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14. ABSTRACT We have developed and characterized a new methodology to generate genotypically and phenotypically diverse mouse models of bladder cancer that may better reflect the heterogeneity of the human disease. We have introduced lentiviral pools encoding gain- and loss-of-function events associated with bladder cancer in multiplexed fashion in the context of a mouse bladder urothelial transformation assay. This led to the generation of multiple tumors models that are polyclonal and exhibit multiple histologies including typical urothelial carcinoma, papillary urothelial carcinoma, squamous cell carcinoma, and sarcomatoid carcinoma. Furthermore, transcriptional profiling studies and the application of published molecular classifiers indicate that these tumors reflect multiple molecular subtypes of bladder cancer. This methodology and the resultant tumor models may be a valuable resource for the field in understanding the interactions of oncogenic events in tumorigenesis and investigating therapeutic strategies in bladder cancer.					
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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.* Local IACUC and USAMRMC ORP ACURO approval were obtained.

Task 2: *Mouse urothelial organoid transformation using pooled and barcoded lentiviruses (months 1-10) Completed.* Five batches of pooled, barcoded lentivirus representing gain-of-function and loss-of-function genetics in bladder cancer (Fig. 1) were collected, concentrated by ultracentrifugation, titered, and cryopreserved. For the initial two batches of lentivirus, we transduced mouse basal bladder urothelial cells in organoid cultures, collected DNA, and performed two-step polymerase chain

reaction (PCR) for barcode amplification and library preparation for next-generation sequencing (NGS). Based on barcode representation from the NGS data, we identified, that short hairpin RNA infectious virions are produced much more readily than open reading frame (ORF) infectious virions (Fig. 2), likely due to differences in size which impact lentiviral packaging efficiency. We therefore adjusted the surface area of lentiviral producer cells to account for differences in barcode representation and repeated this analysis. Using this approach, we were able to nearly normalize the ratio of ORF-to-shRNA infectious virions and overcome this technical issue (Fig. 2). Each batch of pooled, barcoded lentivirus was titered on mouse basal bladder urothelial cells and the mean copy number was determined by a quantitative PCR assay measuring lentiviral *Gfp* copies relative to endogenous *Tfrc* copies. We used tail DNA from a *Gfp* transgenic mouse bearing two copies of *Gfp* as a calibrator sample in our copy number assays.

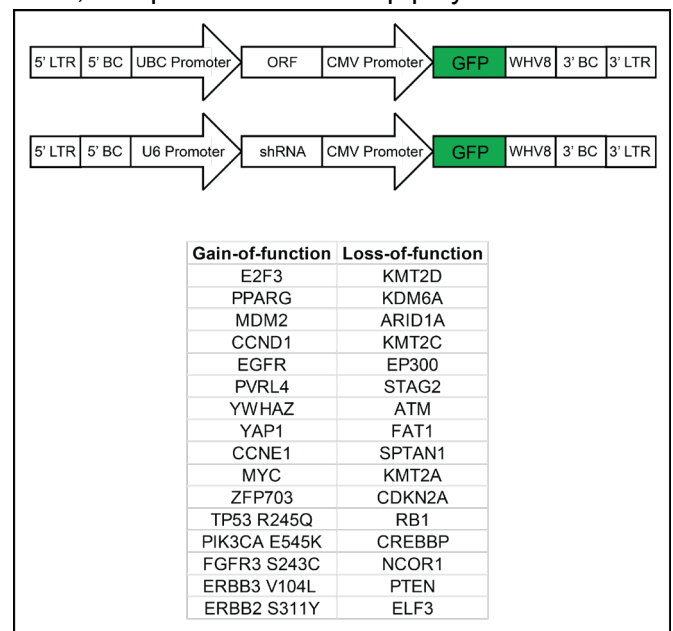


Fig. 1. Barcoded lentiviruses to recapitulate gain-of-function and loss-of-function events associated with bladder cancer. Top, schematic of barcoded lentiviral constructs for the expression of open reading frames (ORF) or short hairpin RNAs (shRNA).

We applied these lentiviral pools to the mouse urothelial organoid transformation assay, harvested transduced urothelial organoids 5-7 days post-transduction, recombined with embryonic bladder mesenchyme, and grafted these subcutaneously into NSG mice. After a latency of 2-4 months, we have collected multiple tumors (~15 thus far) and have completed analyses by histology and immunohistochemistry. Most of the tumors we collected harbored multiple bladder cancer histologies based on review with clinical pathology colleagues. For example, one tumor was found to have three histologically distinct regions (Fig. 3A) comprising urothelial carcinoma with squamous differentiation, papillary carcinoma with inverted growth pattern, and sarcomatoid carcinoma.

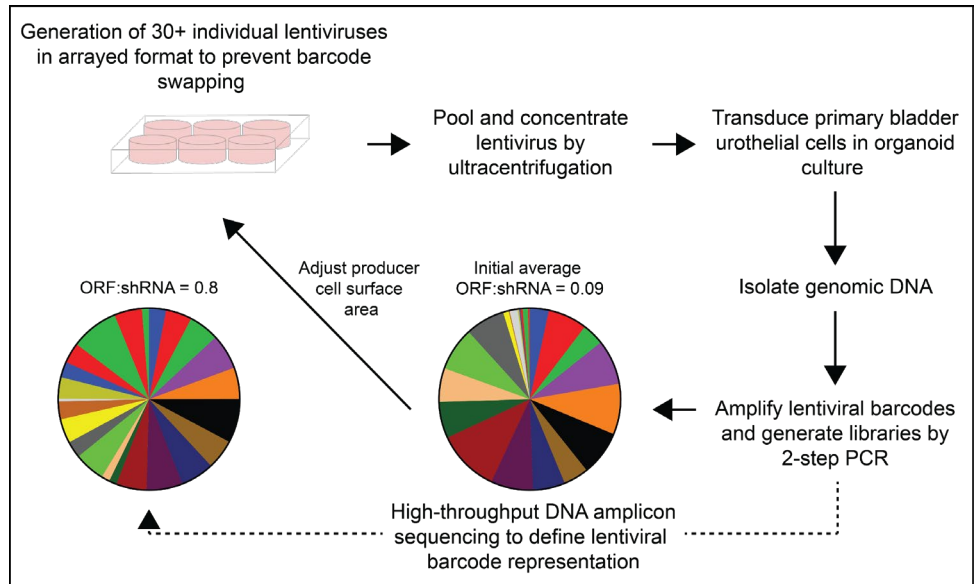


Fig. 2. Schematic of the generation of pooled, barcoded lentiviruses and optimization of barcode representation in infectious virions.

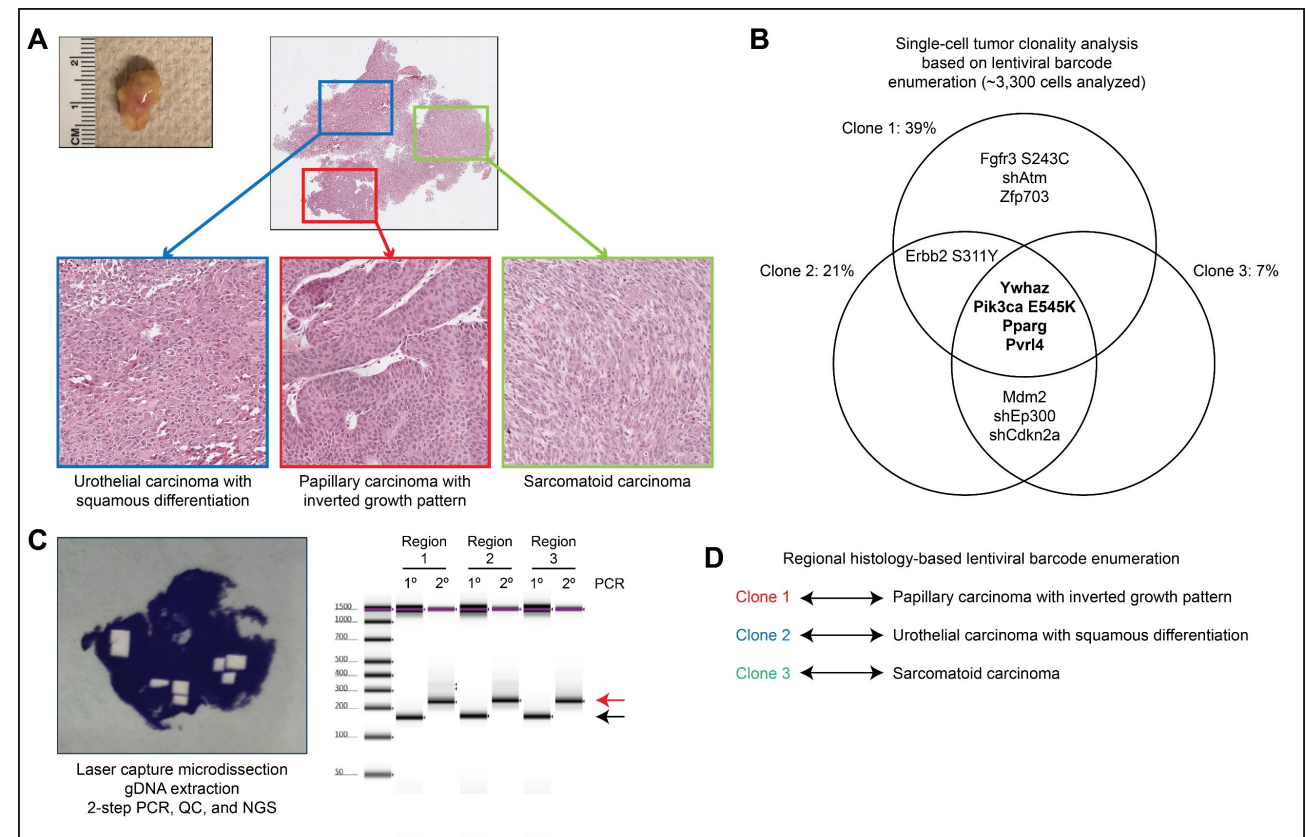


Fig. 3. Generation of genetically and phenotypically heterogeneous mouse bladder cancer models. (A) Photomicrographs of a gross tumor generated from the mouse urothelial transformation assay using the barcoded lentiviral library and H&E-stained sections of the tumor showing three distinct histologies. (B) Clonal architecture of the tumor determined by Tapestry 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 1^o and 2^o PCR (to append NGS adapters) from each region. (D) Association of histologies to clones in B based on LCM and bulk DNA amplicon sequencing.

Task 3: *Lentiviral barcode deconvolution by bulk DNA amplicon sequencing of the engineered urothelial tumors (months 11-14) Completed.* From the tumors collected in Task 2, we performed physical and enzymatic digestion to generate single-cell suspensions for viable cryopreservation. We subjected these cells from each tumor to targeted single-cell DNA amplification and library preparation for NGS using the Mission Bio Tapestry platform and a custom assay that we developed to specifically amplify the lentiviral barcodes that have integrated into each cell. We have completed the library preparation and NGS for 12 tumors. We highlight analysis of the tumor shown in Fig. 3A which revealed three dominant clonal populations (Fig. 3B) which shared multiple genetic perturbations (Ywhaz, Pik3ca E545K, Pparg, and Pvr14) but also had unique genetic perturbations that could differentiate the populations (Fgfr3 S243C, shAtm, Zfp703, Mdm2, shEp300, shCdkn2a, and Erbb2 S311Y).

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

A question we could not immediately answer 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. 3C). This analysis allowed us to then associate lentiviral barcodes present in regional histologies with the single-cell data (Fig. 3D). 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).

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 1). We identified Myc, Pik3ca E545K, and Ccnd1 as the most common genetic alteration enriched in the tumors.

Table 1. Gene alterations based on lentiviral barcodes represented in 10 tumors (31,818 total cells) arising from the mouse 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) Completed.* We have functionally validated our findings in directed experiments using the mouse

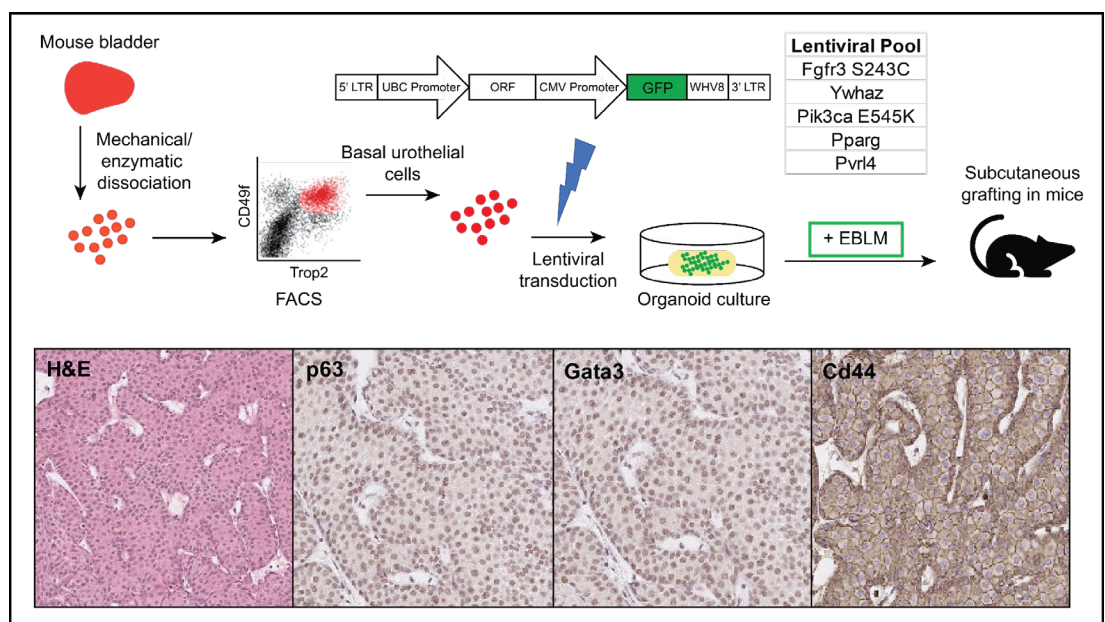


Fig. 4. Initial validation study to examine the role of an activating mutation of Fgfr3 in establishing a papillary urothelial carcinoma phenotype. Top, experimental schematic. Bottom, photomicrographs of H&E and immunohistochemical stains of tumor sections.

urothelial organoid transformation system. Based on the results shown in Fig. 3, we hypothesized that the clonal population harboring the *Fgfr3* S243C likely represented the papillary carcinoma with inverted growth pattern seen by histologic analysis of the tumor. This assumption was based on multiple genomic studies that have shown that *FGFR3* mutations are highly enriched in human luminal papillary urothelial carcinomas (1, 2). To evaluate this hypothesis, we generated a lentiviral pool expressing *Fgfr3* S243C as well as *Ywhaz*, *Pik3ca* E545K, *Pparg*, and *Pvrl4* which represent genes common to the three dominant clones identified from the single-cell DNA amplicon sequencing analysis of the tumor (Fig. 3B). We used this lentiviral pool for in the mouse urothelial organoid transformation assay (Fig. 4) to functionally validate this combination for its competency in driving malignant transformation and a papillary urothelial carcinoma histology. Indeed, histological analysis of the resultant tumor confirmed a papillary urothelial carcinoma with inverted growth pattern (Fig. 4) similar to that seen in the original tumor (Fig. 3A). These data suggest that mutant active *Fgfr3* may be a lineage driver of luminal papillary urothelial carcinoma.

Task 2: *Perform leave-one-out analyses and compare the transcriptome profiles of the resultant tumors (months 25-36) Completed.* We generated a series of eight tumors—two containing multiple histologies—that were subjected to laser capture dissection and RNA-seq for transcriptional profiling. Principal component analysis (Fig. 5) revealed that 1) individual histologies co-existing in the two tumors (B7—papillary, sarcomatoid, squamous with urothelial differentiation; B9—squamous and papillary) were transcriptionally distinct and 2) sarcomatoid and squamous tumors were transcriptionally more similar than urothelial or papillary urothelial tumors.

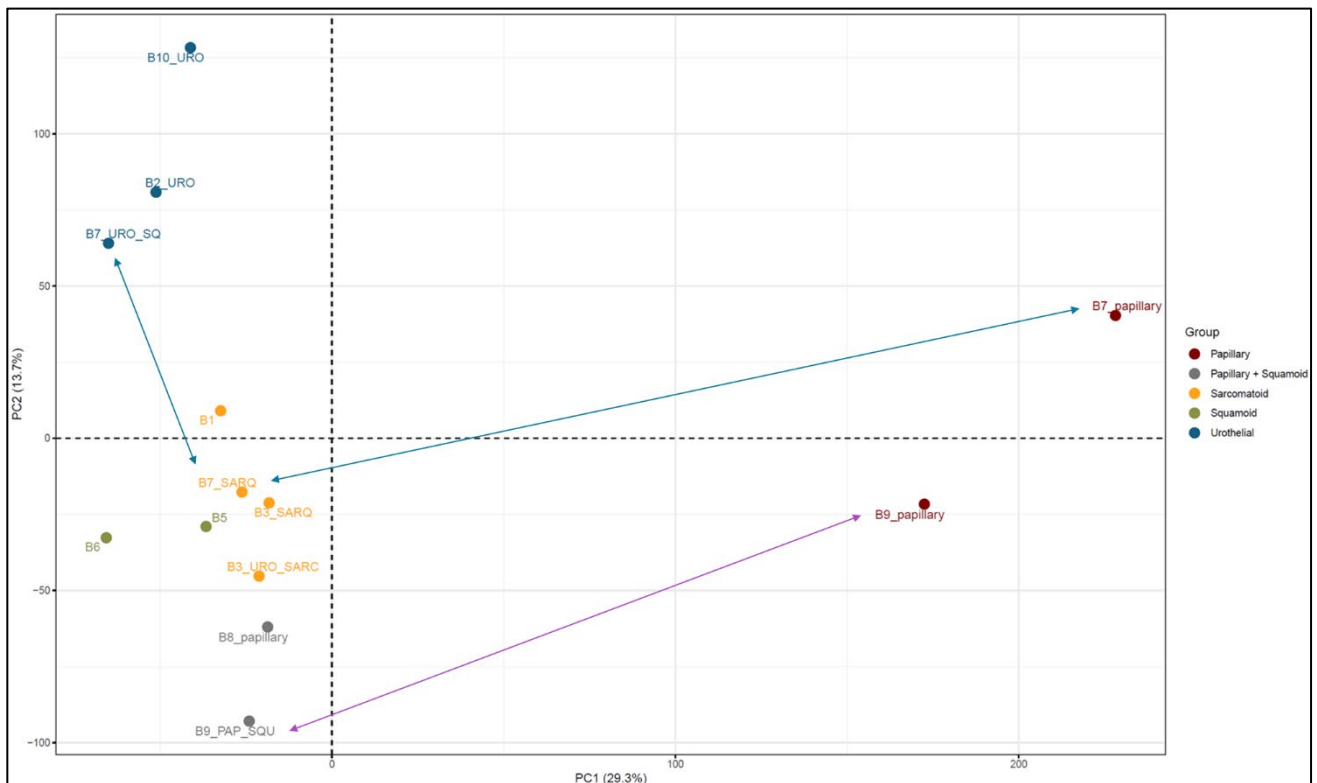


Fig. 5. Principal component analysis (PCA) of mouse bladder cancer models generated from the mouse urothelial transformation assay. Tumors are color-coded based on histologic appearance (papillary, papillary/squamoid, sarcomatoid, squamous, or urothelial). Arrows denote differences in individual cancer histologies within tumors isolated for analysis by laser capture microdissection.

We applied published molecular signatures such as the BASE47 classifier (3) and the consensus molecular classifier (4) to the tumors to define their subtypes. Not surprisingly, the BASE47 classifier called tumors that were papillary or papillary/squamous by histology as luminal while all other urothelial, squamous, and sarcomatoid tumors were called basal

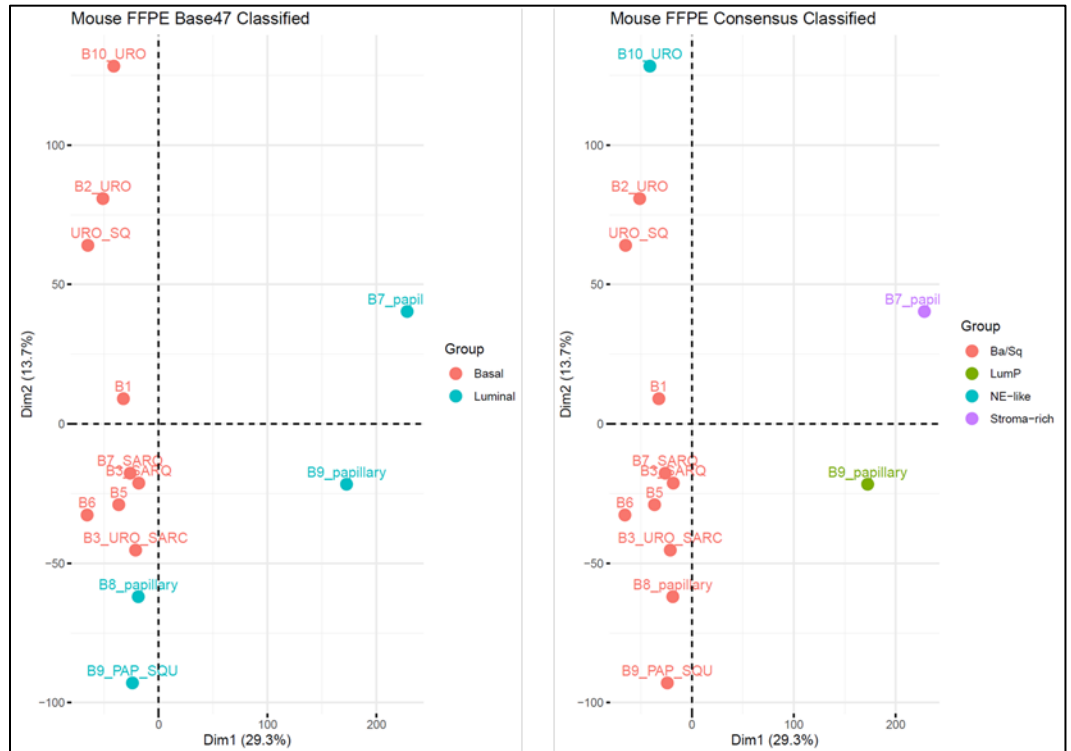


Fig. 6. Application of established molecular classifiers to the mouse bladder cancer models to determine their subtypes. Tumors are shown in PCA plots but color-coded based on their molecular subtype classification using (left) the BASE47 classifier and (right) the consensus molecular classifier.

(Fig. 6). The consensus molecular classifier 1) identified one of the urothelial tumors as being neuroendocrine (NE)-like, 2) called the papillary/squamous tumors as basal/squamous, and 3) classified the papillary tumors as luminal papillary and stroma-rich. Together, these data highlight the molecular diversity of the mouse bladder cancer models that we have established thus far using our novel methodology.

SA 3: 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) Completed. 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 Tapestry, 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. 7). The data revealed a binomial distribution of lentiviral transduction. These data were reassuring as it indicates that the distribution is not skewed.

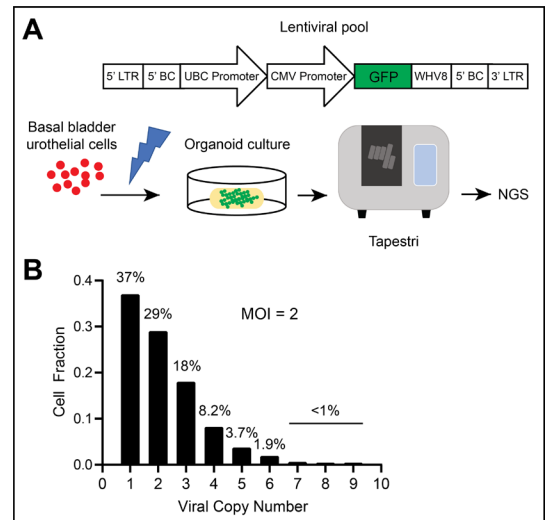


Fig. 7. Lentiviral transduction of mouse bladder urothelial cells in organoid culture follows a binomial distribution. (A) Experimental setup showing transduction of bladder urothelial cells with a lentiviral pool in organoid culture, dissociation for single cell DNA amplicon library preparation using the Tapestry, and NGS. (B) Distribution of viral copy number across cells from the experiment performed at a multiplicity-of-infection (MOI) of 2.

We have performed 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. However, this analysis was limited as the overall clonal diversity from tumors were limited. While we had assumed that most tumors might be highly polyclonal to allow for these analyses, this was not possible in a statistically rigorous way. However, it may be possible to readdress this once we have generated a significantly larger number of tumors.

Task 2: *Investigate potential genetic interactions in bladder cancer using in vitro and in vivo systems (months 21-27) In progress.* While we were unable to define potentially co-occurring and mutually exclusive genetic interactions in SA3 Task 1, we have pivoted to focus on the role of specific factors in lineage specification of bladder cancer. Specifically, we have focused on Pparg due to recurrent activating mutations in luminal bladder cancer (5) and recent work implicating Pparg as a key regulator of bladder cancer subtype (6). We have generated and validated lentiviral constructs expressing wildtype Pparg, constitutively active Pparg, and dominant-negative Pparg to investigate the role of Pparg in luminal differentiation both in the setting of benign urothelial cells and in tumorigenesis. In preliminary studies, we have shown that overexpression of wildtype Pparg or constitutively active Pparg alters the morphology of benign mouse bladder urothelial organoids toward previously described luminal structures (7) (Fig. 8A), significantly upregulates the expression of the luminal marker Upk2 (Fig. 8B), and inhibits cell proliferation (Fig. 8C). These findings are all consistent with luminal differentiation of urothelial cells and provide a tractable model for understanding how Pparg signaling effects a shift in urothelial cell lineage specification.

Furthermore, we have initiated experiments using the mouse urothelial transformation system in which we introduce either wildtype Pparg, constitutively active Pparg, or dominant-negative Pparg in combination with Ywhaz, Pik3ca E545K, and Pvr14. We will investigate the impact of differential Pparg signaling on tumor latency and tumor phenotype based on histology and molecular analyses. As the readouts of these studies are more complex than those that we initially proposed, these experiments will continue well beyond the timeframe of this award.

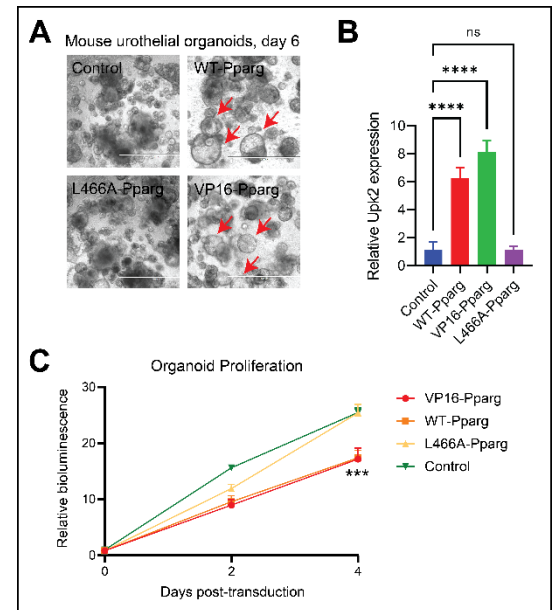


Fig. 8. Phenotypic effects of enforced Pparg activity in benign mouse urothelial organoids. (A) Representative photomicrographs of mouse urothelial organoids on day 6 after mock transduction (control) or transduction with lentiviruses expressing wildtype Pparg (WT-Pparg), constitutively active Pparg (VP16-Pparg), or dominant-negative Pparg (L455A-Pparg). Red arrows indicate luminal organoid structures. (B) qPCR quantification of relative Upk2 expression across the different organoid conditions in A. (C) Plot showing cell viability/proliferation as relative bioluminescence of the organoid conditions in A as determined by CellTiter-Glo analysis.

Key Research Accomplishments:

- Demonstrated the ability to generate multiple genetically and phenotypically diverse tumors from mouse bladder urothelial cells using the proposed functional cancer genomics strategy.
- Established a genetically defined model of luminal papillary urothelial carcinoma which may be functionally driven by mutant activation of Fgfr3.
- 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.
- Transcriptionally characterized mouse bladder cancer models to demonstrate their molecular diversity, akin to that seen in human bladder cancer.

Opportunities for Training and Professional Development:

During the course of the award, I have advanced my professional development by engaging 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 was invited in the last year to co-chair a Preclinical Modeling session at the Bladder Cancer Advocacy Network Think Tank which was met with enthusiasm from attendees. I continue to 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 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. Based on this scientific project, I have been recognized with the Eula and Donald S. Coffey Innovative Research Award from the Society for Basic Urologic Research and a Young Physician-Scientist Award from the American Society for Clinical Investigation. I am also a recipient of the Bladder Cancer Advocacy Network Research Innovation Award.

Dissemination of Results:

These findings were presented recently at an invited virtual talk as part of the Bladder Cancer Research Seminar Series hosted by the Department of Urology at Stanford University on October 14, 2022. In addition, the results of our studies were disseminated at an invited plenary lecture at the 2022 Society for Basic Urologic Research Annual Meeting on November 12, 2022.

We are also actively preparing a manuscript of our findings which we hope to deposit to the pre-print server bioRxiv for immediate visibility to the broad scientific community prior to submission to a journal for peer-review.

Plan for the Next Reporting Period:

Nothing to report. This is the final technical report.

IMPACT

The major impact is that we have established a robust functional genomics platform that enables rapid, multiplex interrogation of genetic alterations in the initiation of bladder cancer and heterogeneous phenotypic subtypes. We have already demonstrated the benefits of this approach in terms of generating new and diverse models and potential assignment of oncogenic function to genetic alterations. Our hope is that the development and distribution of these model systems to the bladder cancer research community will accelerate discovery and therapeutic translation. To this end, we have established early 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 was impacted by the COVID-19 pandemic and diminished workforce between the months of March and June 2020 due to physical distancing and mouse order restrictions at the Fred Hutchinson Cancer Research Center.

A change to SA3 was made due to the inability to acquire statistically robust inferences regarding co-occurrence and mutual exclusivity of genetic interactions with available data. However, we pivoted by delving

into the specific role of Pparg activity in mediating lineage/subtype specification in benign urothelial cells and during malignant transformation.

PRODUCTS

Presentations:

“Applying Higher-order Combinatorial Genetics to Model Genitourinary Malignancies,” 2022 Society for Basic Urologic Research Annual Virtual Meeting, November 12, 2022.

Methodologies:

We have described a methodology to generate diverse models of bladder cancer using lentiviral pools encoding gain- and loss-of-function genetic events and a mouse urothelial transformation assay. All materials associated with this approach including lentiviral constructs will be made available to the scientific community upon request. A detailed methodology will be described in the manuscript in preparation.

Other Products:

We have developed a series of mouse bladder cancer models, as described above, that reflect the genotypic and phenotypic diversity of human bladder cancer.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	<i>Vipul Bhatia</i>
Project Role:	<i>Research Technician</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Bhatia performed generation of lentivirus, isolation of mouse bladder urothelial cells, transformation experiments, and characterization of resultant tumors.</i>
Funding Support:	

Name:	<i>Gerardo Javier</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Mr. Javier performed quality control analysis, alignment of RNA-seq reads to the mouse reference genome, gene-level expression quantification, principal component analysis, and molecular classifier analysis.</i>
Funding Support:	

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

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APPENDICES

None.