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14. ABSTRACT <u>Purpose:</u> The goal of the project is to determine the function of neuroendocrine (NE) cells in the initiation and progression of human prostate cancer <u>Scope:</u> 1) Use a pten null mouse prostate cancer model to determine if ablation of NE cells by selective expression of a toxin in these cells can delay or prevent tumor initiation and/or progression. 2) Use a human tissue recombination model to determine if depletion of NE cells from human epithelial cells can retard the initiation and progression of the recombinant tumor. 3). Demonstrating the origin and molecular basis of human small cell carcinoma <u>Major findings:</u> 1). We have successfully completed the vast majority of the research tasks; 2). We are in the process of restoring transgenic mice expressing toxin to complete task 1c; 3). We are in the process of optimizing tissue collection method to complete task 3c.					
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Introduction:

Despite years of study by clinicians and scientists around the world, there are still many unanswered questions in prostate cancer. A fundamental and clinically important issue is why prostate cancer responds to hormonal therapy initially but becomes resistant eventually in nearly all patients [1]. Prostate cancer is histologically heterogeneous consisting of luminal type tumor cells and a small component of neuroendocrine (NE) cells [2]. Unlike luminal type tumor cells that express androgen receptor (AR) and depend on androgen for proliferation, NE cells lack AR and are androgen-independent [3]. Hormonal therapy, while inhibiting luminal tumor cells, increases the number of NE cells in prostate cancer which is evident in recurrent castration-resistant prostate cancer [4]. In some patients, the recurrent tumor is composed of pure NE cells and is classified as small cell neuroendocrine carcinoma (SCNC) [5]. We hypothesize that NE cells play important roles in the initiation and progression of PC. We also hypothesize that they are the cells of origin for SCNC and p53 is the molecular target. This research proposal has the following specific aims: 1: To determine if NE cells are required for tumor initiation and/or progression in a mouse PC model; 2: To determine if NE cells are required for tumor initiation and/or progression in a human PC model; 3: Cell of origin and the molecular targets of prostatic small cell neuroendocrine carcinoma

Body:

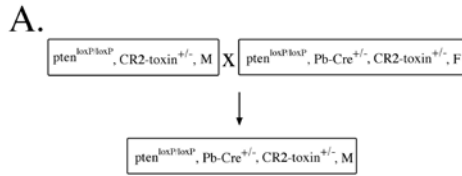
Research accomplishments associated with Task 1: In this task, we will generate *pten*^{loxP/loxP}/pb-Cre/CR2-toxin+ mice by breeding *pten* conditional knockout mice with CR2-toxin mice. We will then observe tumor development and whether the mice develop castration-resistant tumors after castration (Time frame: Months 1 – 36)

1a: Breeding and genotyping (Time frame: Months 1 – 24)

Our goal is to determine the function of neuroendocrine cells in the initiation and progression of prostate cancer. Our approach is to compare the mice with or without NE cells in the prostate their prostate cancer formation. The hypothesis is that in the male mice, the toxin will be expressed in prostate neuroendocrine cells because of the selective activity of CR2 promoter in such cells [6-7], resulting in ablation of the neuroendocrine cells. This will give us an opportunity to definitively determine the function of neuroendocrine cells in prostate cancer.

We have successfully established genotyping protocol for identifying mice that are of the desired genotype. As can be seen from Figure 1B, mice with prostate deletion of *Pten* can be identified through PCR by 3 primers: WT forward 5'TCCCAGAGTTCATACCAGGA3', WT reverse 5'GCAATGGCCAGTACTAGTGAAC3' and an internal primer 5'AATCTGTGCATGAAGGGAAC3'. For *Probasin-Cre* detection, we used the following primers: 5'CAAACAGGTAGTTATTCGG3' and 5'CGTATAGCCGAAATTGCCAG3'. For detection of *CR2-toxin*, we used two rounds

of PCR. In the first round, the following primers were used: 5'ctaacgctttcgctgttc3' and 5'tcgtaccacgggactaaacc3'. The product of the first PCR reaction was diluted 20 fold and used as the input for the 2nd round of PCR with the primers: 5'gctctctggaaaagctggag3', 5'aggaaggctgagcactaca3'.



Since the last report period, we have found a productive strategy to derive mice of desired genotypes after we realized that male mouse with prostate deletion of *Pten* is most likely not fertile. We therefore bred male mouse carrying the floxed *Pten* allele to the female mouse carrying both the floxed *Pten* and *Cre* gene as outlined in Figure 1A.

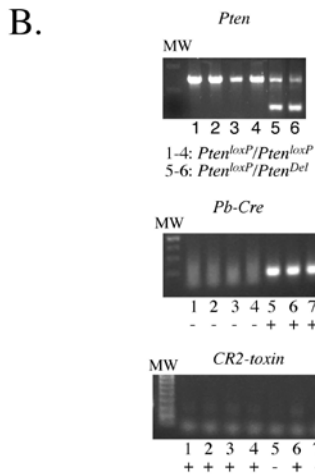


Figure 1. Genotyping strategies to identify mouse of desired genetic composition through PCR.

1b: Observing the development of primary tumors (Time frame: Months 12 – 24)

In the first reporting period, we demonstrated that CR2-toxin gene can ablate the prostate cancer formation in TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse model. Prostate tumors in TRAMP mice are composed exclusively of neuroendocrine cells. Therefore this result demonstrated that our approach to abolish neuroendocrine cells in the prostate with CR2 promoter-driven diphtheria toxin is a valid strategy, which will help us to definitively determine if

neuroendocrine cells play a role in prostate cancer.

Table 1. A collection of mice of various ages.

<i>Pten</i> ^{loxP/loxP} / <i>Pb-Cre</i>	<i>CR2-toxin</i> +	<i>CR2-toxin</i> -
Age (wks)		
54	2	1
43	1	2
38	2	6
33	2	2
29	2	2
25	3	2
21	3	2
total	15	17

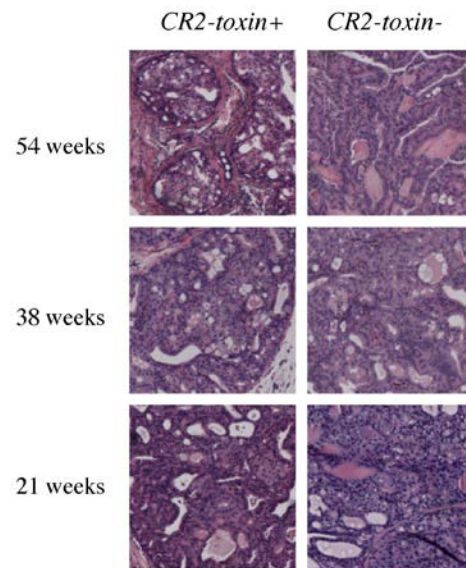


Figure 2. Morphological comparisons among mice with or without diphtheria toxin driven by CR2 promoter.

As outlined in Table 1, we have generated 32 mice with the desired genotypes of various ages. There were 15 mice carrying the diphtheria toxin, likely devoid of neuroendocrine cells as the toxin will kill the target cells. There were 17 mice without the toxin genes. The prostates of those mice were dissected and whole mount H&E analyses were performed on all of those samples. As shown in Figure 2, a sampling of histological analyses of those mice did not show a difference in the morphology of those prostates which uniformly were cancerous.

1c: Castration and observation of the development of castration-resistant tumors (Time frame: Months 24-36)

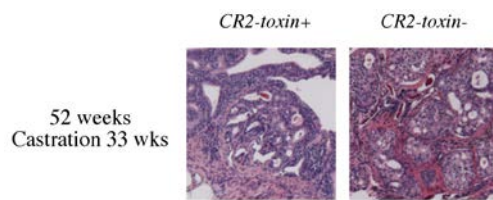


Figure 3. Morphological comparisons among castrated mice with or without CR2-toxin expression.

However, the toxin group seems to have more localized PIN rather than a more developed PIN pathology. Due to the limited number of mice of the castration group, we cannot make a firm conclusion about whether the neuroendocrine cells might play a role in castration-resistant growth of the *Pten*-loss induced prostate cancer.

We also collected 4 mice that were castrated at age of 19 weeks and continued for 33 weeks. There might be an interesting difference between the group carrying the toxin gene vs. the mouse that did not have the toxin gene. It appears that both groups have a smaller prostate due to castration, and the morphology of the prostate is of PIN, rather

We recently discovered that our mice have lost the expression of CR2-toxin transgene before we are able to complete the entire project. Loss of transgene expression is not an infrequent problem encountered by researchers. We have reported this problem to DOD and requested a no-cost extension. We are trying to solve this problem and to continue collecting mice with the desired the genotype of expressing *CR2-toxin* in mice with prostate deletion of *Pten*. If this effort is unsuccessful, we will request the transgenic mice from University of Texas Southwestern medical Center that provided the mice to us originally. We will focus on the impact of castration on the prostate cancer development in these mice, and analyze them at various time periods post castration to dissect the role of neuroendocrine cells in castration-resistant development of prostate cancer.

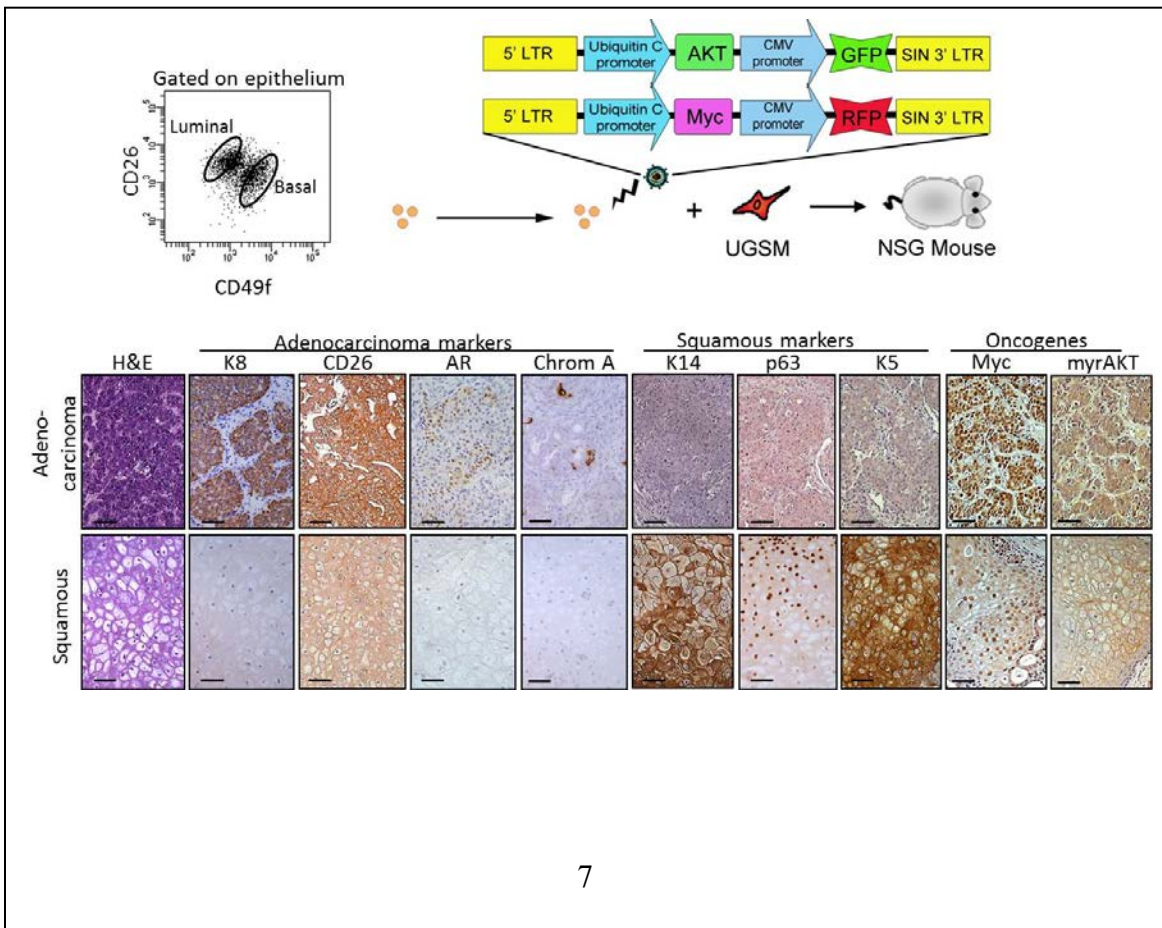
Research accomplishments associated with Task 2: In this task, we will procure fresh human prostate cancer tissue, separate tumor from benign prostate, separate epithelial cells into NE and non-NE cells, and perform tissue regeneration experiments to determine if NE cells are essential in tumor initiation and progression

2a. Procurement of fresh human prostate cancer tissue, separate tumor from benign prostate, separate tumor cells into NE and non-NE tumor cells (Time frame: Months 1 – 36)

We have established a robust system for the procurement of fresh prostate tissue, involving close collaborations among urologists, pathologists, Pathologist Assistants, technical staff from UCLA's Translational Pathology Core Laboratory (TPCL) and basic researchers [8]. There is a seamless workflow starting from when the prostate is removed from the patients and including rapid transportation of the specimen to pathology, gross examination of the prostate, procurement of tissue for research, diagnosis of the procured tissue, separation of the tissue into benign prostate and prostate cancer, preparation of single cell suspension and flow cytometric separation of sub-population of epithelial cells based on cell surface markers. We have published a high profile article describing the technology developed by our group (Goldstein et al. Nat Protoc. 2011; 6:656-67).

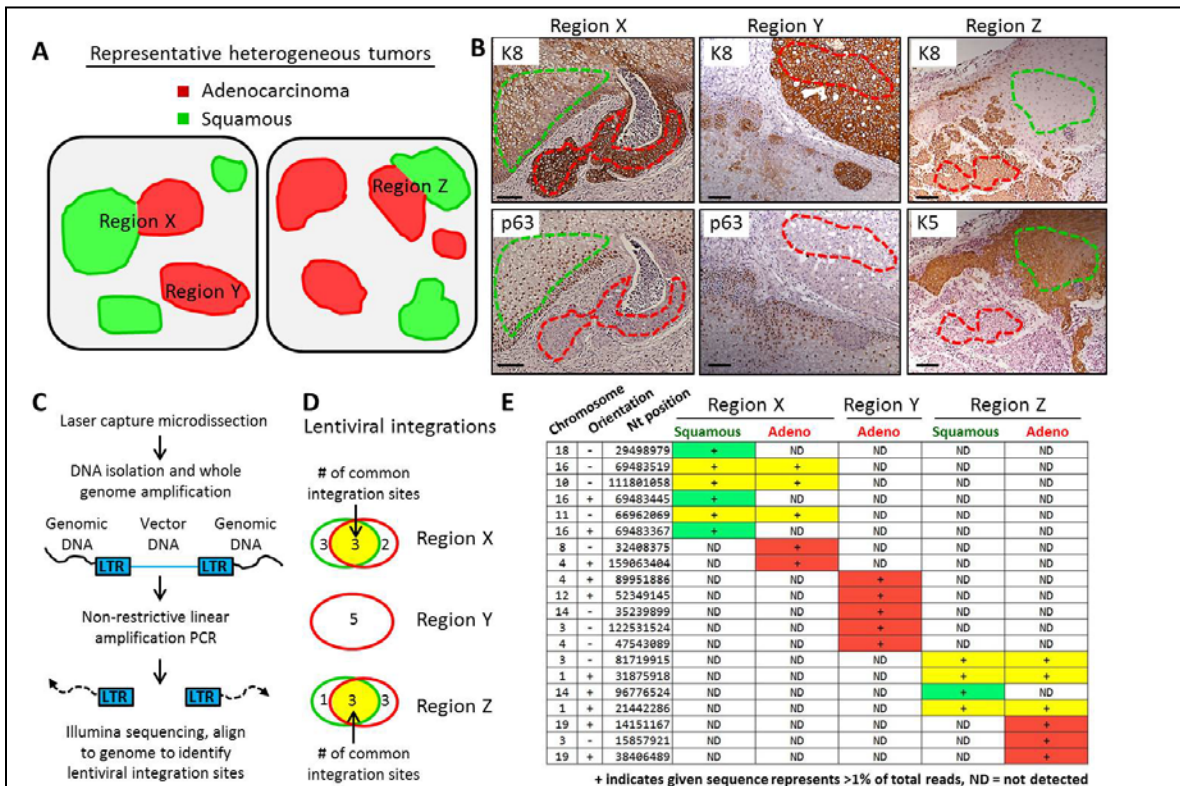
We explored the utility of several candidate cell-surface markers for the isolation/purification of an enriched subset of neuroendocrine cells using Fluorescence Activated Cell Sorting (FACS) on dissociated human prostate tissue preparations. After significant attempts and optimization, we identified the antigen CD56/NCAM (Neural Cell Adhesion Molecule) as the most robust marker for NE cells in primary human prostate. Our gating strategy was based on a negative depletion for the hematopoietic cell-surface marker CD45, positive enrichment for the epithelial antigens EpCAM (Trop1) or Trop2, and further separation into CD56+ (NE-enriched) and CD56- (NE-depleted) cells. Quantitative PCR analysis for classical NE genes including chromogranin A and Neuron-Specific Enolase (NSE) demonstrated significant enrichment for NE-specific transcripts in the CD56+ fraction compared to the CD56- subset.

2b. Tissue regeneration experiment to determine if NE cells are involved in tumor initiation (Time frame: Months 1 – 24)



Schematic of naïve human prostate *in vivo* transformation. Top: CD45-Trop2⁺ epithelial cells were sorted based on CD49f and CD26 into CD49f^{hi}CD26⁻ basal-enriched and CD49f^{lo}CD26⁺ luminal-enriched subsets, transduced with lentivirus carrying Myc, myrAKT or both, combined with UGSM cells and transplanted into NSG mice. Bottom: Representative adenocarcinoma and squamous regions are identified based on staining for H&E and antibodies against luminal markers Keratin 8 (K8), CD26 and androgen receptor (AR), the neuroendocrine marker chromogranin A, and basal/squamous markers Keratin 14 (K14), p63 and Keratin 5 (K5), and oncogenes Myc and myrAKT/pAKT. Scale bars, 100 μ m.

In our attempt to determine the role of distinct epithelial lineages in prostate cancer development and progression, we have generated and characterized a model of human prostate cancer initiating in naïve human prostate epithelial cells transduced with the oncogenes Myc and myristoylated AKT (myrAKT). Transduction of Trop2⁺CD49f^{lo}CD26⁺ luminal cells and Trop2⁺CD56⁺ neuroendocrine cells did not result in any detectable tumors. In contrast, Trop2⁺CD49f^{hi}CD26⁻ basal cells were efficient targets for cancer initiation.

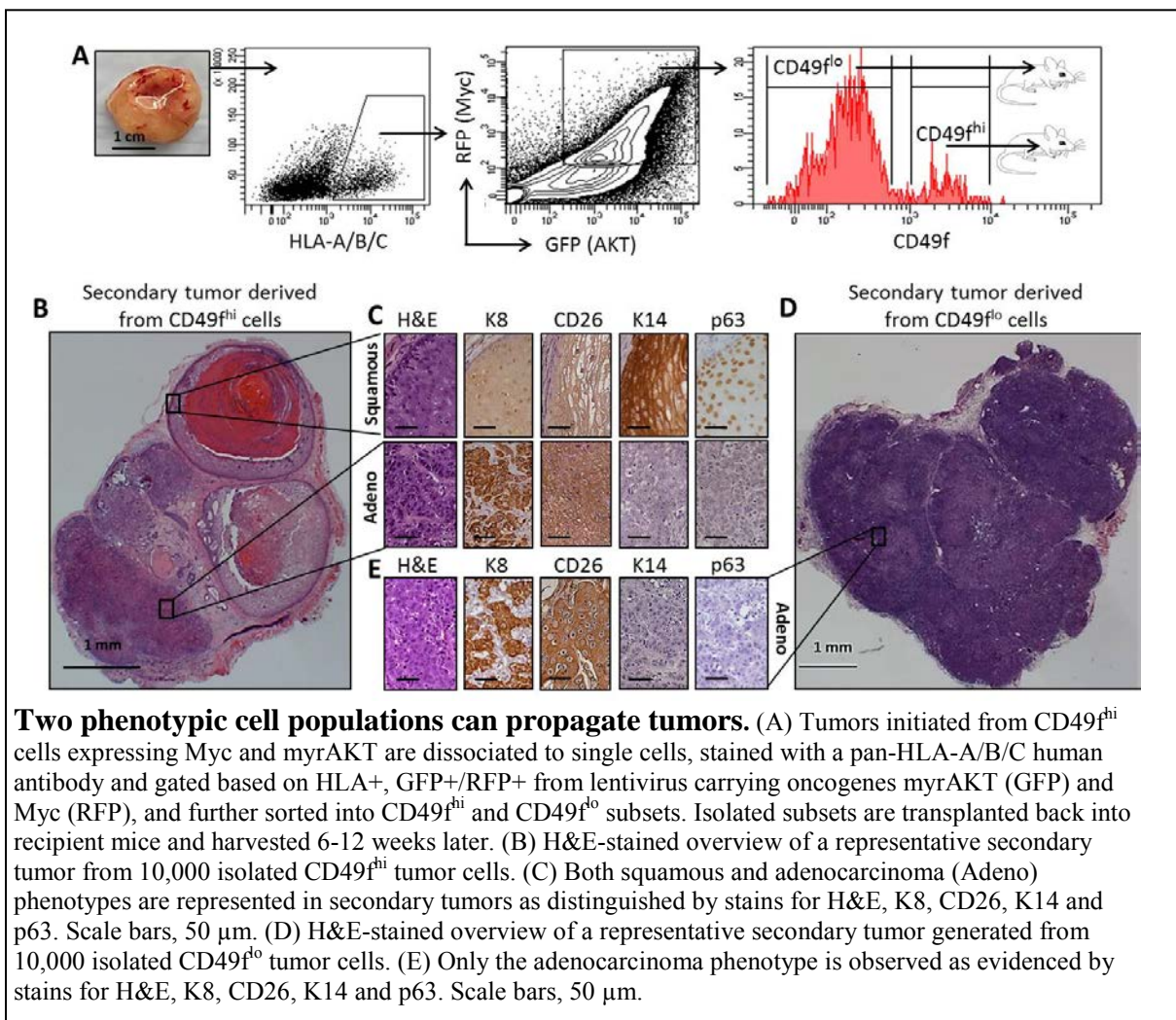


Distinct histological variants in heterogeneous tumors can share a clonal origin. (A)

Schematic of two different heterogeneous tumors containing adenocarcinoma, squamous or both phenotypes. Regions X, Y and Z were further studied for lentiviral integration site analysis. (B) Laser capture microdissection was performed on individual glands containing both squamous and adenocarcinoma phenotypes. Representative regions X, Y and Z are shown with serial tissue sections stained with K8 to highlight adenocarcinoma and either p63 or K5 to highlight squamous regions. Dotted lines indicate region excised using laser capture microdissection. Scale bars, 100 μ m. (C) Schematic of lentiviral integration site analysis. LTR: long terminal repeat (viral DNA), PCR: polymerase chain reaction. (D) Venn diagrams depict shared lentiviral integration sites in DNA isolated and amplified from neighboring adenocarcinoma (red) and squamous (green) phenotypes (region X), distinct adenocarcinoma

gland (region Y), and additional neighboring adenocarcinoma and squamous phenotypes (region Z). (E) Table lists all unique integration sites (IS) with genomic location identifiers (chromosome, orientation, nucleotide position) representing at least 1% of total reads (indicated by +) in each sample. Highlighted rows in yellow represent shared IS between distinct histological phenotypes in the same region, rows in red indicate IS unique to adenocarcinoma, and green represent IS unique to squamous. Note: different regions (X, Y, Z) do not share any IS.

Interestingly, we have found that primary tumors derived from Myc/myrAKT-transformed basal cells exhibit a heterogeneous or mixed tumor response, containing features of both adenocarcinoma and squamous cell carcinoma. Cells bearing both histological phenotypes are human in origin and express the oncogenes Myc and AKT. However, several markers are preferentially expressed in one or the other histological subtype. Expression of luminal-type markers Keratin 8, CD26 and Androgen receptor, and the neuroendocrine marker chromogranin A are found exclusively in adenocarcinoma tumor foci. In contrast, basal cell marker Keratin 5, Keratin 14, and p63 are exclusively expressed in squamous tumor foci.



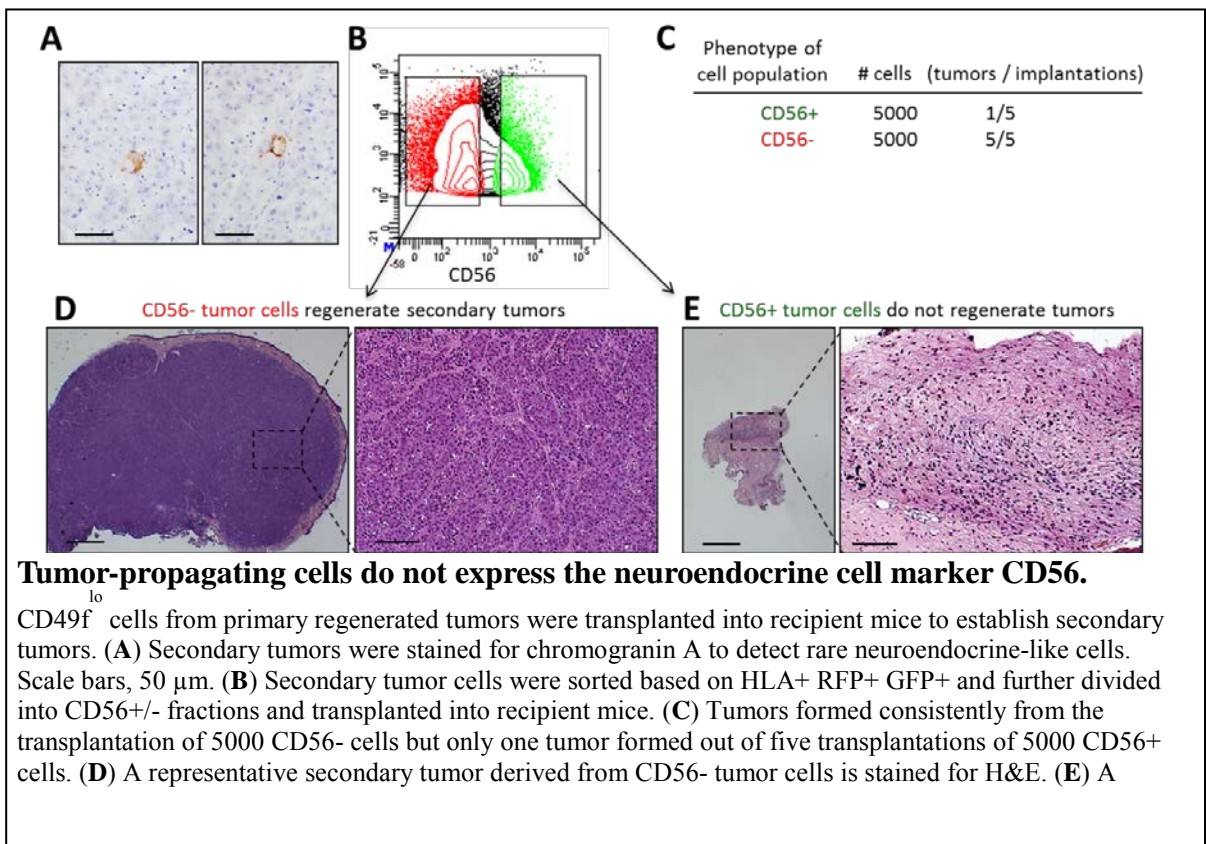
In addition to separate adenocarcinoma and squamous tumor foci, we also found mixed foci containing cells with both histological phenotypes. Since tumors were initiated by

lentiviral delivery of oncogenes into basal cells, the viral sequence randomly integrates into the genome of the target cell and all of its progeny. We isolated DNA from neighboring adenocarcinoma and squamous cells within a mixed tumor foci, and performed PCR extending from the viral DNA into the host genome containing the lentiviral integration site. We then performed deep sequencing and aligned reads to the genome. Our analysis revealed that both histological phenotypes are derived from a common clonal cell of origin.

To determine which cell-types are capable of propagating tumors, we utilized the antigen CD49f. We first showed that CD49f was expressed highly in the basal layers of squamous tumor cells, but not in adenocarcinoma. In contrast, adenocarcinoma cells express low levels of CD49f. We then fractionated CD49f^{hi} and CD49f^{lo} cells and transplanted both into recipient mice. Both phenotypic populations were competent to propagate tumors, however the histologies represented in the tumors differed. CD49f^{hi} cells could propagate heterogeneous mixed tumors, while CD49f^{lo} cells could only transplant adenocarcinoma.

2c. Serial transplantation and tissue regeneration to determine if NE cells are involved in tumor progression

Regardless of the role of NE cells in the initiation of prostate cancer, NE cells are continually found in tumors suggesting a role in tumor progression and maintenance [9]. As described above, we can establish aggressive primary human prostate cancer using lentiviral Myc and AKT transduced primary cells combined with UGSM cells in vivo into immune-deficient mice.



representative graft comprised of mesenchymal cells without a detectable tumor is shown, stained for H&E. Scale bars, 1 mm. Magnified image, 200 μ m.

While high levels of Myc alone or AKT alone were not sufficient to drive full progression to cancer, the combination synergized to initiate large highly-proliferative tumors. Dissociated tumor cells were capable of propagating adenocarcinoma upon transplantation into mice. Tumors maintained a phenotype that was Keratin 8+ and p63- indicating an acinar-type or luminal-like cell. Importantly, staining for chromogranin A indicated continued presence of NE cells in tumors. Therefore, we separated out the CD56+ and CD56- fraction from aggressive prostate tumors initiated by Myc and AKT, and transplanted each subset into mice. After 12 weeks, only the CD56- (NE-depleted) fraction could initiate tumors, demonstrating that in this model, NE cells may not be required for tumor propagation.

Research accomplishments associated with Task 3: In this task, we will procure fresh human prostate cancer tissue, separate tumor from benign prostate, separate epithelial cells into NE and non-NE cells, and perform tissue regeneration experiment to determine if SV40 T antigen induces SCNC in NE cells and if p53 is the molecular targets

3a: Same as 2a

3b. Tissue regeneration experiment to determine if NE cells are the cells of origin for SCNC (Time frame: Months 1 – 24)

Dissociated naïve benign human prostate tissue was separated by FACS into CD56+ (NE-enriched) and CD56- (NE-depleted) fractions and then transduced with lentivirus carrying the SV40 Large T-antigen. Transduced cells were combined with UGSM and transplanted into mice in vivo. Dissociated cells from two different patients were tested and no growths were established from either the CD56+ or CD56- fraction. These findings suggest two possible outcomes. First, the quality of the tissue from these patients may not have been sufficient for continued growth of the cells in the new hosts. Working with primary human tissue can be challenging as the length of time that tissues are kept without a blood supply before being utilized for research can vary greatly. The second possibility is that the SV40 T-antigen is toxic to naïve benign primary human prostate cells when introduced through lentiviral transduction.

3c. Tissue regeneration experiment to determine if p53 is the molecular target for SCNC (Time frame: Months 12 – 36)

We have successfully established tissue regeneration system from fresh human prostate epithelial cells immediately following prostatectomy. This is a generally robust experimental model but there are still challenges. Unfortunately, we have had some problems with our human prostate transformation system in the past year. We were unable to regenerate human structures using the tissue provided to us through the pathology core following radical prostatectomy. The main problem is that many of the surgeries are finished late in the day. Since such tissues need to be processed by

pathology personnel for diagnosis first before any tissue is taken for research, the availability of pathology personnel is crucial. When the prostate arrives in pathology late in the day and cannot be processed same day, the prostate is held in media overnight prior to processing the following day. Although this is perfectly fine for the purpose of histologic diagnosis, it proves to be detrimental to tissue regeneration as cell viability is severely compromised once an extra day is added before tissue recombination and implantation in animals.

We have now changed the protocol to ensure that tissue used for in vivo experiment is processed the same day of surgery or that material is not considered suitable for these expensive and lengthy studies. We have just begun a new round of viral preparation in order to proceed with the proposed experiments now that the assay appears to be working well again. We have published a paper showing that the model can work to generate robust tumor responses using the oncogene combination Myc and AKT which can serve as a positive control for an aggressive subset of tumors derived from benign human prostate epithelium.

Key Research Accomplishments

- 1). The vast majority of the tasks have been accomplished
- 2). We are in the process of restoring the transgenic mice expressing CR2-toxin in order to complete task 1c.
- 3). We are optimizing the tissue collection procedure to improve the robustness of the tissue recombination experiment in order to complete task 3c.

Reportable outcomes

Manuscripts, abstracts, presentations;

Manuscripts:

1. Ren S, Wang F, Shen J, Sun Y, Xu W, Lu J, Wei M, Xu C, Wu C, Zhang Z, Gao X, Liu Z, Hou J, Huang J, Sun Y. Long non-coding RNA metastasis associated in lung adenocarcinoma transcript 1 derived miniRNA as a novel plasma-based biomarker for diagnosing prostate cancer. *European journal of cancer (Oxford, England : 1990)*. 2013;49(13):2949-59. Epub 2013/06/04. doi: 10.1016/j.ejca.2013.04.026. PubMed PMID: 23726266.
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Presentations:

Invited by academic institutions:

1. Neuroendocrine Differentiation in Prostate Cancer, Department of Urology, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China, May 2013
2. Neuroendocrine Differentiation in Prostate Cancer, Department of Urology, West China Hospital of Sichuan University, Chengdu, China, June 2013
3. Recent progress in prostate cancer. Wuxi Second People's Hospital, Wuxi, China, November 2013
4. Recent progress in prostate cancer. National Center of Biomedical Analysis (NCBA), Beijing, China, November 2013

Invited by local, national or international conferences:

1. Neuroendocrine Differentiation in Prostate Cancer, the 2nd Annual PCF China Scientific Symposium, Shanghai, China, May 2013
2. Neuroendocrine Differentiation in Prostate Cancer, Annual Urology Conference, the 2nd Affiliated Hospital of Zhejiang University, Hangzhou, China, May 2013
3. Genitourinary Pathology Case discussion, 2nd Huaxia Pathology Forum, Guilin, China, June 2013
4. Targeted biopsy of prostate cancer, The 7th Annual Forum of Prostate Diseases, Shanghai, China, July 27, 2013
5. Challenges in the Diagnosis of Small Cell Neuroendocrine Carcinoma, The 7th Annual Forum of Prostate Diseases, Shanghai, China, July 27, 2013
6. Pathology and Biology of Neuroendocrine Prostate Cancer, Pathology Workshop for t-NEPC organized by Prostate Cancer Foundation. New York, NY. July 31, 2013

7. Recent progress in prostate cancer. Soochou University Annual Translational Medicine Meeting, Suzhou, China, November 2013
8. Prostate Cancer Stem Cells, Annual Jingmeng Stem Cell Conference, Beijing, China, Feb 15, 2014
9. Aggressive variants of castration-resistant prostate cancer. The Annual Meeting of the Basic Science Division, Chinese urologic Association, April 26, 2014.

Licenses applied for and/or issued:

None

Funding applied for based on work supported by this award

1. National Cancer Institute 1R01CA172603-01A1 (PI: Jiaoti Huang)

Period: 07/01/2013 – 6/30/2018

Title: A Novel Strategy to Identify Prostate Cancer Biomarkers for Patient Management

Direct cost: \$1,037,500 (all 5 years)

Percent effort: 20%

Role: Principle Investigator

CONCLUSION:

We have completed the vast majority of the research tasks. In the past year, we experienced some technical challenges. We are in the process of restoring the transgenic mice expressing CR2-toxin in order to complete task 1c. We are optimizing the tissue collection procedure to improve the robustness of the tissue recombination experiment in order to complete task 3c. The support from DOD has led to many high impact publications, invited presentations in academic institutions as well as local, national and international meetings. Most importantly, continued effort in the study of prostate cancer has resulted in the successful application of RO1 grant awarded to our laboratory this past year.

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Appendices: