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14. ABSTRACT: Prostate carcinogenesis is a multi-step process resulting in the transformation of prostatic epithelial cells into invasive carcinoma and metastasis. In recent years, mouse models have emerged that recapitulate salient features of prostate carcinogenesis found in human disease. These models illuminate the molecular events that result in transformation and disease progression. In addition, mouse models can be used to identify molecular targets and test chemotherapeutic agents that may alter the course of disease. We have generated a new mouse model to further delineate targets that may halt cancer progression and lead to regression of disease. Crossing the TRAMP mouse with the PSCA-TVA transgenic mouse has resulted in the TRAMP-TVA mouse that is destined to develop prostate cancer and expresses the avian viral receptor, TVA, on prostate cancer cells. This new transgenic mouse should enable specific gene transfer of imaging genes and small hairpin nuclear RNAs (shRNAs) resulting in knockdown of specific targets. TRAMP-TVA mice demonstrate PIN lesions at 8 weeks and develop adenocarcinoma at 6-15 months. We have been able to demonstrate PSCA-driven expression of the TVA viral receptor in these lesions. Intraperitoneal injection of virus containing the luciferase gene results in luminescence signal in the prostate. Further development of this model will enable the effect of target gene knockdown via RNA

15. SUBJECT TERMS

Prostate Cancer, TRAMP, shRNA, TVA, viral gene transfer

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INTRODUCTION:

Prostate carcinogenesis is a multi-step process that results in the transformation of prostatic epithelial cells into invasive carcinoma with the ability to metastasize to distant sites. Along the continuum of prostatic cellular transformation, several phenotypes are observed, including benign hyperplasia, prostatic intraepithelial neoplasia (PIN), and invasive carcinoma. In recent years, numerous mouse models have been generated that recapitulate some of the salient features of prostate carcinogenesis that are found in human disease[1-3]. These models are critical to our understanding of the molecular events that result in transformation and disease progression. In addition, mouse models can be used to identify molecular targets and test chemotherapeutic agents that may alter the course of the disease. The overall goal of this proposal is to utilize an established, well characterized mouse model of prostate cancer to further delineate molecular targets that may halt cancer progression and/or lead to regression of metastatic disease. In order to rapidly evaluate a variety of select target genes in the long-term, we have created a new transgenic mouse, the TRAMP-TVA mouse, which allows for efficient and specific gene transfer of imaging genes into prostate epithelial cells that are destined to form cancer. The use of small hairpin nuclear RNAs (shRNAs) in this model may result in target gene knockdown and the ability to assess the effect of this action on prostate cancer development and progression in mice, non-invasively over time.

BODY:

The overall goal of this proposal is to utilize an established, well characterized mouse model of prostate cancer to further delineate molecular targets that may halt cancer progression and/or lead to regression of metastatic disease. In order to rapidly evaluate a variety of select target genes, we created a new transgenic mouse (TRAMP-TVA) to allow for efficient and specific gene transfer of imaging genes with small hairpin nuclear RNAs (shRNAs) that can result in target gene knockdown. The development of this mouse may enable prostate cancer development, progression, and the effect of shRNA introduction to be monitored non-invasively in mice over time.

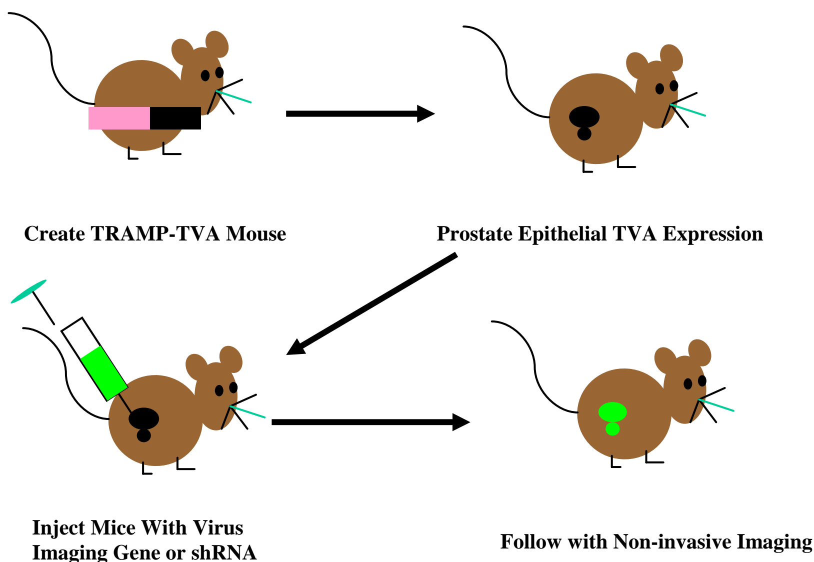

Figure 1: Viral-mediated gene transfer of TRAMP-TVA transgenic mouse. The TRAMP-TVA mice express the TVA avian viral receptor in prostate tissue under the direction of the PSCA promoter. Genetically engineered avian viruses containing the luciferase imaging gene and/or shRNA directed at specific target genes are intraperitoneally or orthotopically injected. Mice are imaged for reporter gene expression with change in tumor development/progression over time.

The concept of the TRAMP-TVA mouse model is shown in Figure 1. We have previously generated a PSCA-TVA mouse that utilizes the PSCA promoter to drive expression of the avian viral receptor, TVA, in prostate epithelial cells (see original proposal). This receptor is required for efficient gene transfer of avian viruses that are genetically engineered to express imaging genes, oncogenes, or shRNAs. The advantages of the TVA system include stability of gene transfer (viral genes integrate into host genome) and the ability to introduce multiple genetic changes into a single cell via multiple rounds of infection. The TRAMP mouse is a well-established mouse model that utilizes the prostate-specific probasin promoter to drive expression of the SV40 large T antigen, resulting in PIN, adenocarcinoma, and eventually, metastatic disease in male mice[1]. Previous work has demonstrated increased PSCA expression in prostate cancer cells from TRAMP mice[4]. Therefore, we predicted that prostate cancer cells from TRAMP-TVA mice would express high levels of the TVA receptor. The creation of TRAMP-TVA mice should enable transfer of genetic information via avian viruses in order to study the effect of shRNA on disease initiation and progression. Combined transfer of imaging genes (luciferase and/or GFP) will allow this process to be followed non-invasively over time. The specific aims of this project are listed below:

AIM 1: Establish a TRAMP-TVA transgenic line with characterization of prostate tumorigenesis as well as TVA expression within prostate cells

AIM 2: Non-invasive imaging of tumor development and progression in TRAMP-TVA mice

AIM 3: Demonstration of viral mediated shRNA delivery to TRAMP-TVA mice cancer cells

Tasks Involved in Accomplishing Aim 1:

1) Cross-breeding of TRAMP and PSCA-TVA mice

2) Confirmation of TVA expression and prostate tumor development in mice generated from the TRAMP-TVA cross

Materials/ Methods/Outcomes (Aim 1): We examined males generated from TRAMP-TVA crossbred mice for development of PIN and progression to prostate adenocarcinoma (Figure 2). Mice were genotyped for the presence of the TVA gene and SV40 large T antigen (data not shown). TRAMP-TVA mice were sacrificed at various timepoints to determine cancer initiation and progression. Immunohistochemistry with anti-TVA antibodies confirmed the presence of the TVA receptor expression in PIN lesions and well-differentiated adenocarcinoma of TRAMP-TVA mice. Ki-67 activity was markedly increased in the TRAMP-TVA mice with PIN. Proliferative activity is an important parameter in determining viral infection efficiency, since the avian retroviruses require cellular proliferation for infection.

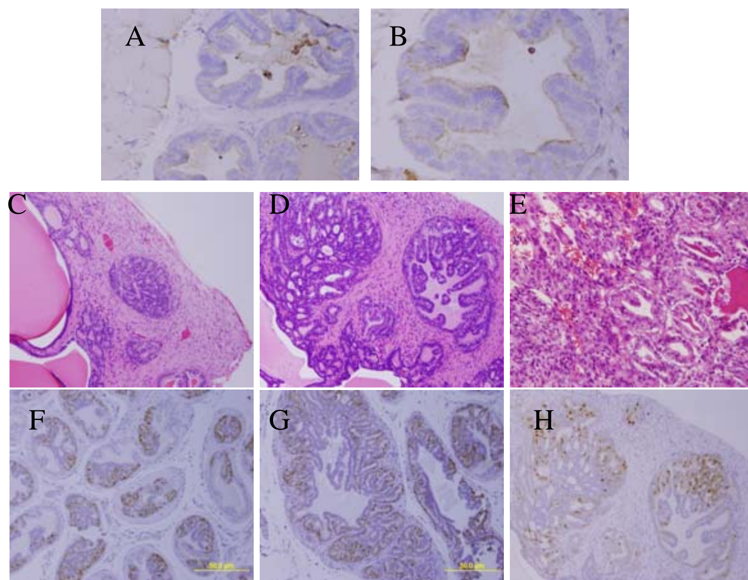

Figure 2: Prostate Epithelial Cell TVA Expression and Tumor Development and Progression in TRAMP-TVA Mice. TRAMP-TVA crossbred mice were confirmed by tissue genotyping and males were sacrificed at 8 weeks to determine TVA expression. The TVA viral receptor was demonstrated by immunohistochemistry on the apical surface of TRAMP-TVA mice (A&B). TRAMP-TVA mice develop PIN lesions by 8 weeks, as shown on H&E sections and with increased Ki-67 staining (C, F). These lesions progress to well-differentiated (D,G, H) and poorly differentiated adenocarcinoma (E) at 4 months and 12-15 months, respectively.

Problems Encountered in Accomplishing Aim 1: One problem that we have faced in generating the TRAMP-TVA mice is a relatively slow time for tumor progression. Many of the TRAMP mice do not progress to advanced prostate cancer with metastasis within 12 months. All of the mice develop PIN and well-differentiated carcinoma within 6 months, but only 2 of 15 mice developed advanced disease by 15 months. This phenomenon is probably due to the fact that the TRAMP-TVA mice that we have created are on the C57/Black6 background of inbred mice. It has been shown by Greenberg that mice bred on the FVB background develop prostate cancer and progress to metastatic disease more rapidly (6-9 months)[5]. Therefore, we are currently beginning to generate TRAMP-TVA mice on this background so that we may be able to perform our gene transfer analysis more rapidly. On the other hand, the slower tumor growth rate noted in the C57/Black6 mice may be advantageous to studying the effect of shRNAs on cancer initiation in that there may be more of a window to intervene. Therefore, will likely continue to use theC57/Blk6 mice fo rthis purpose in shRNA experiments as well.

Tasks Involved in Accomplishing Aim 2:

1) Infection of TRAMP-TVA prostate epithelial cells with viral vectors containing luciferase at various timepoints in development
2) Imaging of infected TRAMP-TVA mice with charged coupled device (CCD) camera over time

Materials/Methods/Outcomes (Aim 2):

Previously we have shown that intraperitoneal and orthotopic injection of avian virus containing luciferase is capable in infecting the prostate of PSCA-TVA mice (see original proposal). In order to evaluate imaging gene transfer into prostate tissue of TRAMP-TVA mice, we attempted IP injections at 2-days, 1 month, and 3 months of age. The IP injections were performed daily for 7 days. Mice were imaged with the CCD camera following the last day of injection (data not shown). CCD imaging over a 4-month period of mice injected as 2-day old pups resulted in durable prostatic luciferase expression (Figure 3). In older mice, however, luciferase gene transfer did not occur, despite escalation in viral titer and increased number of injections (data not shown). The reason for the inability to demonstrate efficient gene transfer in older mice could be due to the mature immune system resulting in neutralization of virus when given IP. Previous experiments using orthotopic injection in our lab have been successful in luciferase gene transfer in mice regardless of age (see original grant proposal). Therefore, orthotopic injection of luciferase virus in TRAMP-TVA mice is currently underway and is likely to result in durable infection in older mice. The advantage of IP injection is its simplicity lack of the need for mouse anesthesia. It also allows for systemic exposure, which would be ideal for targeting metastatic sites. However, based on these findings, IP injection may not be feasible in immunocompetent mice that are more than a few days old. For future experiments targeting metastatic lesions in TRAMP-TVA mice, orthotopic injection may be required. Additionally, in mice that we want to re-inject with different viruses to assess the effect of multiple genes, we will have to perform orthotopic injections instead of IP. Alternatively, the TRAMP-TVA cross could be generated in immunocompromised animals.

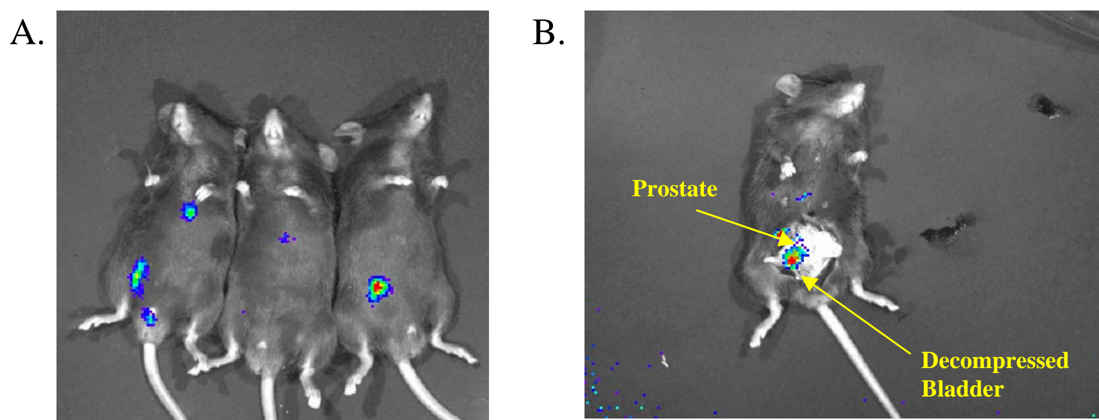

Figure 3: TRAMP-TVA mice intraperitoneally injected with avian virus containing the luciferase gene at 2 days of age were imaged with the CCD camera four months later for stable luciferase activity in the prostate (A). Two/three mice demonstrated signal in the area of the prostate. One of the mice was opened to expose the prostate and confirm the sight of the signal. The bright-red signal was indeed emitted from the prostate and can be compared to the lack of signaling seen from the bladder. TVA is also expressed in the stomach of TRAMP-TVA mice, so some luciferase activity is noted this region as well.

Tasks Involved in Accomplishing Aim 3:

1) Creation of RCAS-shRNA vectors targeted against luciferase and SV40 large T antigen
2) In vitro and in vivo analysis of target gene knockdown by RCAS-shRNA viruses

Materials/Methods/Outcomes (Aim 3):

The goal of this aim is to create oligonucleotide sequences will produce shRNAs capable of target gene knockdown and clone them into the avian viral system for in vivo gene transfer into TRAMP-TVA mice. We have acquired a DNA vector that contains shRNAs against luciferase as well as a GFP reporter. Transient transfection of our viral producer cell lines has demonstrated a >80% knockdown in luciferase signal (Figure 4). We are currently in the process of cloning the DNA fragment containing the luciferase shRNA and GFP into our RCAS viral vector. This RCAS-RNAT vector should enable targeting of prostate epithelial cells in TRAMP-TVA mice that have been previously infected with luciferase (see Figure 3). We anticipate that with sufficient infection efficiency, we will be able to visualize the emergence of GFP signal and the loss of

luciferase. Once the cloning of this vector is complete, we will first confirm infection efficiency and luciferase knockdown capability in vitro in viral producer cells. Following this, we will perform in vivo infections in TRAMP-TVA mice previously infected with luciferase with prominent, stable activity in the prostate. Depending on the success of the luciferase knockdown experiments, we will go on to produce other shRNAs directed at specific molecular targets, such as SV40 large T antigen, and investigate the in vivo effect on prostate cancer initiation and progression using luciferase or GFP as a co-infected imaging gene.

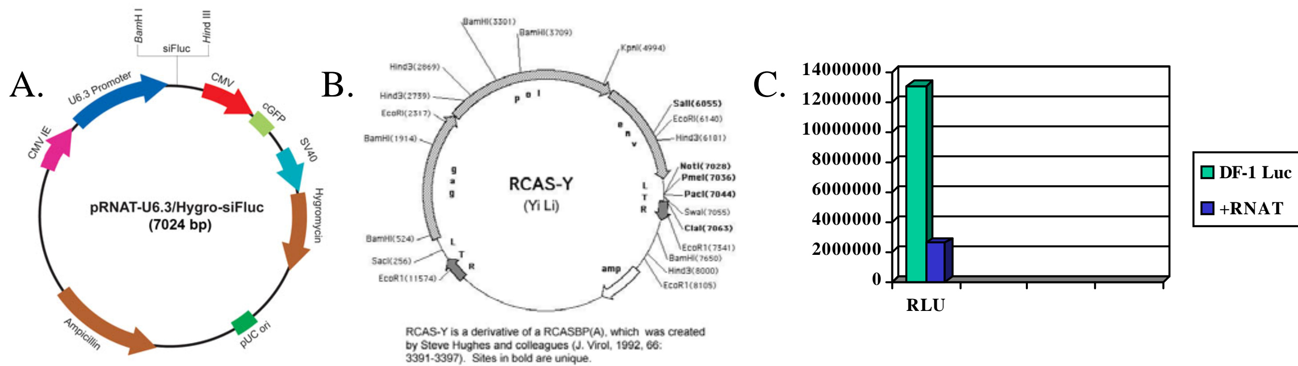

Figure 4: Construction of RCAS vector containing siRNA against Luciferase. A. RNAT vector (Genscript corporation) contains RNAi against luciferase driven by the U6.3 promoter and enhanced with the CMV enhancer. An approximately 2250 bp fragment was amplified from the RNAT backbone and inserted into the Cla and Pac sites (B). Transient transfection of luciferase-infected DF-1 viral producer cells were transiently transfected with RNAT and luciferase activity was measured after 72 hours (C). Greater than 80% reduction in luciferase activity was seen in cells containing RNAT than in controls.

The timeline initially outlined in the statement of work for the proposed experiments is shown below:

Year 1: Perform mouse breeding to generate cross, analyze mice for timeframe of tumor development and metastasis, confirm TVA expression within prostate cells and tumors, infect mice with luciferase and follow with imaging

Year 2: Continue with imaging experiments; optimize imaging protocol for orthotopic and IP injections, Begin shRNA design and cloning

Year 3: Complete cloning of shRNAs, test in vitro and in vivo, and follow mice with optical imaging

KEY RESEARCH ACCOMPLISHMENTS:

Generation of TRAMP-TVA MICE
Analysis of Prostate Cancer Initiation in TRAMP-TVA mice
Confirmation of TVA Expression in Prostate Epithelial Cells of TRAMP-TVA mice
Prostate Viral-Mediated Luciferase Gene transfer of TRAMP-TVA mice
Cloning Initation of Anti-Luciferase shRNA virus

REPORTABLE OUTCOMES: We have not yet submitted our results for publication, however, we are well underway to developing this model as a reproducible and efficient method for viral-mediated gene transfer of imaging genes and shRNAs into prostate epithelial cells. We plan on submitting abstracts to the upcoming American Urological Association Meeting and AACR meeting.

CONCLUSIONS:

Since initiation of funding, we have accomplished many of the goals outlined in the original statement of work in a faster timeframe than originally anticipated. We have established a new mouse line that is capable of multiple rounds of viral-mediated gene transfer of imaging and other target genes. We hope that we will be able to further establish this model of prostate cancer development and progression as a valid assay for testing the in vivo effect of a variety of genes.

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APPENDICES: N/A

SUPPORTING DATA: N/A