AWARD NUMBER: W81XWH-19-1-0569

TITLE: Multiplex Functional Interrogation of Oncogenic Networks in Bladder Cancer

PRINCIPAL INVESTIGATOR: John K. Lee, M.D., Ph.D.

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center

REPORT DATE: AUGUST 2021

TYPE OF REPORT: Annual Technical Report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
AUGUST 2021	Annual Technical Report	1AUG2020 - 31JUL2021

4. TITLE AND SUBTITLE

Multiplex Functional Interrogation of Oncogenic Networks in Bladder Cancer

5a. CONTRACT NUMBER
W81XWH-19-1-0569

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John K. Lee, M.D., Ph.D.

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Fred Hutchinson Cancer Research Center

1100 Fairview Ave N, J6-300
Seattle, WA 98109-4433

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this reporting period, additional tumors were generated using the mouse urothelial organoid transformation assay using barcoded lentiviral pools. In this system, single cell DNA amplicon sequencing resolves clonal architecture in the tumors but does not allow *in situ* association with cancer histologies. A workflow of laser capture microdissection of stained tissue sections and DNA amplicon sequencing was established to overcome this issue. Tumor models of histologic variants of bladder cancer were analyzed and lentiviral barcodes enumerated to identify the putative genetic alterations associated with each. Validation studies to re-generate these tumors and leave-one-out analyses to examine the role of each genetic alteration in cancer initiation/phenotype are ongoing. Lastly, lentiviral transduction of mouse urothelial cells in organoid culture was found to follow a (non-skewed) binomial distribution which supports the potential for co-occurrence and mutual exclusivity analyses.

15. SUBJECT TERMS
Bladder cancer, urothelial carcinoma, tumor initiation, functional genomics

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

INTRODUCTION

Background: The declining cost of high-throughput sequencing has prompted a flood of cancer genome sequencing data. This information has provided substantial insights into the intrinsic genetic and molecular complexity of bladder cancer. Unfortunately, the functional consequences of most of these genetic abnormalities in bladder cancer have not been explored. Current technologies to define genetic function in relevant tumor models are too slow and costly to effectively confront the complexity of this problem. The objective of these studies is to develop a rapid functional genomics approach using a mouse bladder urothelial organoid transformation system to enable the multiplex interrogation of genetic aberrations and their contributions to bladder cancer subtypes.

KEYWORDS

Bladder cancer, urothelial carcinoma, tumor initiation, functional genomics

ACCOMPLISHMENTS

To summarize the research accomplishments to date, the tasks described in the proposed Statement of Work are itemized here with a brief update for each task.

SA 1: Nominate groups of genetic aberrations that initiate bladder cancer from the pooled, multiplex lentiviral transduction of mouse urothelial organoids.

Task 1: Obtain regulatory approval for studies (months -5-3) Completed during prior reporting period.

Task 2: Mouse urothelial organoid transformation using pooled and barcoded lentiviruses (months 1-10) Completed during prior reporting period.

Task 3: Lentiviral barcode deconvolution by bulk DNA amplicon sequencing of the engineered urothelial tumors (months 11-14) Completed. We previously highlighted a tumor generated from the mouse urothelial organoid transformation assay using pooled and barcoded lentiviruses that simultaneously exhibited three clinically relevant histologies consistent with a) urothelial carcinoma with squamous differentiation, b) papillary urothelial carcinoma with inverted growth pattern, and c) sarcomatoid carcinoma (Fig. 1A). We performed single cell DNA amplification and library preparation using the Mission Bio Tapestri and next-generation sequencing (NGS) which revealed three dominant clonal populations that shared multiple genetic perturbations (Ywhaz, Pik3ca E545K, Pparg, and Pvrl4) but also had unique genetic perturbations that could differentiate the populations (Fgfr3 S243C, shAtm, Zfp703, Mdm2, shEp300, shCdkn2a, and Erbb2 S311Y) (Fig. 1B).

A question we could not immediately address from this data was how the clonal architecture relates to histology. To address this, we performed laser capture microdissection of each histologically distinct region, DNA isolation, two-step PCR for amplification and to append NGS adapters, and NGS (Fig. 1C). This analysis allowed us to then associate lentiviral barcodes present in regional histologies with the single cell data (Fig. 1D). We were reassured to identify that the Fgfr3 S243C mutation specifically associated with the papillary urothelial carcinoma as this alteration has been shown to be enriched in luminal papillary urothelial carcinomas (*1, 2*).

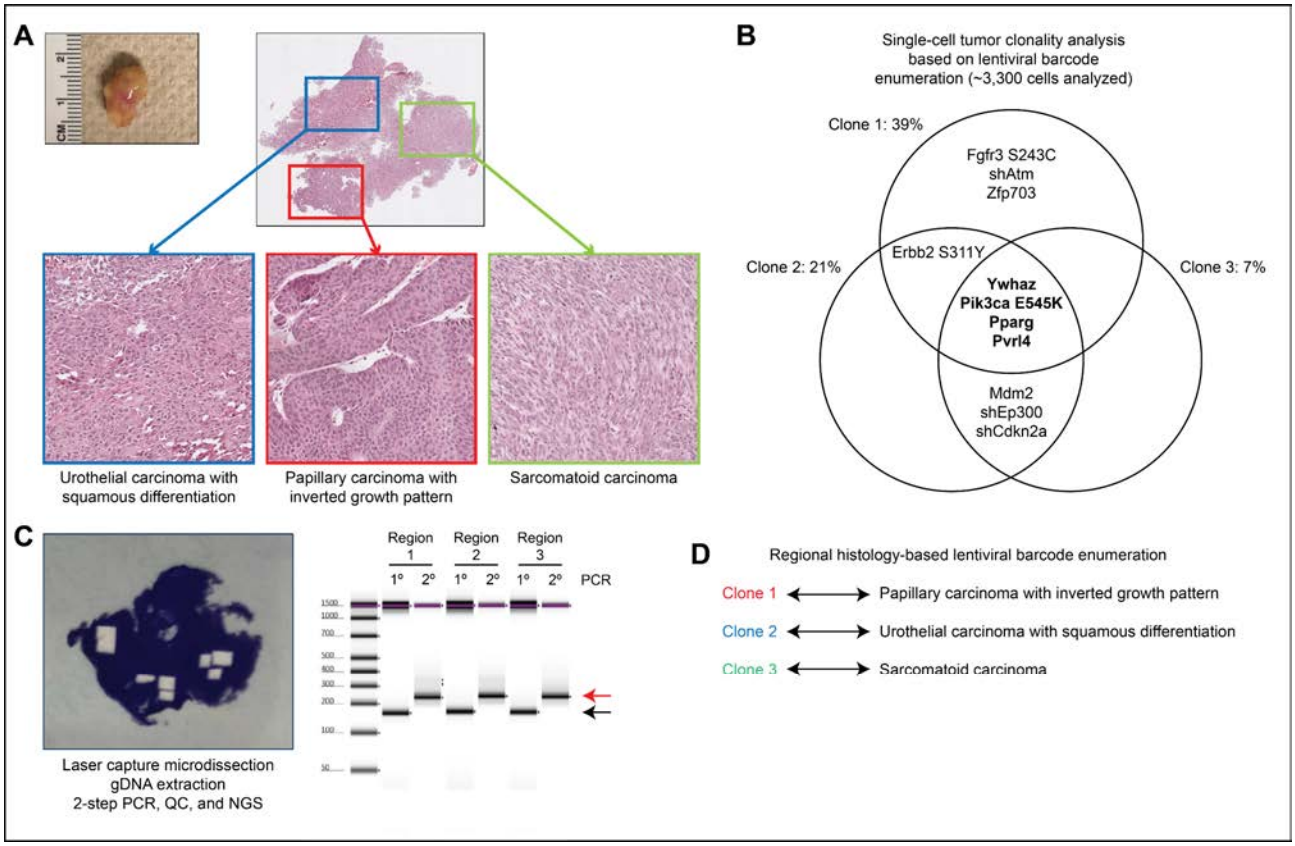

Fig. 1. Generation of genetically and phenotypically heterogeneous mouse bladder cancer models. (**A**) Photomicrographs of a gross tumor generated from the mouse urothelial transformation assay using the barcoded lentiviral library and H&E-stained sections of the tumor showing three distinct histologies.(**B**) Clonal architecture of the tumor determined by Tapestri analysis with depiction of the genetic events associated with the three dominant clones. (**C**) *Left*, tumor tissue section after laser capture microdissection (LCM) of the histologically distinct regions. *Right*, Tapestation results of 1º and 2º PCR (to append NGS adapters) from each region. (**D**) Association of histologies to clones in **B** based on LCM and bulk DNA amplicon sequencing.

Prior to encountering this problem, we admittedly did not foresee the need to resolve clonal architecture with respect to architecture. In future studies (beyond the scope of this project), we may consider instituting 1) Tapestri single cell DNA/protein multi-omics (currently available) or 2) future Tapestri single cell DNA/RNA multi-omics (currently under development). The use of a panel of antibodies targeting cell surface proteins (akin to Cellular Indexing of Transcriptomes and Epitopes by Sequencing or CITE-seq) or marker genes that could accurately distinguish subtypes of bladder cancer may provide additional indexed single cell data that could be

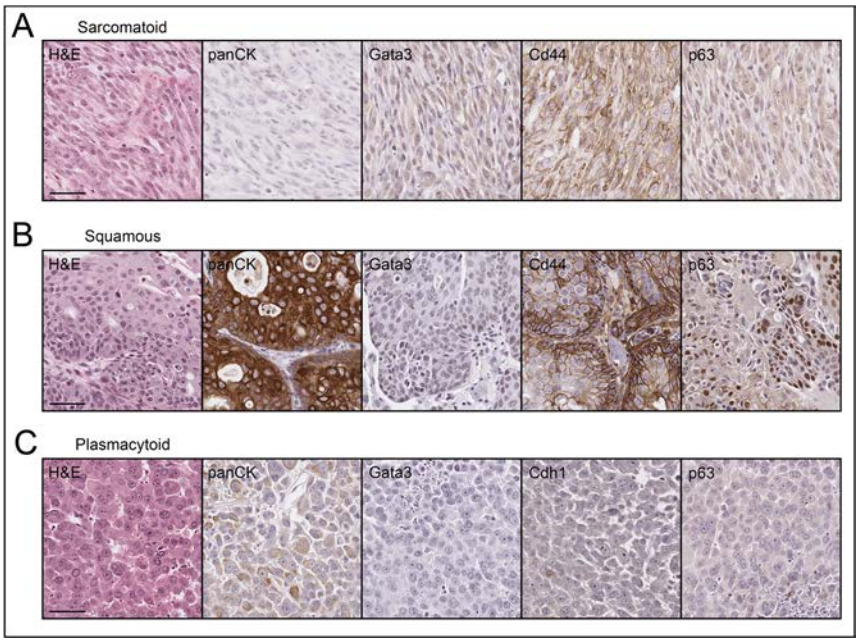

Fig. 2. Phenotypic characterization of histologic variants of bladder cancer arising from the experimental system. Photomicrographs of H&E- and IHC-stained tumor tissue sections are shown from (**A**) sarcomatoid carcinoma, (**B**) squamous cell carcinoma, and (**C**) plasmacytoid urothelial carcinoma (PUC) which were generated from the mouse urothelial transformation assay using the barcoded lentiviral library. Scale bar = 50 μm.

readily associated with histologic phenotype.

We have also generated other tumor models with histologies consistent with squamous cell carcinoma (SCC), plasmacytoid urothelial carcinoma (PUC), and sarcomatoid carcinoma (SC) (Fig. 2). In addition to morphology, these models retain phenotypic features such as p63 expression in SCC, loss of Cdh1 expression in PUC (*3*), and SC with focal expression of cytokeratins (*4*) (Fig. 2). We have performed single cell DNA amplicon sequencing to determine the gene alterations associated with these tumors (Table 1). These represent first-in-field genetically defined models that will be valuable for the field as histological variants of bladder cancer lack preclinical models and demonstrate aggressive clinical behaviors with resistance to standard therapies.

Plasmacytoid urothelial carcinoma	Squamous cell carcinoma	Sarcomatoid carcinoma
Pik3ca E545K	Pik3ca E545K	Pik3ca E545K
Erbb2 S311Y	Ywhaz	Erbb2 S311Y
Myc	Pvrl4	Mdm2
shATM	Pparg	Ywhaz
shKMT2A		Pvrl4
shKMT2C		Pparg
		shCdkn2a
		shEp300

Table 1. Gene alterations linked to lentiviral barcodes associated with each histologic variant bladder cancer model.

Task 4: *Classify the most common sets of genetic aberrations detected from single cell DNA barcode amplicon sequencing (months 6-18)* Completed. We have categorized the most common and least common genetic aberrations identified from a total of 31,818 cells across 10 tumors generated using the methodology (Table 2). We identified *Myc*, *Pik3ca* E545K, and *Ccnd1* as the most common genetic alteration enriched in the tumors.

Interestingly, we have not seen significant overrepresentation of the dominant-negative *Tp53* R245Q lentivirus despite common inactivation and loss of p53 in human bladder cancer. We do see the *Mdm2* lentivirus (Table 1, sarcomatoid carcinoma), and Mdm2 suppresses p53 activity. We have previously characterized the representation of our lentiviral pools based on lentiviral barcode sequencing and demonstrated that the *Tp53* R245Q lentivirus is present and capable of infecting target cells. This has prompted us to validate this construct by sequencing, expression, and functional analysis. Should the construct continue to be problematic, we may design a lentiviral short hairpin RNA (shRNA) construct targeting *Tp53* to replace the *Tp53* R245Q construct.

Gene	Percentage
mMYC	20.40
mPIK3CA(E545K)	15.79
mCCND1	11.50
mERBB3(V104L)	8.18
mERBB2(S311Y)	6.04
mPPARG	4.91
mshATM	3.35
mCCNE1	2.97
mYAP1	2.64
mFGFR3(S243C)	2.44
mEGFR	2.34
mZFP703	2.20
mPVRL4	1.95
mshCDKN2A	1.76
mYWHAZ	1.75
mMDM2	1.71
mshKMT2C	1.65
mE2F3	1.37
mshKMT2A	1.33
mshKDM6A	1.20
mTRP53(R245Q)	1.13
mshPTEN	0.71
mshRB1	0.55
TRC control	0.42
EmptyControl	0.39
mshCREBBPB	0.37
mshEP300	0.23
mshSTAG2	0.19
mshSPTAN1	0.18
mshKMT2D	0.16
mshARID	0.11
mshNCOR1	0.06

Table 2. Gene alterations based on lentiviral barcodes represented in 10 tumors (31,818 total cells) arising from the mouse bladder urothelial transformation assay.

SA 2: Validate and characterize the functional contribution of specific genetic alterations to bladder cancer initiation by leave-one-out analyses.

Task 1: *Functionally validate identified combinations of genetic alterations by mouse urothelial organoid transformation (months 19-25)* In progress. In the prior reporting period, we validated the role of Fgfr3 S243C in driving luminal papillary urothelial carcinoma in combination with Pik3Ca E545K, Pparg, Ywhaz, and Pvrl4.

We have now focused on validating the histologic variant bladder cancer models that we generated based on the identified genetic alterations from lentiviral barcode enumeration. Individual lentiviruses encoding each factor (Table 1) were made, concentrated, and titered. These were introduced in a directed fashion in the mouse urothelial organoid transformation assay, mixed with embryonic bladder mesenchyme (EBLM), and implanted subcutaneously into the flanks of male NSG mice. Multiple replicates of these grafts were established and we are awaiting tumor outgrowth.

Task 2: *Perform leave-one-out analyses and compare the transcriptome profiles of the resultant tumors (months 25-36)* In progress. In conjunction with the studies in SA 2 Task 1, we used the leave-one-out approach to generate a series of grafts in which a single factor was iteratively removed from the set of genetic alterations for the PUC, SCC, and SC models (Table 3 shows an example of the conditions tested for the PUC model). Multiple replicates were established and we are awaiting tumor outgrowth. Once we have collected the tumors, we will assess phenotype by analyzing morphology, immunohistochemistry, and gene expression by RNA-seq.

<u>SA 3:</u> Identify potential cooperative and essential genetic interactions in bladder cancer.

Task 1: Analyze co-occurrence and mutual exclusivity based on the single cell DNA amplicon sequencing of lentiviral barcodes from the engineered urothelial tumors (months 18-20) <u>In progress.</u> A concern that arose about the feasibility of performing co-occurrence and mutual exclusivity analysis was the assumption that lentiviral infection in the context of organoids would follow a normal distribution. We evaluated this question by performing lentiviral transduction of mouse urothelial organoids at a multiplicity-of-infection of ~2 using a lentiviral pool marked by diverse barcodes, collecting organoids after three days, preparing single cell DNA amplicon libraries on the Tapestri, and performing NGS (Fig. 3A). We analyzed the NGS data to identify the viral copy number per cell based on lentiviral barcode enumeration and plotted viral copy number against cell fraction (Fig. 3B). The data revealed a binomial distribution of lentiviral transduction. These data were reassuring as it indicates that the distribution is not skewed.

We have started the co-occurrence and mutual exclusivity analysis using the DISCOVER Python package and data from available tumors that have undergone lentiviral barcode enumeration from single cell DNA amplicon sequencing. We anticipate having mature data within the next few months.

Task 2: Investigate potential genetic interactions in bladder cancer using in vitro and in vivo systems (months 21-27) <u>Not yet started.</u>

Plasmacytoid urothelial carcinoma	LOO 1	LOO 2	LOO 3	LOO 4	LOO 5	LOO 6
Pik3ca E545K	Erbb2 S311Y	Pik3ca E545K	Pik3ca E545K	Pik3ca E545K	Pik3ca E545K	Pik3ca E545K
Erbb2 S311Y	Myc	Myc	Erbb2 S311Y	Erbb2 S311Y	Erbb2 S311Y	Erbb2 S311Y
Myc	shATM	shATM	shATM	Myc	Myc	Myc
shATM	shKMT2A	shKMT2A	shKMT2A	shKMT2A	shATM	shATM
shKMT2A	shKMT2C	shKMT2C	shKMT2C	shKMT2C	shKMT2C	shKMT2A
shKMT2C						

Table 3. Example of leave-one-out (LOO) conditions for the plasmacytoid urothelial carcinoma model.

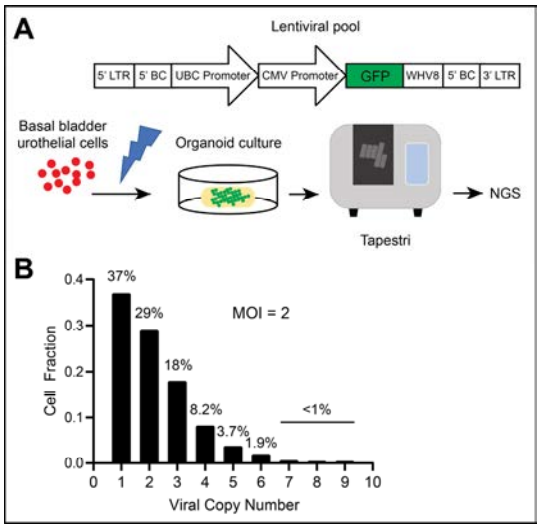

Fig. 3. Lentiviral transduction of mouse bladder urothelial cells in organoid culture follows a binomial distribution. (**A**) Experimental setup showing transduction of bladder urothelial cells with a lentiviral pool in organoid culture, dissociation for single cell DNA amplicon library preparation using the Tapestri, and NGS. (**B**) Distribution of viral copy number across cells from the experiment performed at a multiplicity-of-infection (MOI) of 2.

Key Research Accomplishments:

- Developed a system to implement laser capture microdissection and bulk DNA amplicon sequencing to associate clonal architecture from single cell lentiviral barcode enumeration with histology.
- Established new histologic variant bladder cancer models: squamous cell carcinoma, plasmacytoid urothelial carcinoma, and sarcomatoid carcinoma.
- Demonstrated that lentiviral transduction of mouse urothelial cells in the context of organoids follows a binomial distribution.

Opportunities for Training and Professional Development:

This reporting period included my third year as an independent investigator in the Human Biology Division at Fred Hutch. My laboratory now includes four post-doctoral fellows, two graduate students, two technicians, and

a staff scientist that I oversee and mentor. I meet with my junior faculty mentoring committee consisting of Drs. Pete Nelson, Valeri Vasioukhin, and Bob Eisenman on a yearly basis.

I have advanced my professional development by continuing to engage in multiple groups and meetings including the Bladder Cancer Advocacy Network Think Tank, Society for Basic Urologic Research, and American Association for Cancer Research to expand my peer networks in the field of bladder cancer research. I meet with my clinical and scientific colleagues in the Bladder Cancer Group at Fred Hutch and the University of Washington on a monthly basis to provide updates and receive feedback on the progress of bladder cancer projects in my laboratory. I have established new scientific collaborations with bladder cancer investigators locally and nationally and have started to submit partnering grant applications based on these efforts. I have also met with my co-Career Guides Drs. Eric Holland and Peter Black to provide updates on scientific progress as well as my professional growth.

In the past year, I was selected by the Early Career Reviewer Program from the NIH Center for Scientific Review and served on the Molecular Oncogenesis (MONC) study section in February 2021. I have also been nominated to become a Federal Advisory Committee Act (FACA) member of the Veterans Affairs Oncology A (ONCA) panel. I successfully competed for a Bladder Cancer Advocacy Network (BCAN) 2021 Bladder Cancer Research Innovation Award. I have also been nominated to present research related to this project at the American Urological Association (AUA) Early-Career Investigators Showcase. Lastly, I am a finalist for the prestigious 2021 NIH Director's (DP2) New Innovator Award and have received an unofficial notice of award with the official announcement to be made in October 2021.

Dissemination of Results:

Due to the nature of the COVID-19 pandemic and institutional restrictions on travel, the findings of the ongoing research were presented virtually at 1) the Fred Hutch Genitourinary Oncology Seminar Series on October 1, 2020; 2) a plenary lecture at the 2020 Society of Basic Urologic Research Annual Meeting on November 12, 2020; and 3) the American Society for Clinical Investigation Young Physician-Scientists Award Poster Session on February 2, 2021.

In addition, the results of our studies will be disseminated during a virtual presentation at the American Urological Association Early-Career Investigators Showcase on September 12, 2021.

Plan for the Next Reporting Period:

In the next reporting period, we will continue to analyze tumors arising from leave-one-out analyses to determine how genetic factors contribute to cancer phenotype. We have already stated preparing a technical manuscript describing the methodology of the higher-order combinatorial genetics assay to transform primary epithelial organoids and intend to submit this for peer-review and publication.

IMPACT

We have established a robust functional genomics platform that enables rapid, multiplex interrogation of genetic alterations in the initiation of bladder cancer. We continue to generate new models of bladder cancer including, more recently, histologic variants of bladder cancer that have otherwise been poorly modeled in the past. We will leverage these unique preclinical model systems to delve further into understanding how histologic variants diverge from conventional urothelial carcinoma and evaluate potential therapeutic strategies. In addition, we have started to establish collaborations with other investigators in the bladder cancer field to use and share these models to accelerate discovery and therapeutic translation.

CHANGES/PROBLEMS

The project has been mildly impacted by the COVID-19 pandemic related to physical distancing restrictions at the Fred Hutch which affected the productivity of laboratory members. While the COVID-19 pandemic is still evolving and future closures may be unforeseen, I still believe that we are still on track to complete the proposed milestones.

No changes to the original proposal are indicated.

PRODUCTS

None.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*John K. Lee*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-6570-2180
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Lee has performed data analysis and overseen the conduct of the study.*
Funding Support:	

Name:	*Alicia Wong*
Project Role:	*Research Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Ms. Wong has performed all molecular cloning, generation of lentivirus, isolation of mouse bladder urothelial cells, transformation experiments, and characterization of resultant tumors.*
Funding Support:	

Name:	*Vipul Bhatia*
Project Role:	*Post-doctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Dr. Bhatia performed generation of lentivirus, isolation of mouse bladder urothelial cells, transformation experiments, and characterization of resultant tumors.*
Funding Support:	

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

REFERENCES

1. A. G. Robertson *et al.*, Comprehensive Molecular Characterization of Muscle-Invasive Bladder Cancer. *Cell* **171**, 540-556.e525 (2017).
2. B. D. Robinson *et al.*, Upper tract urothelial carcinoma has a luminal-papillary T-cell depleted contexture and activated FGFR3 signaling. *Nature communications* **10**, 2977 (2019).
3. H. A. Al-Ahmadie *et al.*, Frequent somatic CDH1 loss-of-function mutations in plasmacytoid variant bladder cancer. *Nature genetics* **48**, 356-358 (2016).
4. C. C. Guo *et al.*, Dysregulation of EMT Drives the Progression to Clinically Aggressive Sarcomatoid Bladder Cancer. *Cell reports* **27**, 1781-1793.e1784 (2019).

APPENDICES

None.