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14. ABSTRACT

Heterogeneity, variable and often unpredictable clinical course are fundamental challenges in management of patients with prostate cancer. To make rapid advances in understanding of disease mechanism that can be translated to clinical care in short order, there is an immediate need for innovative in vivo disease models that accurately recapitulate human disease at cellular level. We propose to develop an innovative and hitherto not attempted in vivo prostate cancer model that will delineate the exact cell of origin through different stages of prostate cancer development and progression. We propose to study possible cell(s) of origin for prostate cancer by combinatorial expression of florescent magenta, cyan and yellow primary color proteins in prostate at development in mice. The study includes (1) Construction of "Prorainbow" plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters. (2)Establish mouse line with the resulting "Prorainbow" construct and generation of transgenic mice by crossing with Cre mice. (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions. The study is expected to produce in vivo animal of prostate cancer with unique capabilities.

15. SUBJECT TERMS
Prostate cancer, in vivo model, Prorainbow, tumor development

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Annual Report

Title: Tracking Origins of Prostate Cancer – an Innovative *in vivo* Modeling

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Introduction

Prostate cancer is the second most frequently diagnosed cancer of men and the fifth most common cancer overall. The cancer cells may metastasize from the prostate to other parts of the body, particularly the bones and lymph nodes. About 20% of patients undergoing radical prostatectomy develop metastasis beyond 5 years, suggesting metastasis is an early event and removal of primary tumor does not significantly decrease the rate of metastasis. Thus, understanding the role of the genetic changes leading to origination and development of primary tumor and metastasis would provide for a targeting strategy to clinical therapy. The goal of this project is to study the origin of cancer cells within the prostate. Since development of human prostate cancer proceeds through a serious of defined states, we would utilize a newly developed fluorescent protein labeling technique, Brainbow, which has been used to study the nervous system development in Brain[1]. Similar to the 'Brainbow' concept we propose 'Prorainbow' modeling to track prostate cell proliferation and differentiation by labeling individual early prostate precursor cell a unique color. In case of a tumor or metastasis, we can track down the ancestor normal cell by matching to the tumor cell color. We can then track these color distributions and pattern changes with time course, which will build up a dynamic vision of prostate cancer progression. Also, we want to examine functions of Protein Kinase D1 (PKD1) and Phosphatase and Tensin homolog (Pten) in conditional knockout mice in the development of cancer formation and metastasis in the prostate. Successful development of florescent labeled *in vivo* animal model will be unique in the field of prostate cancer research and provide much needed advance to understand progression of prostate cancer.

We proposed to testify the stated hypothesis with following aims:
1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.
2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.
3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

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Aim (1) Construction of 'Prorainbow' plasmid with fluorescent proteins (XFPs) under control by prostate epithelial and basal cell-specific promoters.

Task I. Generation of *Probasin* promoter controlled XFP.

Probasin is a prostate specific and androgen-regulated protein, which can be used as a marker of prostate differentiation. The rat probasin promoter (ARR2PB), which is 455 bp in size, has been successfully cloned and used in transgenic mice to target high-level, prostate-specific expression of down-stream transgenes[2], and the expression regulated by Cre recombinase is in both basal and luminal epithelial cells.

We generated the Pro-rainbow construct PB-XFP and checked the DNA plasmids by restriction enzyme digestion (Fig 1A) and DNA sequencing. We confirmed that cells transfected with those plasmids can express fluorescent proteins under the control of Probasin promoter by testing the expression of this construct in human prostate LnCap cells (Fig 2A), as well as the expression in other human cell lines (NIH 3T3, HEK 293, MCF7, MS1, normal kidney primary cells and smooth muscle primary cells). This part is done.
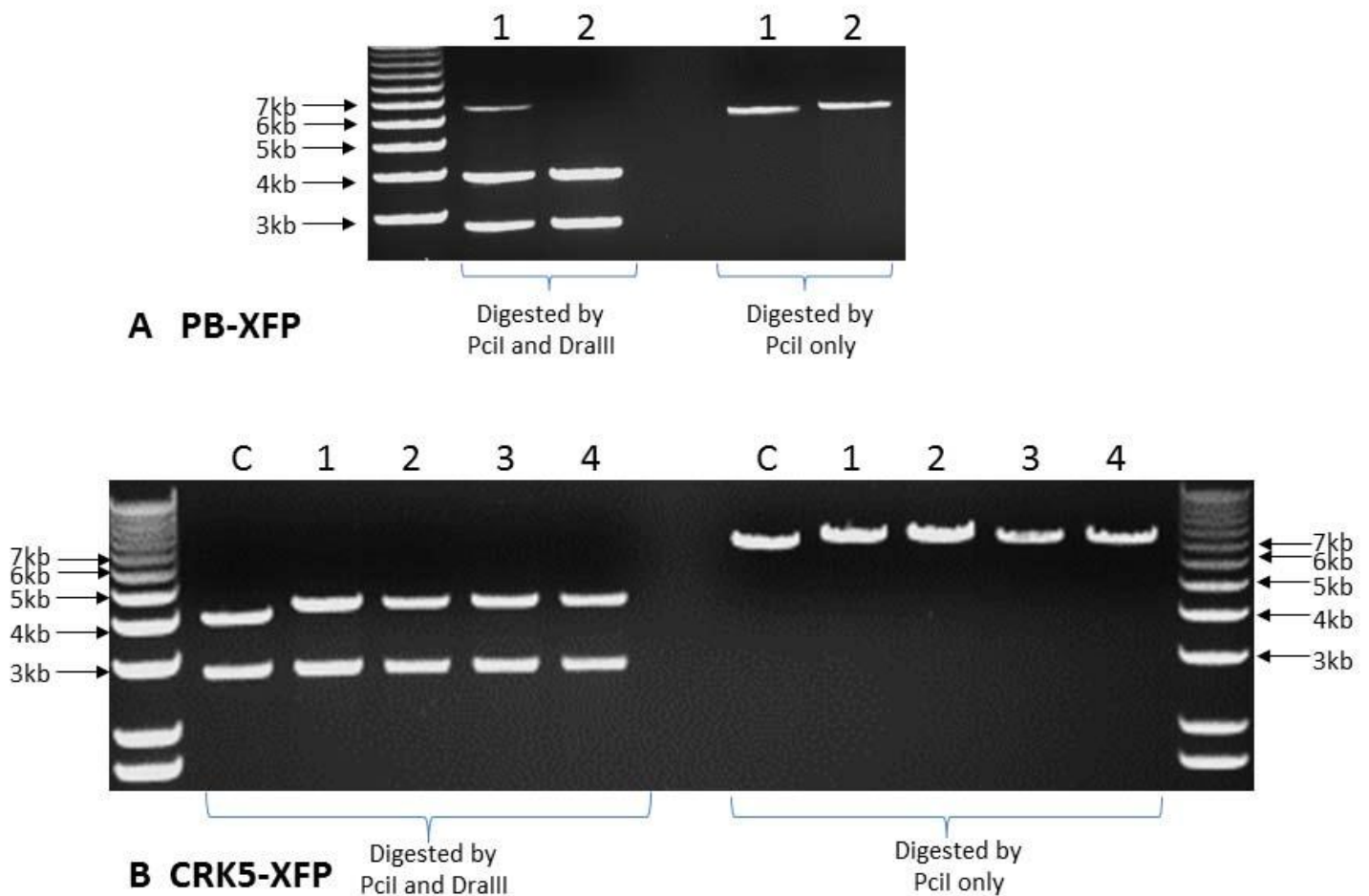
Figure1 PB-XFP and CRK5-XFP DNA constructs were successfully produced. In A, 1 and 2 are DNA construct candidates for PB-XFP. 2 is of correct size, checked by single digestion with PciI (right two lanes) or double digestion with both PciI and DraIII (left two lanes). In B, 1~4 are DNA construct candidates for CRK5-XPF, and all four of them are of correct size, checked by single digestion with PciI (right panel) or double digestion with both PciI and DraIII (left panel). C is the control DNA construct which is CMV-XFP (Brainbow 1.0L). Expected sizes: linearized PB-XFP (single digested by PciI), 6737bp; linearized CRK5-XFP (single digested by PciI), 7179bp; linearized CMV-XFP (single digested by PciI), 6856bp; transgene with Probasin promoter (digested by PciI and DraIII), 3686bp; transgene with Cytokeratin 5 promoter (digested by PciI and DraIII), 4128bp; backbone of the plasmid (digested by PciI and DraIII), 3051bp.

Task II. Generation of *Cytokeratin 5* promoter controlled XFP.

Cytokeratin (CRK) 5 is expressed specifically in basal layer of all stratified squamous epithelia[3-5]. After the differentiation, CRK5 expression would be gradually lost[6], which makes CRK5 promoter an ideal candidate for direction of XFP expression.

We generated the Pro-rainbow construct CRK5-XFP and checked the DNA plasmids by restriction enzyme digestion (Fig 1B) and DNA sequencing. We confirmed that cells transfected with those plasmids can express fluorescent proteins under the control of Cytokeratin 5 promoter by testing the expression of this construct in human prostate LnCap cells (Fig 2B), as well as the expression in other human cell lines (NIH 3T3, HEK 293, MCF7, MS1, normal kidney primary cells and smooth muscle primary cells). This part is done.
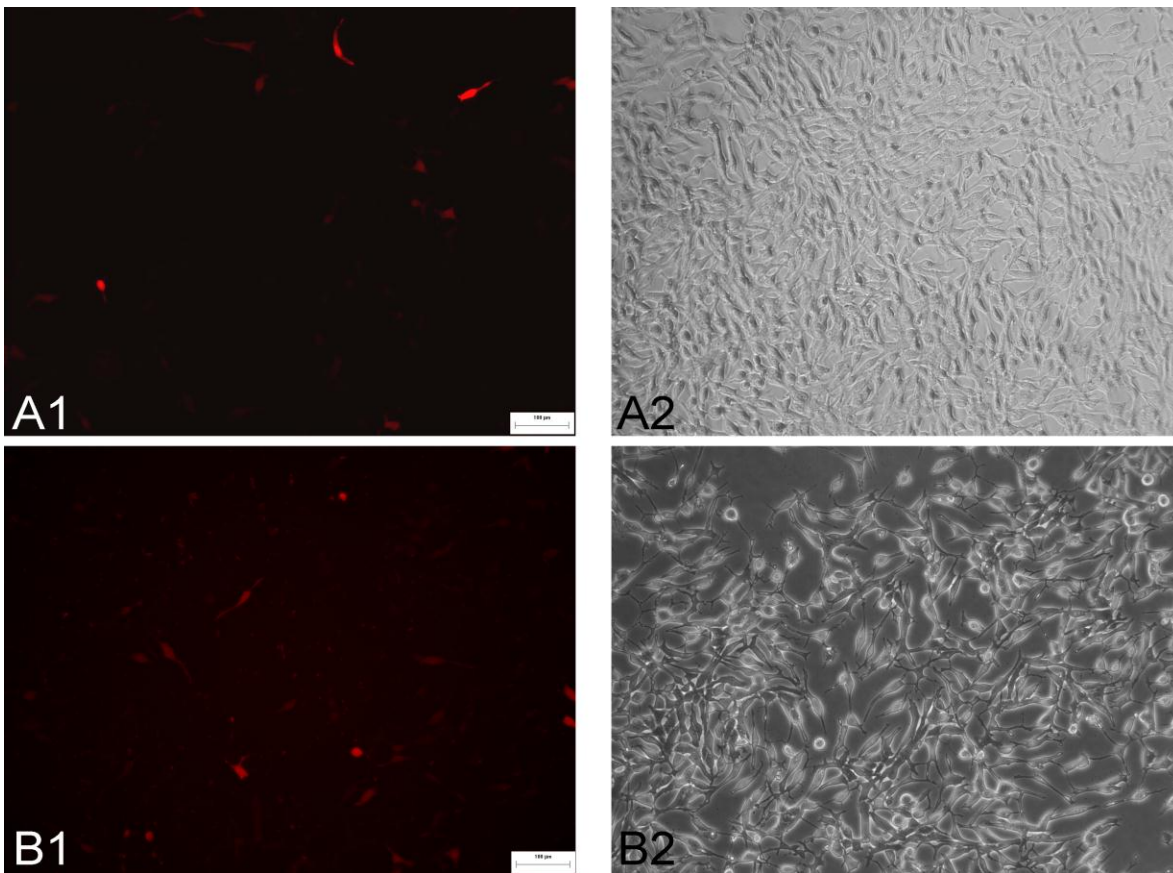
Figure 2 Transfection of Prorainbow constructs in LnCap cells. A, PB-XFP transfection. A1 is red fluorescent view and A2 is bright field view. B, CRK5-XFP transfection. B1 is red fluorescent view and B2 is bright field view. Scale bar, 100 μm.

Aim (2) Establish mouse line with the resulting 'Prorainbow' construct and generation of transgenic mice by crossing with Cre mice.

Task I. Obtain institutional approval for animal study.

This part is done and was reported in prior annual report.

Task II. Generation of Prorainbow construct expressing transgenic mice (n=3~5).

We prepared the Prorainbow fragment and the pronuclear injection was done by University of North Carolina (UNC) Animal Models Core. Nine PB-XFP founder animals (Fig.3) and eight CRK5-XFP founder animals were generated after genotyping (Fig. 4). Primers RPBPF (5'-TCTGATTGGAGGAATGGATAAT AGTCATC-3') and tdTomato-R2 (5'-CACCTTGAAGCGCATGAACTCTTTGATG-3') were used for PB-XFP founder sequencing, and primers HCK5P-F (5'- GCAAGGCAAGGTTATTTCTAACTGAGCA-3') and tdTomato-R2 (5'-CACCTTGAAGCGCATGAACTCTTTGATG-3') were used for CRK5-XFP founder sequencing. Mouse *Rag1* primers F2 (5'-TTCTGCCGCATCTGTGGGAATC-3') and R2 (5'-CTTCACATCTCCACCTTCTTCTTTGTCAG-3') were used in PCR as DNA loading controls. The PCR cycles used in genotyping were: 95 °C, 2 min; 95°C, 30 sec, 58 °C, 30 sec, 72 °C, 1min, 35 cycles; 72°C, 10min.
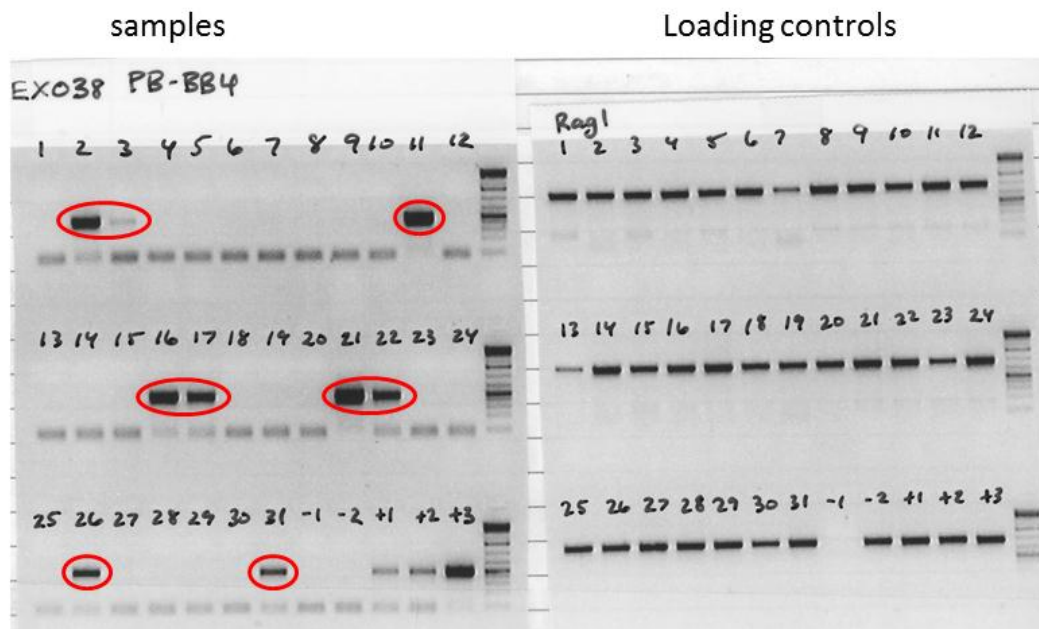
Conclusion: animals #2, 3, 11, 16, 17, 21, 22, 26 and 31 are positive for the transgene

Figure 3 Genotyping results of PB-XFP founder mouse candidates. Samples:#1-21, samples from potential founder animals. Controls: -1, dH$_2$O template; -2, wt DNA; +1, PB-XFP transgene diluted in wt DNA at 0.1 copy/genome; +2, PB-XFP transgene 1 copy/genome; +3, PB-XFP transgene 10 copies/genome. Left panel used primer pair RPBPF and tdTomato-R2 in PCR, and expected product size is 492bp; right panel used primer pair Mouse *Rag1* primers F2 and R2 in PCR, and expected product size is 960bp.
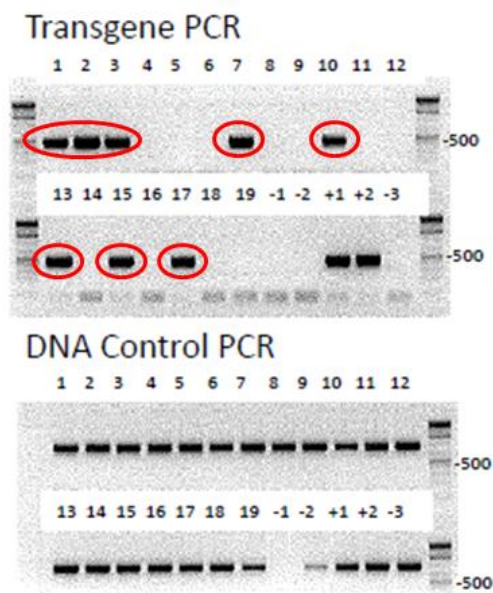
Conclusion: Animals 1, 2, 3, 7, 10, 13, 15 and 17 are positive for the transgene

Figure 4 Genotyping results of CRK5-XFP founder mouse candidates. Samples:#1-19, samples from potential founder animals. Controls: -1, dH$_2$O template; -2, wt DNA; +1, CRK5-XFP transgene 1 copy/genome; +2, CRK5-XFP transgene 10 copies/genome; -3, PB-XFP transgenic animal DNA. Left panel used primer pair hCK5P-F and tdTomato-R2 in PCR, and expected product size is 452bp; right panel used primer pair Mouse *Rag1* primers F2 and R2 in PCR, and expected product size is 960bp.

We are crossing PB-XFP founders with Cre mice to test the expression level of fluorescent proteins in prostate and will screen for three lines from them. CRK-XFP founders are still in quarantine phase after they were transferred from UNC and will be screened after they are released from quarantine. We plan to finish the founder line screening in the following 3-6 months.

*Task III.* Cross-breeding of Cre mice with Prorainbow transgenic mice.
Three different crossings are planned to be set up to validate the Prorainbow system and to study normal prostate development.
1) ROSA-CreER X PB-XFP
2) PB-Cre4 x CRK5-XFP
3) PB-Cre4 x CMV-XFP

ROSA-CreER mice were ordered from JaxMice (stock 008463, B6.129-Gt(ROSA)26 Sor tm1(cre/ERT2)Tyj/J) and arrived at our facility recently. We will start the breeding with PB-XFP founders and check the founder lines. The offspring from the first mating method are expected to show color spectra in whole prostate (both basal and luminal epithelial cells) with tamoxifen induction.

PB-Cre males are available in our lab and we keep an active breeding line of those animals. We are waiting for the CRK5-XFP founders to be released from quarantine phase and we will start the breeding as soon as they are available to check the fluorescent protein expression. The offspring from the second mating method are expected to show color spectra prostate stem cells which reside in basal layers. Also, the fluorescence will decrease in daughter cells after each cell cycle, given the fact that CRK5 promoter activity diminishes with progressive differentiation.

We have got several male mice express CMV-XFP under Probasin promoter driven Cre induction based on genotyping results (Fig. 5). PB-F and PB-R primers were used in genotyping for Probasin promoter (PB-F, 5'-AGTC ATTAAT AAGCTTCCACAAGTGCATTTAGCCTCTCC-3'; PB-R, 5'- AGTC GCTAGC CTGTAGGTATCTGGACCTCACTGAC-3'). Primers 11341, oIMR8545 and oIMR8916 were used in genotyping for Brainbow2.1 transgene (11341, 5'-GAATTAATTCCGGTATAACTTCG-3'; oIMR8545, 5'-AAAGTCGCTCTGAGTTGTTAT-3'; oIMR8916, 5'-CCAGATGACTACCTATCCTC-3'). Their genotype is PB-Cre; Brainbow2.1/+ or PB-Cre; Brainbow2.1/Brainbow2.1 (Fig.5). The offspring from the third mating method is expected to have unique color spectra in prostate only.
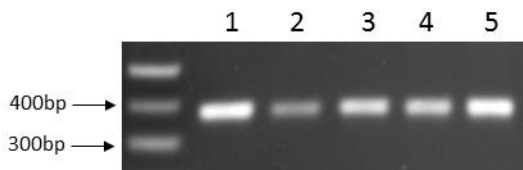
Pb-Cre: 393bp   WT: no band
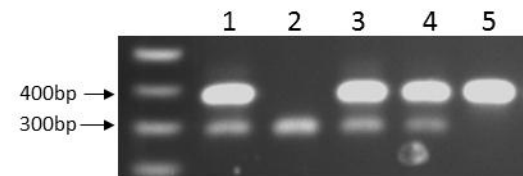
Brainbow2.1 homozygous: 300bp; WT: 386bp
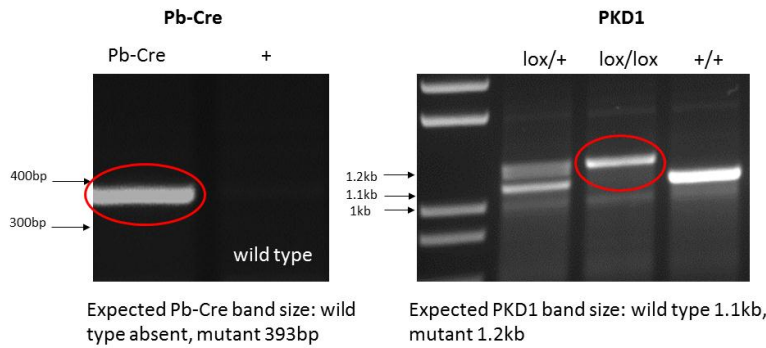Heterozygous: both 300bp and 386bp bands

Figure 5 Genotyping results of PB-Cre; Brainbow 2.1/+ or PB-Cre; Brainbow 2.1/Brainbow 2.1 mice. Mice #1, 3 and 4 are PB-Cre; Brainbow 2.1/+. Mouse #2 is PB-Cre; Brainbow 2.1/Brainbow 2.1. Mouse #5 is PB-Cre only. Mice #1-4 will express fluorescent color spectra in prostate. Mouse#5is a negative control, as it won't express fluorescent proteins in prostate.

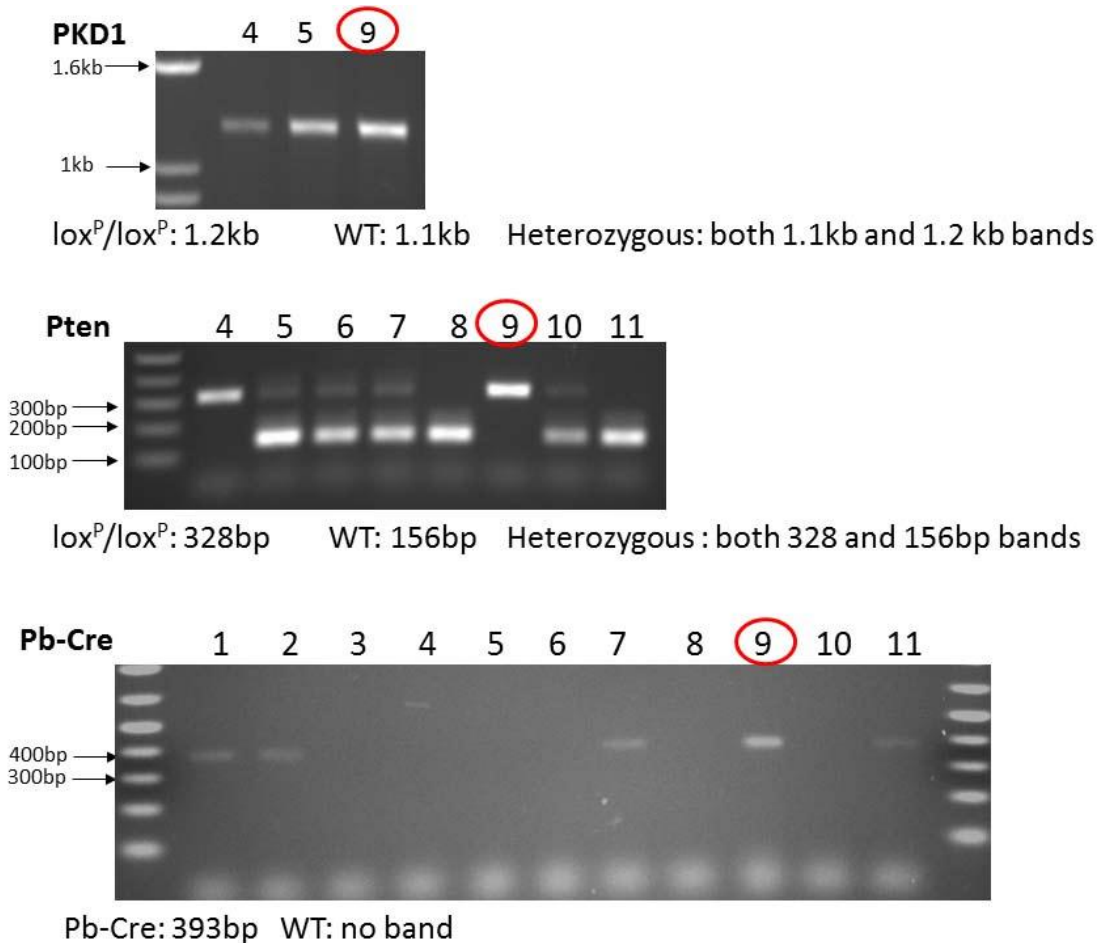We plan to finish this part in the following 6-9 months.

Task IV. Cross-breeding of Prorainbow transgenic mice for cancer research.

We generated PKD1 prostate-specific knock-out male mice based on genotyping results (Fig.6) and keep an active breeding line of those animals. PKD1#2 and PKD1#3 rev primers were used for genotyping of PKD1 loxP insertion (PKD1#2, 5'-TGTTCTCCCCAGTGGCAT-3'; PKD1 #3 rev, 5'-AAACGGAAATGCTCACAGAAATAT-3'). We are doing the breeding to generate PKD1 and PTEN double knockout (specifically knocked out in prostate) mice by crossing PB-Cre4; PKD1$^{lox/lox}$ and PTEN$^{lox/lox}$ mice, and check whether the double knockout animals would have increased possibility to initiate primary tumor or start metastasis. So far we got one animal of expected genotype (genotyping results, Fig.7). Primers oIMR 9554 and oIMR 9555 were used for genotyping of PTEN loxP insertion (oIMR9554, 5'-CAAGCACTCTGCGAACTGAG-3'; oIMR9555, 5'-AAGTTTTTGAAGGCAAGATGC-3').

Expected Pb-Cre band size: wild type absent, mutant 393bp

Expected PKD1 band size: wild type 1.1kb, mutant 1.2kb

Figure 6 Genotyping results of PKD1 knock-out mouse. Red circles indicating the expected genotype of PKD1 knock-out animal, which is Pb-Cre; PKD1 lox/lox.

lox^P/lox^P: 1.2kb   WT: 1.1kb   Heterozygous: both 1.1kb and 1.2 kb bands

lox^P/lox^P: 328bp   WT: 156bp   Heterozygous : both 328 and 156bp bands

Pb-Cre: 393bp   WT: no band

Figure 7 genotyping results of PKD1 PTEN double knock-out mouse. Mouse #9 (highlighted by red circle) has the expected genotype of double knock-out animal.

We are working on the breeding to get PTEN or PKD1 knock-out mice expressing fluorescent proteins in prostate for cell lineage labeling, as well as PKD1 PTEN double knock-out mice with fluorescent protein expression for cell lineage labeling. We plan to finish the work in following 6-9 months.

Aim (3) Study the transgenic Prorainbow mice under normal and oncogenic conditions.

*Task I.* Evaluate combinatorial expression of XFP in prostate of Prorainbow mice.

For PB-Cre4 x CMV-XFP breeding, we got several mice with PB-Cre; Brainbow 2.1/+ mice. We studied the fluorescent protein expression in frozen sections of prostate by both direct observation (Fig. 8) and immunohistochemistry (IHC, Fig. 9). Due to the limit of equipment we only checked green and red fluorescent proteins at the moment. We plan to check all colors in the later study.

Currently we are testing the Prorainbow mouse founder lines (PB-XFP and CRK5-XFP). We will study the anatomical distribution of color spectra in the prostate to see whether the same lineage cells localize focally or diffusely. Also, we will study the number of different spectra can be seen in a prostate gland to check the minimal number of stem cells that is required for gland formation.

We plan to finish this part in the following 6-12 months.
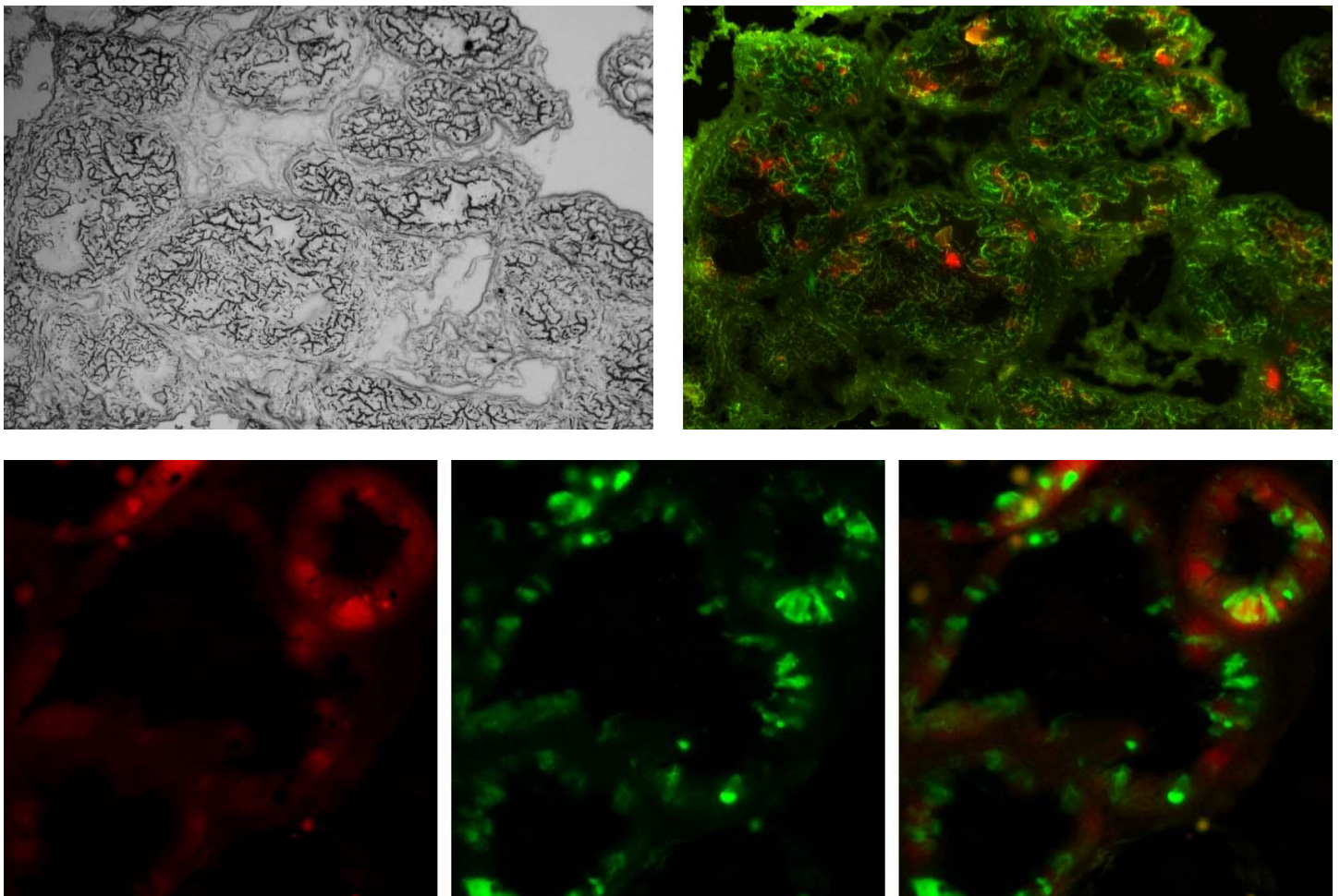
Figure 8 Fluorescent protein expression in frozen section of PB-Cre; Brainbow 2.1/+ mouse prostate. Upper left, bright field view. Upper right, fluorescent view, merged signals of GFP and RFP. Lower panel, RFP (left), GFP (middle) and merged image (right).
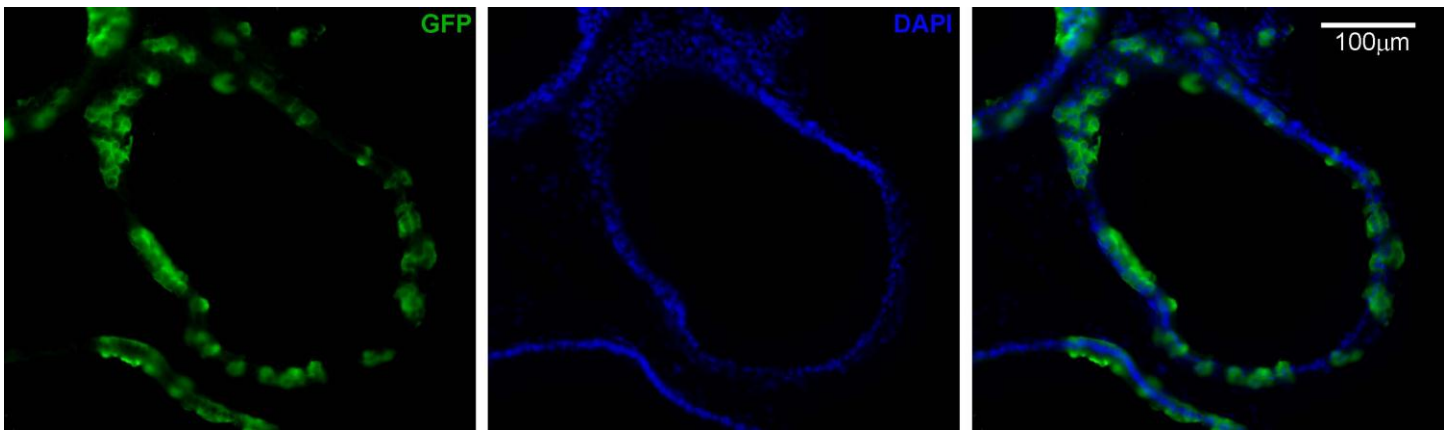
Figure 9 Fluorescent protein detected by immunostaining in PB-Cre; Brainbow 2.1/+ mouse prostate. The sectioned prostate tissue was stained with both anti-GFP antibody (left panel) and DAPI (middle panel). Right panel is the merged image. Scale bar, 100 μm.

Task II. Study of Prorainbow and PTEN or PKD1 knock-out mice hybrids.

We have generated PKD1 prostate-specific knock-out male mice. We euthanized those animals, harvested prostate tissue from them and confirmed that the knock-out of PKD1 protein at both RNA level (Fig. 10) and protein level is significant and tissue specific (Fig. 11). PKD1#1 primer and PKD1#5 rev primer were used for RT-PCR to detect the wild type PKD1 mRNA level (PKD1#1 primer, 5'-AAGTGACCATCAATGGAG-3'; PKD1#5 rev primer, 5'-AAA TGA AGA TGT CGC AAA-3'). We will detect metastasis in PKD1 PTEN double knockout mice by flow cytometry analysis of tumor cells in circulation system (which are XFP positive under the expression control of PB promoter, and are of prostate origin). PTEN knockout mice will be used as control. In case the tumor cells are detected in circulation, the mouse will be sacrificed and the organs known for prostate cancer metastasis (e.g. bone marrow, lymphoid, lung, liver) will be examined for XFP positive cells. If metastatic cells are found, we will establish whether these cells have the same color spectrum(monoclonal) or diverse color spectra (polyclonal). We can also track back the prostate progenitors of the metastatic cells by matching the color spectra.

We plan to finish this part in the following 6-12 months.
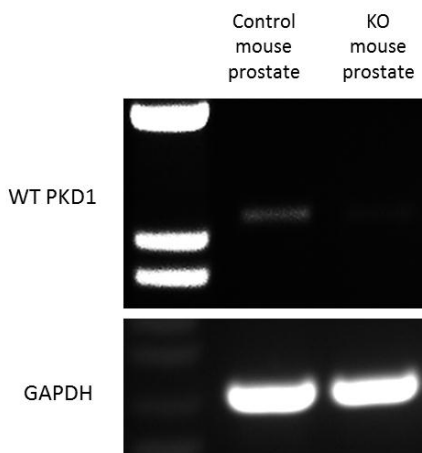
Figure 10 Wild type PKD1 mRNA level is significantly decreased in PB-Cre; PKD1^lox/lox mouse prostate, comparing to wild type mouse prostate. Expected PCR product from wild type PKD1 mRNA amplification is 1.13kb. GAPDH level was used as loading control.
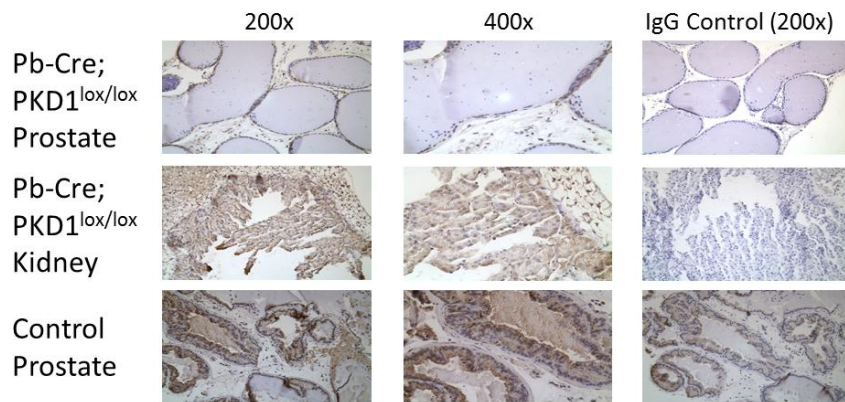
Figure 11 Wild typePKD1 protein level is significantly reduced in PKD1 knockout animal prostate, but not in other organs in the same animal or in wild type animal. Antibody PKC μ (C-20) (sc-639, Santa Cruz Biotechnology Inc.) was used as primary antibody for mouse PKD1 detection. In control experiment mouse IgG was used instead of anti-PKD1 antibody.

Key research accomplishments

After construction of PB-XFP and CRK5-XFP prorainbow DNA plasmids, we successfully produced PB-XFP and CRK5-XFP prorainbow mouse founders. We confirmed the fluorescent protein expression in normal mouse prostate by both direct observation and immunohistochemistry. We successfully produced prostate specific PKD1 knock-out mouse and PKD1 PTEN double knock-out mouse. We confirmed that PKD1 expression is decreased in PKD1 knock-out mouse at both mRNA level and protein level, and that the gene knock-out is prostate specific.

Reportable outcomes

None.

Conclusion

Probasin promoter controlled fluorescent protein expression can be successfully detected in transgenic mouse prostate. Prostate-specific PKD1 knock-out mouse lacked the wild type expression of PKD1, both at RNA and at protein levels.

References

1. Livet, J., et al., Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature, 2007. 450 (7166): p. 56-62.
2. Wu, X. et al., Generation of a prostate epithelial cell-specific Cre transgenic mouse model for tissue-specific gene ablation. Mech Dev, 2001. 101 (1-2): p. 61-9
3. Moll, R. et al., The Catalog of Human Cytokeratins: Patterns of Expression in Normal Epithelia, Tumors and Cultured cells. Cell, 1982 Nov (31): p. 11-24
4. Sun, T.-T., et al., Classification, expression, and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. In The Cancer Cell: The Transformed Phenotype, A. Levine, W. Topp, G. Vande Woude, and J. D. Watson, eds. (New York, Cold Spring Harbor Lab.) (1984), pp. 169-176.
5. Tyner, AL and Fuchs, E. Evidence for posttranscriptional regulation of the keratins expressed during hyperproliferation and malignant transformation in human epidermis. J Cell Bio, 1986. 103(5), pp. 1945-1955.
6. Vassar, R. et al. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. PNAS, 1989. 86 (5): p. 1563-7.