AWARD NUMBER: W81XWH-20-1-0083

TITLE: Multiplex functional interrogation of combinations of genetic alterations in prostate cancer

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CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center

REPORT DATE: MAY 2022

TYPE OF REPORT: Final 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

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE
MAY 2022

2. REPORT TYPE
Final Technical Report

3. DATES COVERED
1FEB2020 - 31JAN2022

4. TITLE AND SUBTITLE
Multiplex functional interrogation of combinations of genetic alterations in prostate cancer

5a. CONTRACT NUMBER
W81XWH-20-1-0083

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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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
During the grant period, we have developed a higher-order, combinatorial genetics strategy using lentiviral (LV) libraries of cancer-associated genetic events introduced into benign epithelial cells that are subsequently engrafted in vivo for tumorigenic selection. We have generated several tumors using this approach that demonstrate clinically relevant, mixed cancer histologies. We incorporated massively parallel single-cell LV barcode sequencing to identify genetic alterations that contribute to the diverse phenotypes represented in the tumors. Validation of the findings is currently ongoing. These initial studies provide proof-of-principle of this powerful approach to rapidly investigate genotype-phenotype relationships in cancer initiation starting from primary epithelial cells. Our ultimate goal is to generate genetically—defined models of prostate cancer that recapitulate the diversity of human prostate cancer.

15. SUBJECT TERMS
Prostate cancer, heterogeneity, organoid transformation, single-cell sequencing

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Background: Prostate cancer (PCa) is genetically diverse and composed of multiple histologic and molecular subtypes. Next-generation sequencing of PCa genomes has identified a large number of genetic alterations, but current methodologies to define drivers of cancer in genetically-defined mouse models are slow, costly, and do not allow facile manipulation of more than two or three genes. New approaches in functional cancer genomics are needed to rapidly understand the genetic interactions that initiate and confer heterogenous phenotypes of cancer. The purpose of this research is to develop an innovative and powerful approach to rapidly interrogate the genetic interactions that initiate and confer heterogenous phenotypes using a biologically relevant assay. This platform may not only provide important insights into the biology of PCa, but also lead to the development of diverse genetically-defined models that recapitulate the molecular phenotypes of PCa.

KEYWORDS

Prostate cancer, heterogeneity, organoid transformation, single-cell sequencing

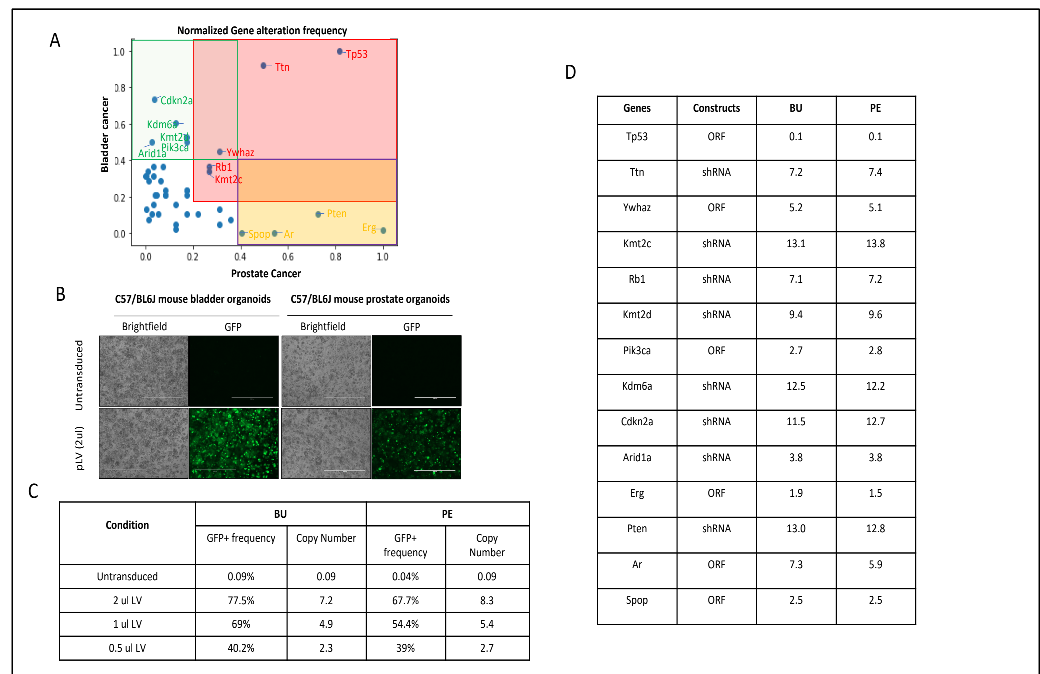
ACCOMPLISHMENTS

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.

<u>Specific Aim 1:</u> Characterize prostate tissue-specific responses to oncogenic factors (months 1-12)

Major Task 1：Generate barcoded lentiviral constructs encoding gain-of-function and loss-of-function genetic alterations associated with PCa and BCa (months 1-3) ***<u>Completed.</u>***

We selected a total of 14 tumor suppressor and oncogenes that are recurrently altered in either prostate cancer or bladder cancer or both and cloned barcoded lentiviral (LV) constructs that express either open reading frames or short hairpin RNAs to mimic gain-of-function or loss-of-function events (**Fig.1A**). We have generated concentrated LV pools and optimized LV production by adjusting the relative ratios of the surface area of producer cells to ensure equivalent representation of each lentivirus. We have demonstrated highly efficiently LV transduction of prostate epithelial (PE) and bladder urothelial (BU) organoids using these concentrated LV pools (**Fig. 1B and 1C**). We observed fairly uniform representation of LV barcodes in mouse PE and BU organoids after transduction as determined by bulk DNA amplicon sequencing (**Fig. 1D**).



Condition	BU		PE	
	GFP+ frequency	Copy Number	GFP+ frequency	Copy Number
Untransduced	0.09%	0.09	0.04%	0.09
2 ul LV	77.5%	7.2	67.7%	8.3
1 ul LV	69%	4.9	54.4%	5.4
0.5 ul LV	40.2%	2.3	39%	2.7

Genes	Constructs	BU	PE
Tp53	ORF	0.1	0.1
Ttn	shRNA	7.2	7.4
Ywhaz	ORF	5.2	5.1
Kmt2c	shRNA	13.1	13.8
Rb1	shRNA	7.1	7.2
Kmt2d	shRNA	9.4	9.6
Pik3ca	ORF	2.7	2.8
Kdm6a	shRNA	12.5	12.2
Cdkn2a	shRNA	11.5	12.7
Arid1a	shRNA	3.8	3.8
Erg	ORF	1.9	1.5
Pten	shRNA	13.0	12.8
Ar	ORF	7.3	5.9
Spop	ORF	2.5	2.5

Fig.1. Characterization of a lentiviral pool recapitulating prostate cancer or bladder cancer-associated genetic alterations in prostate or bladder organoids. (A) Representation of recurrent prostate cancer and bladder cancer genetic alterations. (B) Mouse prostate and bladder organoids at 72 hrs post-transduction with PEBU-lentiviral pool. Scale bar = 1mm. (C) Quantification of the percentage of GFP positive cells as measured by flow-cytometry and GFP copy number by qPCR-based copy number assay in mouse prostate or bladder organoids. (D) Lentiviral representation in the pool by bulk DNA amplicon sequencing.

Major Task 2：Identify tissue type-specific genetic alterations that modulate cellular fitness in the process of mouse prostate epithelial and bladder urothelial organoid transformation (months 1-12) ***<u>In progress, 80% completion.</u>***

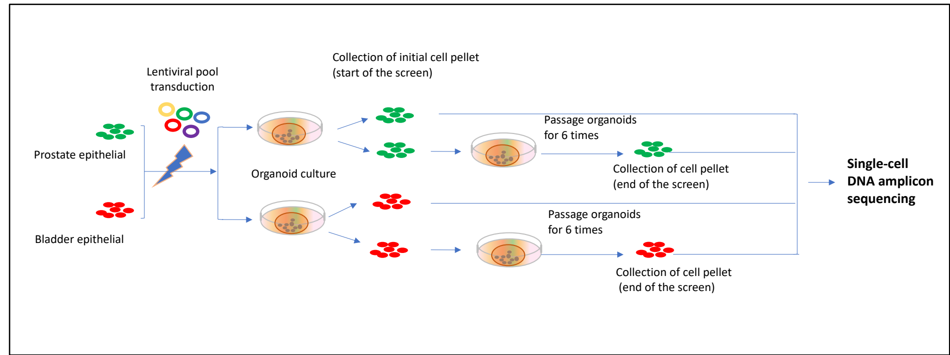


Fig.2. Schematic of a tissue type-specific assay to identify genetic alterations that modulate cell fitness.

We have isolated basal PE and BU cells from male C57Bl/6J mice and transduced cells with the LV pool generated in *Task1.* We performed a pilot tissue type-specific assay as shown in **Fig.2**. We did not observe significant clonal differences in cell fitness after six passages (data not shown). This may be because of insufficient time and cell passages to allow for clonal enrichment. We were planning to repeat the tissue type-specific assay and to collect cells for analysis after ten or more passages. But due to the shortage of Matrigel and Tapestri scDNA sequencing library preparation kit during the pandemic, we postponed the experiments.

<u>Specific Aim 2:</u> Functionally interrogate oncogenic drivers and genetic interactions involved in PCa initiation and progression (months 1-24)

Major Task 1: Generate and characterize a lentiviral pool recapitulating PCa-associated genetic alterations (months 1-6) ***<u>Completed.</u>***

We selected 23 recurrently altered genes found in at least 3% of human PCa and cloned 23 barcoded lentiviral constructs that express either open reading frames or short hairpin RNAs to mimic gain-of-function or loss-of-function events (**Fig. 3A and 3B**). From these constructs, we generated a LV library by pooling the individual LV produced in arrayed format in a fixed surface area, followed by concentration by ultracentrifugation, and titering on basal PE organoids. We isolated basal cells from mouse prostate tissue based on a Lin$^-$ (CD45$^-$CD31$^-$Ter119$^-$) EpCAM$^+$CD49f^{high} immunophenotype by fluorescence activated cell sorting and propagated them in organoid cultures (**Fig. 3C**). We demonstrated the ability to efficiently transduce basal PE cells and propagate them in organoid cultures (**Fig. 3D and 3E**). The representation of individual LVs in the pool was examined by performing two-step PCR amplification of the lentiviral barcodes from the genomic DNA of transduced organoids and adapter

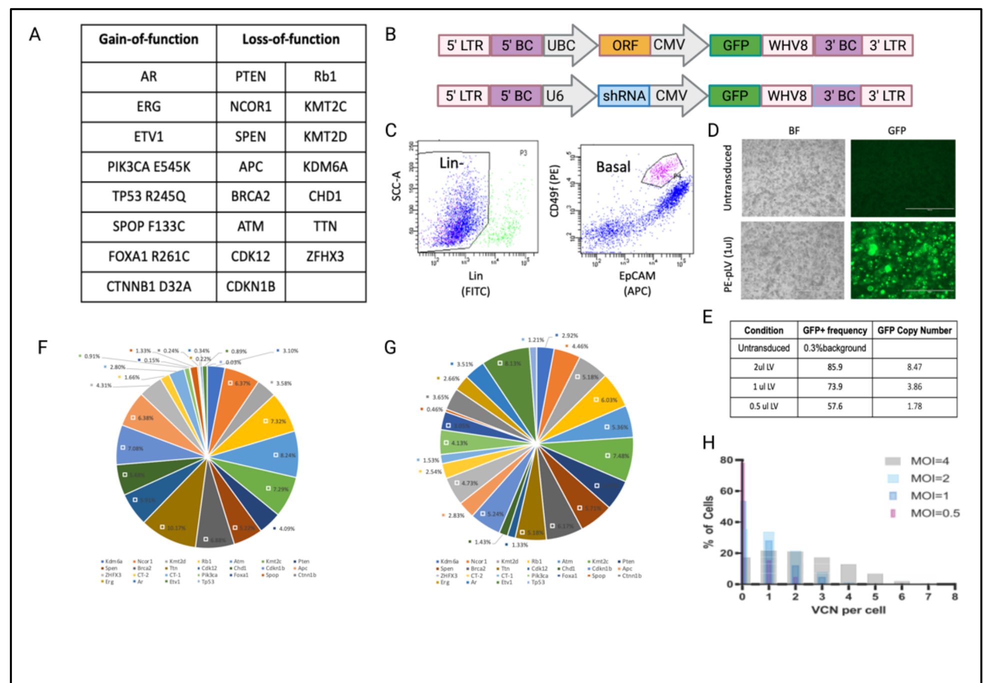


Fig.3. Characterization of a lentiviral pool recapitulating PCa-associated genetic alterations. (A) 23 recurrently mutate genes associated with PCa. (B) Schematic of barcoded lentiviral backbones used to express gain-of-function and loss-of-function. (C) Isolation of mouse prostate epithelial basal cells by fluorescence-activated cell sorting. (D) Representative image of mouse prostate organoids at 72 hrs post-transduction. Scale bar = 1mm. (E) Quantification of the percentage of GFP positive cells as measured by flow-cytometry and GFP copy number by qPCR-based copy number assay in mouse prostate organoids. (F and G) Optimization of lentiviral representation in the pool (F and G). (H) The distribution of viral copies per cell and relative fraction of mouse epithelial cells transduced with lentiviral pool at varying MOI.

step PCR amplification of the lentiviral barcodes from the genomic DNA of transduced organoids and adapter

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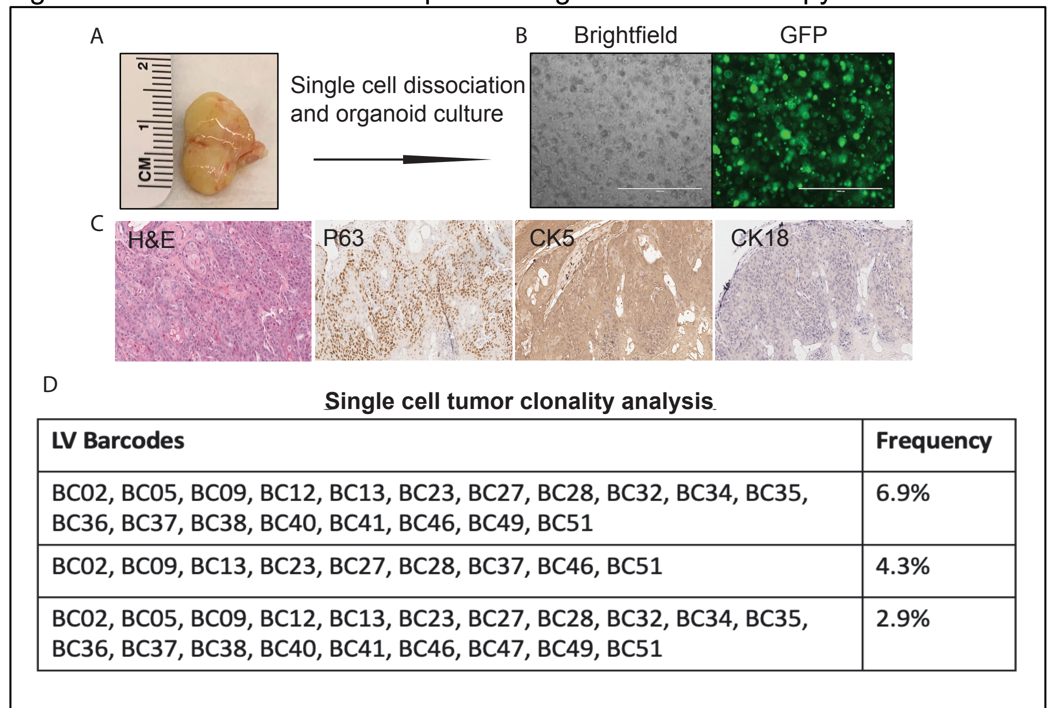
ligation followed by bulk DNA amplicon sequencing (**Fig.3F**). We discovered that the LV distribution was skewed toward significant overrepresentation of short hairpin RNAs relative to open reading frames, likely due to their smaller size. We optimized LV production of the pool by adjusting the relative ratios of the surface area of producer cells to ensure equivalent representation of each lentivirus. Ultimately, we observed uniform representation of LV barcodes in mouse PE organoids after transduction (**Fig. 3G**).In addition, the primary mouse epithelial cells were transduced with the LV pool at varying multiplicities-of-infection (MOI) and single-cell amplicon sequencing showed relatively normal distributions of viral copies per cell based on the presence of unique LV barcodes (**Fig. 3H**)

Major Task 2: Deconvolute combinations of genetic alterations involved in prostate tumorigenesis (months 6-24) ***<u>In progress, 80% completion.</u>***

We initially transduced mouse PE organoids with the barcoded LV pool to target a mean viral copy number of 4. After a brief period of organoid culture (up to a week), the intact transduced organoids were collected and grafted subcutaneously into immune-deficient mice to biologically select for tumorigenic clones. Mice were supplemented with a slow-release testosterone pellet. We harvested initial tumors approximately four months after implantation, and successfully propagated the tumors cells in organoid culture after tumor dissociation (**Fig. 4A and 4B**). In addition, we performed histologic and immunohistochemical analyses of these tumors. Histologic analyses were consistent with squamous cell carcinoma, supported by strong p63 and CK5 expression and lack of luminal cell marker CK18 expression (**Fig. 4C**). Single-cell tumor clonality analysis of the resultant tumors by targeted single-cell DNA amplicon sequencing showed diverse clonal populations with distinct combinations of genetic perturbations.



LV Barcodes	Frequency
BC02, BC05, BC09, BC12, BC13, BC23, BC27, BC28, BC32, BC34, BC35, BC36, BC37, BC38, BC40, BC41, BC46, BC49, BC51	6.9%
BC02, BC09, BC13, BC23, BC27, BC28, BC37, BC46, BC51	4.3%
BC02, BC05, BC09, BC12, BC13, BC23, BC27, BC28, BC32, BC34, BC35, BC36, BC37, BC38, BC40, BC41, BC46, BC47, BC49, BC51	2.9%

Fig.4. Characterization of representative tumors derived from lentiviral pool transduced organoids. (A) Gross images of the tumors derived from lentiviral pool transduced prostate organoids. (B) Organoids culture post dissociation of prostate tumor. Scale bar = 1mm. (C) Histological analysis of prostate tumor and IHC for basal- and luminal-cell markers. (D)Single cell tumor clonality analysis.

In clinical prostate cancer, the squamous cell carcinoma phenotype is relatively rare. Given that we are injecting transduced mouse PE organoids subcutaneously, we questioned whether they may lack and appropriate microenvironment to enable basal to luminal differentiation during transformation. We thus isolated murine urogenital sinus mesenchyme (UGSM) cells based on a Lin$^-$(CD45$^-$CD31$^-$Ter119$^-$) EpCAM$^-$ Trop2$^-$ immunophenotype by fluorescence activated cell sorting (**Fig. 5A and 5B**) for use as an inductive stromal element to recombine with PE organoids for subcutaneous injection. We have harvested several tumors from this set of experiments and performed histologic analyses on these tumors (**Fig. 6A**). We observed mixed tumor phenotypes in a single xenograft initiated from lentiviral pool transduced PE organoids. As shown in **Fig. 6B** the prostate tumors showed a high-grade prostatic cancer morphology including some areas with pleomorphic giant cells, others with poorly-differentiated adenocarcinoma, and focal areas of

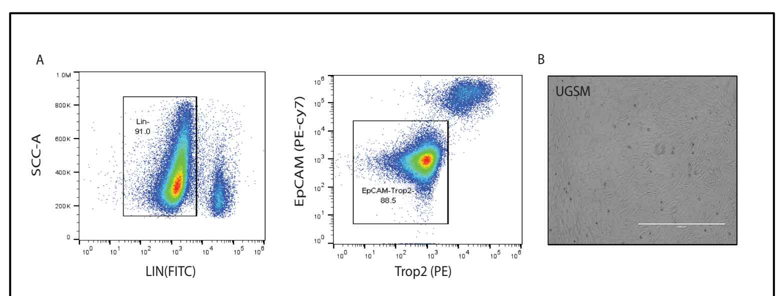


Fig.5 Isolation (A) and culture (B) of UGSM cells.

squamous cell differentiation. We have set up independent experiments using the optimized organoid transformation assay and have so far generated two tumors showed high-grade prostate adenocarcinoma with interspersed pleomorphic giant cells, a rare variant of prostate cancer associated with a very poor prognosis which may contribute to therapeutic resistance. Immunostaining showed HOXB13, AR, and pronounced

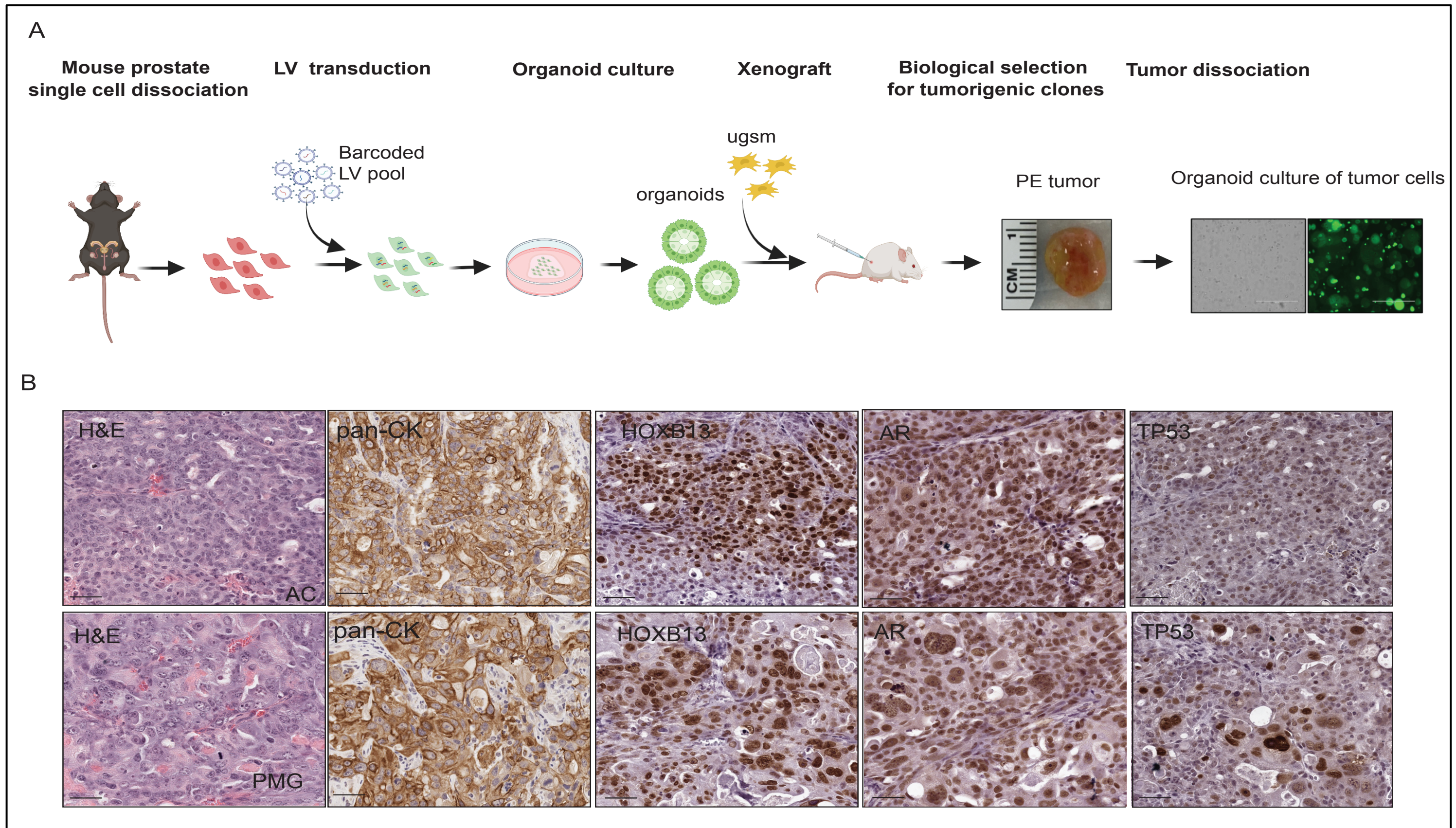


Fig.6 Characterization of representative tumors derived from lentiviral pool transduced organoids. (A) Scheme of prostate organoid transformation assay (B) Histological analysis of prostate tumor and IHC for pan-CK, HOXB13, AR and TP53.

nuclear p53 expression in the pleomorphic giant cells (**Fig. 6B)**.

The identification of genetic alterations that contribute to the pathogenesis of pleomorphic giant cells is important as growing evidence now indicates the polyploid cancer cells are functional actuators of therapeutic resistance. We isolated the pleomorphic giant cells from the prostate tumors using a flow cytometry-based strategy dependent on forward and side scatter and propagated the normal and large tumor cells in organoid culture separately. We then further purified a large tumor cell population based on cell DNA content using Hoechst 33342 staining (**Fig. 7A**). We performed the scDNA-sequencing to deconvolute the genetic alterations that contribute to pleomorphic giant cell phenotype using Mission Bio Tapestri Platform. The analysis revealed enrichment of shKMT2C in pleomorphic giant tumor cells compare to the normal tumor cell population (**Fig. 7B**) indicating loss of KMT2C may promote the pleomorphic giant cell feature in prostate tumors. To validate these findings, we performed a mouse prostate organoid transformation experiment in which we transduced the mouse prostate epithelial with shKMT2C lentivirus in combination of shTTN and ETV1 based on scDNA sequencing data or commonly identified alterations in prostate cancer AR and AKT. We are currently monitoring tumor growth and will perform histological analyses on the resultant tumors to further determine whether loss of KMT2C will promote the pleomorphic giant cell phenotype in prostate cancer.

A

Large cell population

normal cell population

Cell expanding

Large cell

scDNA amplicon library preparation

NGS

B

Genetic alteration	%
shKMT2C, shTTN, ETV1	11.8
shKMT2C, shTTN, SPOP(F133C), ETV1	5.5
shKMT2C, shTTN, ERG, ETV1	5.1
MYC, ETV1	4.7
SPOP(F133C), ETV1	4.5
shKMT2C, ETV1	4.1
shKMT2C, shTTN, SPOP(F133C), ERG, ETV1]	2.7
shKMT2C, SPOP(F133C), ETV1	2.7
MYC, shKMT2C, shTTN, ETV1	2.7
MYC, SPOP(F133C), ETV1	2.3
shKMT2C, SPOP(F133C)', ERG,, ETV1	2.1
MYC, shKMT2C, ETV1	1.9
shKMT2C, PIK3CA(E545K), SPOP(F133C), ERG, ETV1	1.9
shKMT2C, ERG, ETV1	1.8
MYC, shKMT2C, SPOP(F133C), ETV1	1.7
shKMT2C, PIK3CA(E545K), shTTN, ERG, ETV1	1.7
shKMT2C, PIK3CA(E545K), shTTN,ETV1	1.5
shKMT2C, PIK3CA(E545K), SPOP(F133C), ETV1	1.4
shKMT2C, PIK3CA(E545K), shTTN, SPOP(F133C),ETV1	1.4
MYC,shTTN, ERG, ETV1	1.4
MYC, shKMT2C, shTTN, ERG, ETV1	1.3
MYC, shKMT2C, shTTN, SPOP(F133C),ETV1	1.2
MYC, shKMT2C, shTTN, shCDKN1B, ETV1	1.1

Genetic alteration	%
ETV1	34.3
SPOP(F133C),ETV1	26.1
shZFHX3	5.0
SPOP(F133C),shZFHX3	2.9
MYC, shZFHX3	2.6
MYC , SPOP(F133C), shZFHX3	2.2
ETV1, shZFHX3	1.9
MYC, ETV1	1.9
SPOP(F133C), ETV1, shZFHX3	1.6
ETV1, shZFHX3, TP53(R245Q)	1.5
MYC , SPOP(F133C), ETV1	1.2
MYC , SPOP(F133C), ETV1,shZFHX3	1.0
MYC , ETV1,shZFHX3	1.0
MYC , SPOP(F133C), ETV1,TP53(R245Q)	1.0
SPOP(F133C), ETV1, shZFHX3, TP53(R245Q)	1.0

C

Lentiviral pools

shKMT2C/shTTN/ETV1

shKMT2C/myrAKT/AR

UGSM

Organoid culture

Subcutaneous grafting

Fig.7 Deconvolution of genetic alterations contributing to pleomorphic giant cell phenotype in prostate tumor. (A) Isolation of pleomorphic giant cell population based on cell size and DNA content by flow cytometry. (B) Summary of tapestry scDNA analysis results. (C) Mouse prostate organoid transformation experiment to validate the genetic alterations in promoting pleomorphic

In addition, we knocked down KMT2C in the prostate cancer cell line PC3 by shRNA delivery and performed DNA content analysis to investigate whether loss of KMT2C in prostate cancer cell lines will increase the formation of pleomorphic giant cells. We observed an increased frequency of polyploid cells in PC3 cells with KMT2C knockdown (**Fig. 8B**) compared those subjected to a control (shTRC) vector (**Fig.8A**). We are currently testing the role of KMT2C in other prostate cancer cell lines, such as DU145, 22Rv1 and MDA PCa 2b.

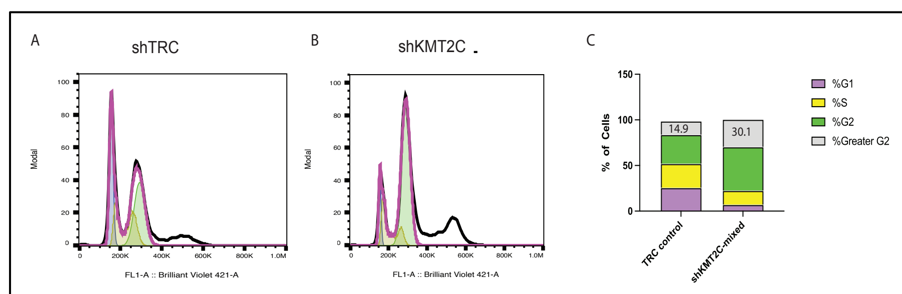


Fig.8 DNA content analysis of prostate cancer cell line PC3 with shTRC (A) and shKMT2C (B). (C) Quantification of cell cycle analysis.

<u>Specific Aim 3:</u> Examine the sequential acquisition of specific genetic alterations to understand how the context-specific temporal order of perturbations impacts progression to PCa (months 1-24)
Major Task1: Establish prostate epithelial organoids with a set of genetic alterations introduced in different sequences (months 1-5) ***<u>In progress.</u>***
We have generated all 23 lentiviral constructs encoding gain-of-function and loss-of function genetic alterations associated with PCa including alterations in Pten, Tp53 and Tmprss2-Erg. All the individual lentivirus from the library have been generated and titered.

Major Task 2: Determine whether the order of acquisition of genetic alterations impacts PCa phenotype (months 5-24) <u>Not yet started due to the Matrigel shortage during the COVID-19 pandemic.</u>

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Key Research Accomplishments:

1. We have generated and optimized lentiviral libraries recapitulating prostate- and bladder cancer-associated genetic alterations.
2. We have developed a higher-order, combinatorial functional genomics assay to generate several tumors with clinically relevant, mixed cancer phenotypes.
3. We are able to deconvolute the genetic alterations contribute to the phenotype of prostate cancer using single cell sequencing.

Training-Specific Tasks:

Major Task1: Training and educational development in prostate cancer research

Subtask1: Scientific coursework and workshops on biocomputational analyses (months 1-24).
I have completed the online course "Genomic Data Science".

Subtask2: Presentation monthly at Fred Hutch prostate cancer program: Attend Multi-Institutional prostate cancer SPORE retreat and present research yearly (months 1-24).
Completed during the grant period.

Subtask3: Attend national and international scientific meeting relating to cancer biology (months 1-24)
I was selected to present an abstract as a short talk at the AACR Special Conference on Advances in Prostate Cancer Research. However, the conference was canceled due to the COVID-19 pandemic.

Training and professional development:

Nothing to report.

Disseminated to communities of interest:

Nothing to report.

IMPACT

Prostate cancer is genetically diverse and next-generation sequencing technologies have identified numerous genetic alterations associated with prostate cancer. However, most genomic alterations have not been functionally annotated and thus their individual and contextual contributions to PCa initiation and progression remains largely unknown. In the past two years, we have developed and optimized a combinatorial strategy to rapidly interrogate the context-specific genetic interactions that initiate and confer heterogeneous phenotypes of PCa. The major impact of this work is to establish multiple diverse, genetically defined models of PCa that can better recapitulate the different molecular phenotypes of PCa. These will hopefully provide important insights into PCa biology and be important tools for the PCa research community in the era of personalized medicine.

CHANGES/PROBLEMS

The project was impacted by the COVID-19 pandemic as 1) the research laboratory at Fred Hutchinson Cancer Research Center was limited to minimal maintenance activities between the months of March and June 2020 and work limitations for several months in 2020 and 2021 due to physical distancing restrictions; 2) plenty of experimental materials (e.g., Matrigel) were delayed due to logistical issues (labor and supply shortage) during the pandemic 2021-2022.

PRODUCTS

None.

PARTICIPANTS & OTHER COLLABORATING ORGANOIZATIONS

Name	Shan Li
Project Role	PI
Researcher Identifier (e.g. OR-CID ID):	
Nearest person month worked:	24
Contribute to Project:	Dr.Li has designed the study, performed the experiments and data analysis
Funding Support:	W81XWH-20-1-0083

SPECIAL REPORTING AND REQUIREMENTS

List of equipment purchased with award funds:

None.

List of residual inventory of unused supplies exceeding $5,000 in value:

None.

Transition plan or other documents as specified in the agreement document:

Not applicable.

APPENDICES

Final DD882, Report of Inventions and Subcontracts