2. KEYWORDS:

Colorectal cancer, CIMP, CDKN2A, DNA methylation

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals for this reporting period are outlined in the following table.

Specific Aim 1: Conduct genetic and chemical screens to identify epigenetic regulators of CIMP-specific tumor suppressor genes	**Timeline**	**Notes**
Major Task 1: to determine the effects of targeting DNA methyltransferase in RKO-knock-in reporter cell lines	Months	
Subtask 1: construction of sgRNA library for epigenetic regulator	1-3	Completed
Subtask 2: generation of stable RKO-*CDKN2A*-GFP-knock-in reporter cell line expressing cas9	1-3	Completed
Subtask 3: transfection and characterization of CRISPR variants by measuring restored *CDKN2A*-GFP signal using FACS	3-6	Completed
Subtask 4: Initiate local animal ethics (IRB/IACUC approved protocol) and human ethics (HRPO/ACURO approved protocol) applications	1-8	Completed
Achieved milestone 1a complete construction and validation of CRISPR variants	**6**	**Completed**
Major Task 2: to determine the effects of co-targeting DNA methyltransferase and other epigenetic regulators in RKO-*CDKN2A*-GFP-knock-in reporter cell lines		
Subtask 1: drug treatment with epigenetics compound library on constructed CRISPR variants	6-9	Completed
Subtask 2: cell viability measurement on treated cells	6-9	Completed
Subtask 3: qRT-PCR and western blotting on treated cells for restored synergistic *CDKN2A* expression after co-targeting DNA methyltransferase and other epigenetic regulator	9-12	50%
Achieved milestone 2a: obtain Local IRB/IACUC approval and HRPO/ACURO approval	**8**	**Completed**
Achieved milestone 2b: complete combination drug treatment and validation	**9**	**Completed**
Milestone 2c: complete gene expression analyses of restored tumor suppressor genes in vitro	**12**	**50%**
Major Task 3: to examine the effects of restored tumor suppressor genes on apoptosis, proliferation and invasion		

Subtask 1: Annexin V/PI staining for flowcytometric-based apoptosis assay on CRISPR variants with restored tumor suppressor gene expression	9-12	0%
Subtask 2: Alamar Blue cell proliferation assay	9-12	Completed
Milestone 3a: complete phenotypic analyses and validation of restored tumor suppressor genes expression	**12**	**50%**

What was accomplished under these goals?

Widespread DNA CpG island hypermethylation is a characteristic of CIMP-positive (CIMP+) colorectal cancer, which results in the silencing of a number of classic tumor suppressor genes including *CDKN2A*, *MLH1*, *MGMT* and *TIMP3*. This has been demonstrated by the presence of both methylated DNA and the absence of acetylated histone marks at the promoter region of those tumor suppressor genes. For our proposed project, we hypothesized that epigenetic regulators, other than the classical histone deacetylase, could work synergistically with DNA methylation in inhibiting the activity of tumor suppressor genes in CIMP+ colorectal cancer. We have previously generated several *CDKN2A*-GFP-knock-in reporter cell lines, where a T2A-GFP cassette had been inserted in frame with the last exon of *CDKN2A* (Fig. 1A top panel). The constructed reporter cells were single cell cloned and treated with 5-Azacytidine, an inhibitor for DNA methyltransferase activity, for 7 days. When RNA was extracted from the treated cells after the course of drug treatment and qRT-PCR was performed, we observed re-activation of both *p14* and *p16* expression (gene products of *CDKN2A* gene) (Fig. 1A bottom panel) indicating an active epigenetic silencing mechanism on its promoter region through DNA methylation mechanism. The restoration of the *CDKN2A* gene products also correlated with the eGFP expression, demonstrating the functional relevance of our reporter cell line.

To identify epigenetic regulators that could potentially inhibit CIMP-specific tumor suppressor genes, we employed a pooled CRISPR-based-loss-of-function (Clustered Regularly Interspaced Short Palindromic Repeats) screen method. We developed our own sgRNA library using lentiviral system by designing sgRNAs targeting 779 epigenetic regulators, which we called **EpiCK** (**Epi**genetic **C**RISPR **K**nock-Out) library. We also designed sgRNAs targeting well-known oncogenes (93 genes), tumor suppressor genes (45 genes), genes regulating general transcriptional and translational machinery (333) and 99 non-targeting control (Fig. 1B). Due to our smaller EpiCK library size compared to the commercially available genome-wide sgRNA library e.g. GecKO and Brunello library, we designed more sgRNA copies per gene (8 – 13 sgRNAs/gene) to improve the specificity of our library. Introduction of EpiCK library and cas9-endonuclease was performed in a 2-step process. We used lentivirus to generate RKO-*CDKN2A*-GFP reporter cells that stably expresses cas9 and subsequently transduced the cells with EpiCK library. Infected cas9-expressing RKO-*CDKN2A*-GFP cells were selected with puromycin 24 hour after the EpiCK library transduction and cell viability was measured with crystal violet 7 days after transduction (Fig. 1B). Notably, we observed a decreasing cell viability consistent with the decreasing concentration of lentiviral particles.

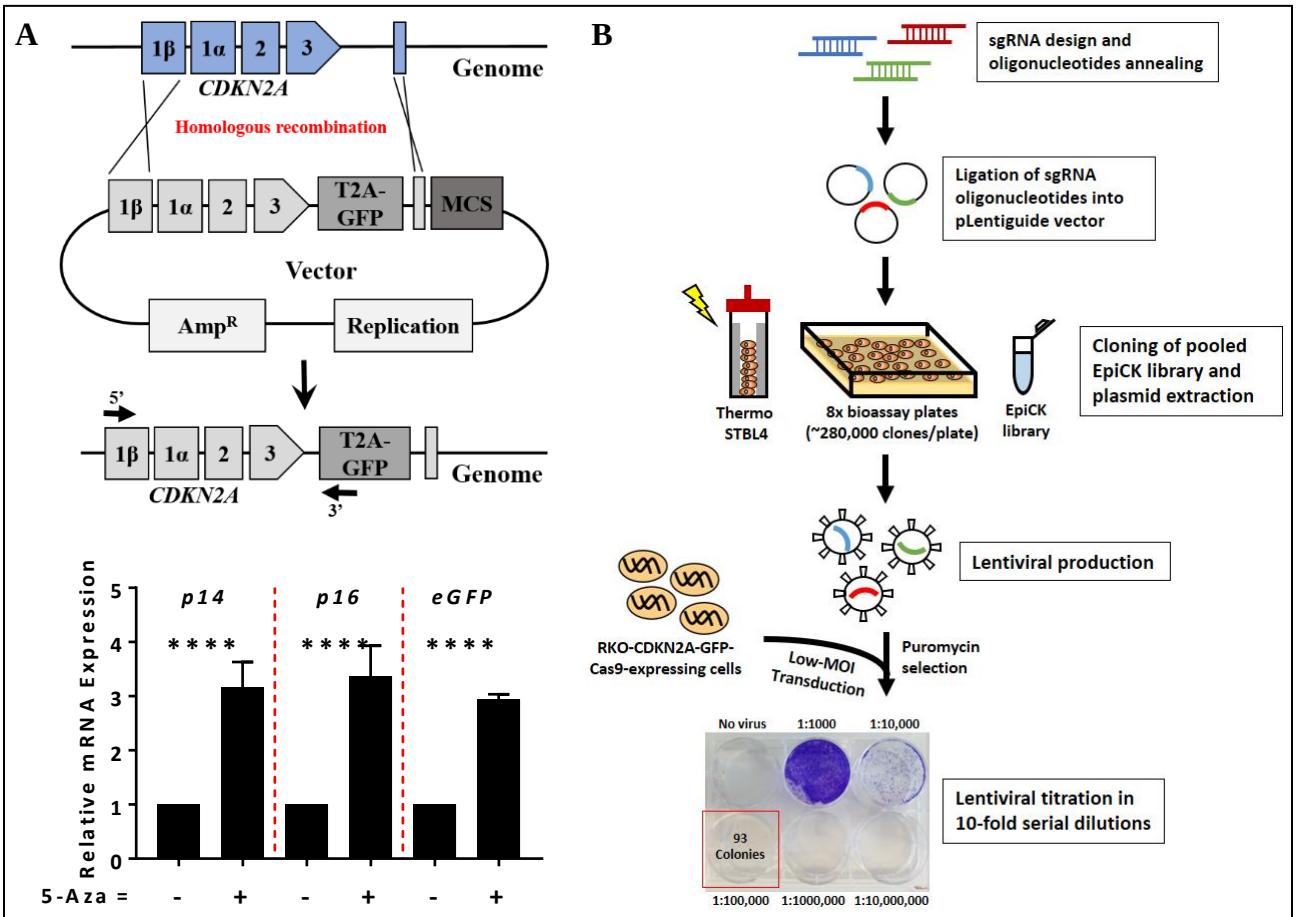


Fig. 1. Generation of Cas9-expressing cells and pooled EpiCK library. (*A, top panel*) Schematic diagram of *CDKN2A*-GFP-knock-in expression vector with primer sequences. **(*A, bottom panel*)** qRT-PCR analysis of the indicated genes in RKO-*CDKN2A*-GFP treated with or without 5-Azac treatment. Relative gene expression normalized to untreated cells are shown. Two-tail student T-test was used for statistical analysis. All data represent mean ± SD; ****$P \leq 0.0001$. **(*B*)** Schematic diagram illustrating the generation of EpiCK sgRNA library in a lentiviral system, and successful transduction into RKO-*CDKN2A*-GFP-cas9 expressing cells as shown by lentiviral titration. 5-Aza treatment was performed over a duration of 7 days with daily drug refreshment at concentration 100nM.

Previous studies have documented the synergistic restoration of CIMP-specific tumor suppressor genes by inhibiting the activity of both DNA methyltransferases and histone deacetylases. Our proposed project seeks to uncover other histone modifiers that could synergistically work together with DNA methyltransferases in silencing CIMP-specific tumor suppressor genes. To achieve this, we transduced cas9-expressing RKO-*CDKN2A*-GFP cells with EpiCK library in the presence or absence of 5-Azacytidine treatment followed by flow-cytometric analysis for GFP signal as a read-out for re-activation of *CDKN2A* gene. As 5-Azacytidine treatment resulted in the re-activation of GFP expression in a dose dependent manner (Fig. 2A), transduction with EpiCK library would, in theory, further enhanced GFP expression and those GFP-positive cells would be isolated subsequently to identify the candidate epigenetic regulators. However, we did not observe further enhancement of GFP expression in EpiCK transduced cells treated with 5-Azacytidine treatment (Fig. 2B), preventing the isolation of transduced cells for further characterization.

Whilst the pooled CRISPR screen approach failed to identify potential epigenetic regulators working synergistically with DNA methyltransferase in silencing *CDKN2A* expression, we sought to utilize an epigenetic compound library as an alternative approach. RKO-*CDKN2A*-GFP cells were pre-treated with 5-Azacytidine followed by a drug screening using an epigenetic compound library containing 151 inhibitors.

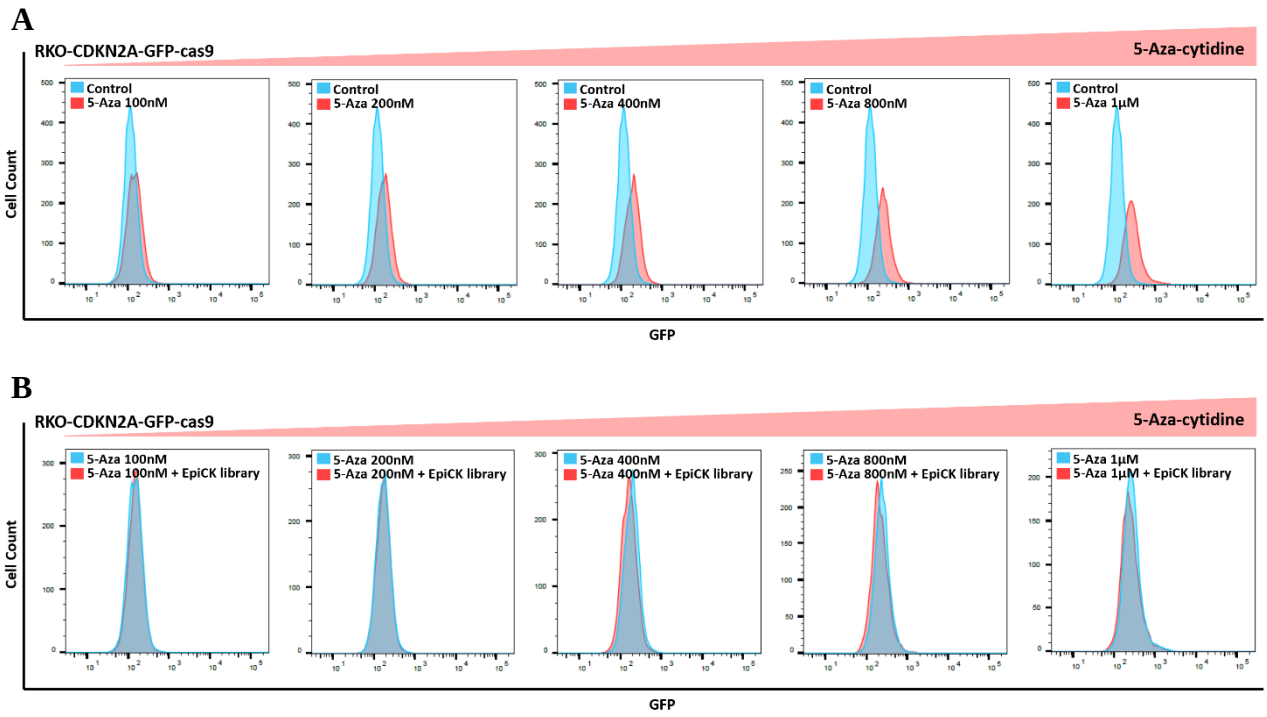
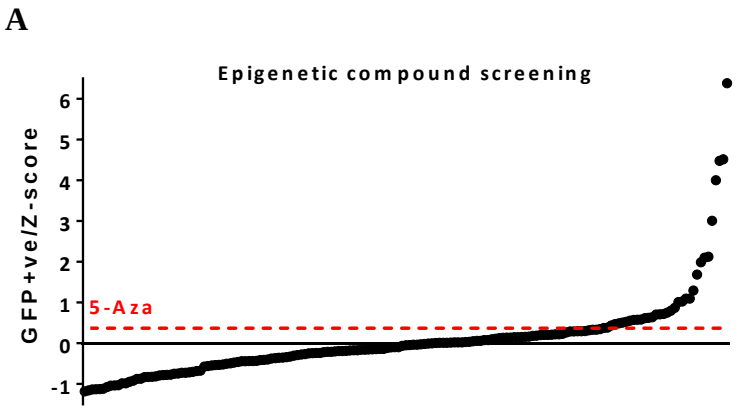


Fig. 2. Flow cytometric analysis for restoration of CDKN2A-GFP expression. (*A*) cas9-expressing RKO-CDKN2A-GFP cells were treated with 5-Aza with increasing concentration showed enhanced GFP signal indicating restoration of *CDKN2A* expression in a dose dependent manner. **(*B*)** 5-Aza treated cas9-expressing RKO-CDKN2A-GFP cells were transduced with EpiCK library did not show further enhancement of GFP signal compared to their respective un-transduced cells. 5-Aza-cytidine treatment was performed over a duration of 7 days with daily drug refreshment.

Flow-cytometric analysis for GFP-positive cells were carried out for each combination treatment and normalized to its respective Z-score for cell viability (Fig. 3A). Our analysis of the drug screen identified 5 classes of inhibitor (Fig. 3B) that re-activated *CDKN2A*-GFP signal using the criteria that at least 1 inhibitor must have GFP-positive/Z-score of more than that of 5-Azacytidine single treatment, which was denoted as dotted red lines in Figure 3A. Among these targets were known inhibitors such as HDAC inhibitors, which was consistent with previously documented studies.

In order to validate the inhibitor hits from the drug screening, we correlated the restoration *CDKN2A* expression for each classes of inhibitors. We pre-treated RKO-*CDKN2A*-GFP cells with 5-Azacytidine followed by combination treatment with one commonly used inhibitor from each classes of inhibitors. RNA was extracted from the treated cells after 7 day of drug treatment and qRT-PCR was performed to assess for the expression of *p14*, *p16* and e*GFP*. Strikingly, only HDAC inhibitor (Romidepsin) was able to enhance the expression of *p14*, *p16* and e*GFP* compared to a single treatment with 5-Azacytidine and not for the rest of the inhibitors (Fig. 3C).



B

Class	Inhibitor	Target
1	Tofacitinib AZD1480 Filgotinib AT9283 Ruxolitinib Fedratinib	JAK signaling
2	Veliparib Talazoparib Olaparib UPF1069 AZD2461	PARP signaling
3	Romidepsin Dacinostat Quisinostat Panobinostat CUDC-907	Histone deacetylases
4	I-BET-762 JQ1 MS436 CPI-203	BET (Bromodomain and Extra Terminal domain) family
5	Hesperadin Tozasertib AMG-900 MK-5108	Aurora kinases

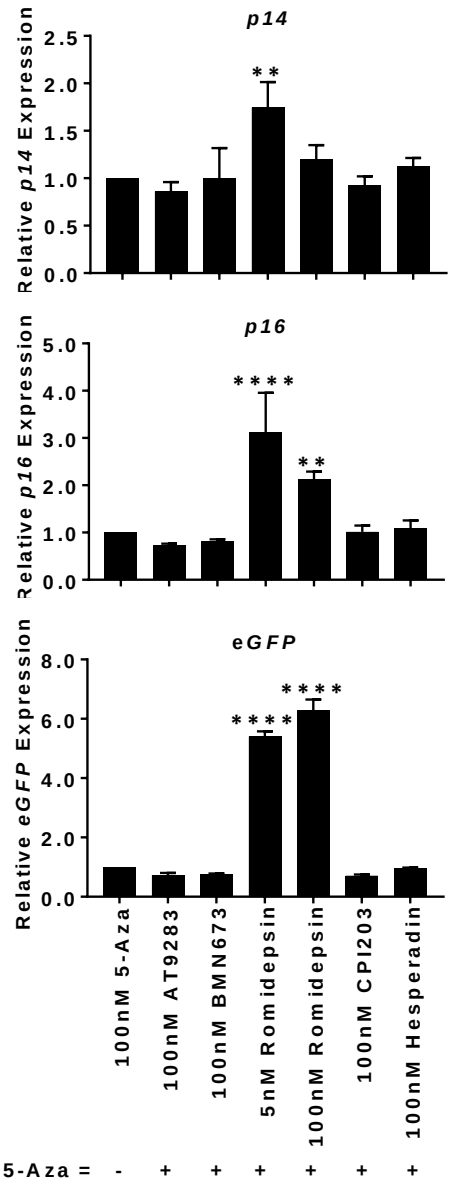


Fig. 3. Epigenetics drug screening identifies 5 classes of inhibitor for re-activation of CDKN2A-GFP expression. **(*A*)** RKO-CDKN2A-GFP cells were pre-treated with 100nM 5-Aza followed by 100nM epigenetics drug screening. Distribution curve shows GFP-positive cells normalized with its respective Z-scores for cell viability for all inhibitors in the drug library. Dotted red lines (GFP-positive/Z-score = 0.4) indicated the cutoffs used for determining whether an inhibitor scored as a hit. (*n = 2*). **(*B*)** Table outlining the inhibitors identified from the drug screening and their target. **(*C*)** qRT-PCR analysis of the indicated genes in RKO-CDKN2A-GFP treated with single arm or combination 5-Aza with indicated inhibitors. Relative gene expression normalized to single arm 5-Aza treatment are shown. Two-tail student T-test was used for statistical analysis. All data represent mean ± SD; $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$.

The hypothesis of our proposed project centered on the presence of two different events of epigenetics aberration underlying inactivation of CIMP-specific tumor suppressor genes. The prevailing dogma for the inactivation of CIMP-specific tumor suppressor genes is that it involves dysregulated DNA methyltransferases and histone deacetylases, which is consistently observed in our proposed project

where combination treatment of DNA methyltransferase inhibitor (5-Azacytidine) and histone deacetylase inhibitor (Romidepsin) restored CIMP-specific *CDKN2A*-GFP. Furthermore, we also proposed to identify other candidate of dysregulated epigenetics regulators that work synergistically with DNA methyltransferases in inactivating CIMP-specific tumor suppressor genes, which is unmet based on our pooled CRISPR screen and drug screening method.

This prompted us to question whether the restored CIMP-specific tumor suppressor genes will still have a critical function in attenuating the disease progression. To address this question, we over-expressed *p16*, a CDKN2A gene product, in RKO cell using a doxycycline-inducible system. We did not use the constitutive overexpression construct as it might have a deleterious effect in the cell. After successful generation of RKO overexpressing inducible *p16*, RKO-*p16*-tet, we treated the cells with 1 µg of doxycycline for 24 hours and extracted RNA to assess for the expression of *p16*. We observed 400-fold of *p16* expression in RKO-*p16*-tet cells compared to its respective empty vector cells (Fig. 4A). In contrast, we did not observe any *p14* expression in these cells demonstrating the specificity of our inducible system. We further investigated the phenotypic effect of restored *p16* expression by measuring cell proliferation using Alamar Blue assay. Surprisingly, there was no changes on cell proliferation (Fig. 4B) despite significant overexpression of *p16* exceeding the levels seen in the combination treatment of 5-Aza and HDAC inhibitors i.e. 400-fold in inducible overexpression system and 3-fold in combination treatment.

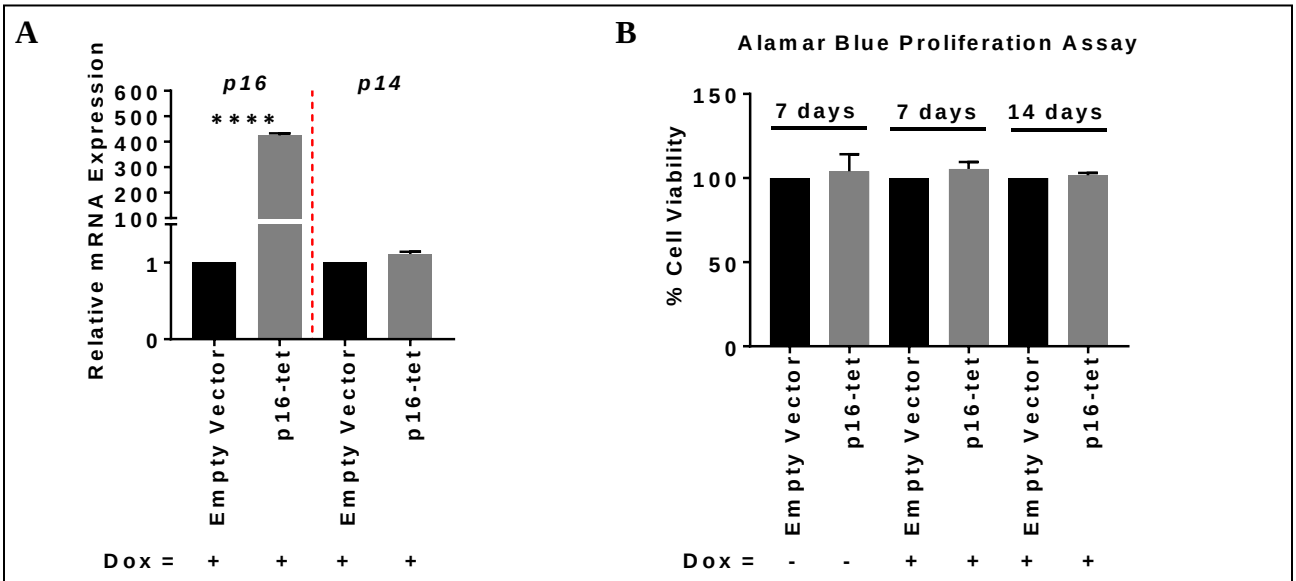


Fig. 4. Overexpression of *p16* does not have any effect in proliferating capacity. (*A*) qRT-PCR analysis of the indicated genes in RKO overexpressed with inducible p16 construct or empty vector treated with 1 µg Doxycycline for 24 hours. Relative gene expression normalized to empty vector-expressing RKO cells are shown. Two-tail student T-test was used for statistical analysis. All data represent mean ± SD; ****$P \leq 0.0001$. **(*B*)** Proliferating capacity for indicated cells are performed using Alamar Blue assay.

Taken together, our data suggest that restoration of CIMP-specific tumor suppressor *CDKN2A* gene is not sufficient to inhibit proliferating capacity of CIMP-positive cells. Although we observed reduced cell numbers in the combination treatment of 5-Azacytidine and HDAC inhibitors through flowcytometric analysis, the reduction in cell numbers could be the reflection of the inhibitors

targeting other biological pathways. Furthermore, CIMP-specific tumor suppressor genes are inactivated at early onset of disease and CIMP-positive cells will undergo further accumulation of genetic mutations. It is possible that CIMP-positive cells have been de-sensitized toward tumor suppressor inactivation that restoring this specific pathway is no longer critical in disease amelioration. In addition, we were not able to identify any epigenetics regulators working synergistically with DNA methyltransferases, other than histone deacetylases, in inactivating CIMP-specific tumor suppressor *CDKN2A* gene indicating that mode of mechanism is exclusively via aberration of DNA methyltransferases and histone deacetylases.

We did not complete 2 tasks outlined in our original SOW;
1) Major task 2 – subtask 3: western blotting on treated cells for restored synergistic *CDKN2A* expression after co-targeting DNA methyltransferase and other epigenetic regulator
2) Major task 3 – subtask 1: Annexin V/PI staining for flowcytometric-based apoptosis assay on CRISPR variants with restored tumor suppressor gene expression.

These tasks were proposed to be completed by the 12th month prior to annual report. At the time of our annual report preparation, it was clear to us that our original hypothesis would not be met. Our qRT-PCR analysis showed a significant restoration of *CDKN2A* (Fig. 3C and 4A), however this restoration had no effect in the proliferating capacity of CIMP-positive cells (Fig. 4B). Performing western blot and Annexin V staining would be redundant as our data contradicted our proposed hypothesis. Hence, we did not procure p16 and p14 antibodies required for western blotting and Annexin V FITC antibody required for flowcytometric assay.

What opportunities for training and professional development has the project provided?

Mentoring:
As outlined in my original proposal, I have established a one-on-one mentoring with a senior scientist, A/Prof. Ron Firestein. I have continued to meet with mentor Firestein on weekly basis, who advises on scientific direction, problem-solving and budget. He also plays a crucial role in my professional development. He has created many opportunities for me to engage with other collaborators to support my project. Mentor Firestein not only broadens my technical skills but also my interpersonal skills, both attributes are crucial for maturation into an independent researcher.

How were the results disseminated to communities of interest?

Nothing to Report

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

The finding generated in this reporting period show definitively that the co-targeting DNA methyltransferases and histone deacetylases is able to re-active CIMP-specific tumor suppressor *CDKN2A* expression. Although the genetic restoration (inducible *p16*-tet overexpression system), which is more specific in studying the gene function than the therapeutic restoration (combination treatment of 5-Azacytdine and Romidepsin), shows no effect on the proliferating CIMP-positive cells, but it provides an evidence that restoration of CIMP-specific suppressor genes is not critical in disease amelioration. Despite unable to meet the objective of our proposed hypothesis, we have developed a pooled CRISPR library targeting epigenetic regulators that has a significant impact in studying gene function in a high-throughput manner.

What was the impact on other disciplines?

The innovation i.e. EpiCK library developed from this project have implications for more targeted functional genomics study that aims to dissect epigenetic regulation. EpiCK library is likely to have broad utility in other biological discipline such as stem cell biology and cellular development, where epigenetics events play crucial roles.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Taken together, our findings have proven that restoration of CIMP-specific tumor suppressor has no effect in disease amelioration, which contradicted our proposed hypothesis. In addition to our endogenous *CDKN2A*-GFP reporter cells, we had also made endogenous c-*MYC*-GFP reporter cells. CIMP-positive cells are not only characterized by global hypermethylation of CpG island but also increased proliferating capacity. One of the well-known oncogenic drivers in aberrant proliferation pathway is c-*MYC* hence, the basis for the generation of endogenous c-*MYC*-GFP reporter cells. We included both *CDKN2A*-GFP and c-*MYC*-GFP reporter cells during initial pooled CRISPR screening using our EpiCK library. Although we were not able to identify any candidates in *CDKN2A*-GFP reporter cells, surprisingly we identified interesting epigenetic candidates in c-*MYC*-GFP reporter cells, both previously reported candidates and a novel candidate, *KMT2A* gene.

KMT2A gene (protein name = Mll1) is a well-known histone modifier associated with gene activation. It often undergoes chromosomal rearrangement resulting in a fusion oncogene in the context of blood cancer. In contrast, dysregulated full-length Mll1 has not been documented in the context of colorectal cancer. Our preliminary data showed that both genetic (CRISPR knock-out) and therapeutic (inhibitors targeting Mll1 currently in clinical trials) perturbation had an effect in disease amelioration. Furthermore, selective inhibition of its methyltransferases activity has no effect in disease progression, suggesting a novel function of Mll1 beyond its canonical role as an epigenetic regulator in the context of CIMP-positive cells. We are preparing a revised SOW at this reporting period to undertake a major change in our initial research approach.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Nothing to Report

- **Website(s) or other Internet site(s)**

  Nothing to Report

- **Technologies or techniques**

  Nothing to Report

- **Inventions, patent applications, and/or licenses**

  Nothing to Report

- **Other Products**

  We have developed our own sgRNA library targeting 779 epigenetic regulators, 93 oncogenes, 45 tumor suppressor genes and 333 genes regulating transcriptional and translational machinery which we called EpiCK library.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report

QUAD CHARTS:

Nothing to Report

9. APPENDICES:

Nothing to Report