

AWARD NUMBER: W81XWH-13-1-0162

TITLE: Using a Novel Transgenic Mouse Model to Study c-Myc Oncogenic Pathway in Castration Resistance and Chemoresistance of Prostate Cancer

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REPORT DATE: October 2015

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE October 2015		2. REPORT TYPE Annual		3. DATES COVERED 15 Sep 2014 - 14 Sep 2015	
4. TITLE AND SUBTITLE Using a Novel Transgenic Mouse Model to Study c-Myc Oncogenic Pathway in Castration Resistance and Chemoresistance of Prostate Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0162	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Feng Yang, Ph.D. E-Mail: fyang@bcm.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine One Baylor Plaza Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 We previously generated a transgenic model (renamed as PB-Cre4/Ai-Myc) to allow Cre-induced and androgen-independent expression of c-Myc and Luc2 in prostate epithelia. This allows concisely studying castration response and CRPC. However, most PB-Cre4/Ai-Myc mice never developed significant prostate tumors, and knockout of p53 (Year 1) led to tumorigenesis of prostate and epididymis. In Year 2, we performed detailed study on tumor progression in the PB-Cre4/Ai-Myc/p53loxP/loxP mice. They developed unexpected lethal epididymis tumors in 3-5 months. About 30% of them also developed significant prostate tumors by death or the time of euthanization due to epididymis tumor burden. Accordingly, we have proposed and received approval for additional animal studies. We performed early castration on these mice to eliminate concern of epididymis tumors, and performed implantation of testosterone pellets to continue support prostate tumor growth. Our recent data suggest that these surgically operated mice rapidly developed prostate tumors w/o evidence of epididymis tumors, which strongly supports continuing to use our model to study CRPC and chemoresistance of CRPC.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 12	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

Annual Progress Report

W81XWH-13-1-0162

**Using a Novel Transgenic Mouse Model to Study c-Myc Oncogenic Pathway in
Castration Resistance and Chemoresistance of Prostate Cancer**

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W81XWH-13-1-0162 "Using a Novel Transgenic Mouse Model to Study c-Myc Oncogenic Pathway in Castration Resistance and Chemoresistance of Prostate Cancer"

Introduction

c-Myc is the most significantly amplified oncogene in human prostate cancer (PCa)^{1, 2}, and its gene amplification is associated with advanced disease grade and worse prognosis³. In addition, c-Myc overexpression is common in PCa as early as PIN⁴. These indicate its critical roles in PCa progression as well as in the development of therapy-resistance, such as castration resistance and chemoresistance. Transgenic models are widely used in cancer research. Dr. Sawyer's group has developed the widely used Hi-MYC model using an enhanced probasin promoter to drive c-Myc overexpression in prostate epithelia. These Hi-Myc mice develop invasive prostate carcinomas that share molecular features with human PCa⁵. However, since probasin promoter activity is crucially dependent on androgen, the Hi-Myc tumors lose c-Myc expression after castration⁵. Therefore, the tumor regression in these androgen-depleted Hi-Myc mice represents the mixed effects of an artificial direct effect from loss of oncogene expression and a potential real effect from tumor responses to castration. These greatly complicate the system and make it difficult to concisely study c-Myc oncogenic pathway in androgen signaling, castration-responses, and the development of castration-resistant PCa (CRPC). Accordingly, we have generated the CAG-SMIL (renamed as Ai-Myc model for expression of c-Myc in any tissue in a Cre-inducible manner, Figure 1). P_{CAG} is an enhanced β -actin promoter that can drive universal expression of transgene in mice. The *loxP-Stop-loxP* cassette located between P_{CAG} and the *c-Myc-IRES-Luc2* (an enhanced luciferase from Promega) cassette abolishes the otherwise ubiquitous expression of c-Myc and Luc2. IRES allows bicistronic expression of both genes. After crossing with PB-Cre4 mice overexpressing Cre in prostate epithelium⁶, Cre will remove the *loxP-Stop-loxP* cassette to specially turn on c-Myc and Luc2 expression in the prostate of the male *PB-Cre4/CAG-SMIL* mice. Importantly, once turned on, the c-Myc and Luc2 expression will be driven by the P_{CAG} promoter independent on androgen. This will allow us to concisely study c-Myc signaling pathway in (1) castration induced prostate tumor response, (2) the recurrence of CRPC tumors, and (3) the development of chemoresistance in CRPC tumors. The Luc2 expression will label the tumors in this model, which allows real-time *in vivo* bioluminescence imaging (BLI) for prostate tumor progression, tumor response to various therapeutic agents, and tumor relapse after the development of therapy resistance including castration resistance and chemotherapy resistance. Furthermore, by crossing *PB-Cre4/Ai-Myc* mice with mouse lines carrying loxP flanked gene of interest, such as *p53*, we will be able to concisely and efficiently knock out the gene of interest and turn on the expression of c-Myc and Luc2 within the same cell population. We observed delayed prostate tumor progression as well as apparent prostate epithelial cell death in our *PB-Cre4/Ai-Myc* model. mPIN, but not invasive prostate tumors were formed in most *PB-Cre4/CAG-SMIL* transgenic mice up to 2 year of age. Since c-Myc overexpression may induce p53 activation and lead to cell senescence or apoptosis⁷, the subject of this grant is to cross our *PB-Cre4/CAG-SMIL* mice with *p53^{loxP/loxP}* mice⁸ to conditional knock out *p53* and overexpress c-Myc in prostate for a rapid onset and progression of prostate tumors, and use these mice to study castration resistance and chemoresistance of PCa. Finally, although mutation or loss of *p53* is not a very common event in human PCa at early stage, it is strongly correlated to PCa disease stages, metastasis, and castration-resistance^{9, 10}. In

fact, p53 is the most significantly altered gene in metastatic castration resistant prostate cancer¹¹. Therefore, conditional ablation of *p53* in the *PB-Cre4/Ai-Myc* mice is a valid approach to model a significant fraction of advanced PCa, which is the exact PCa type that should be targeted for our proposed studies on castration-resistance and chemoresistance of PCa.

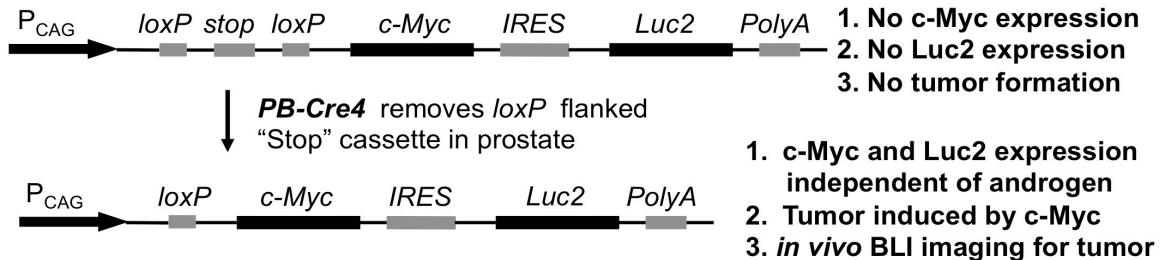


Figure 1. Diagram of the **Ai-Myc** transgenic model (expression of **c-Myc** in any tissue in a Cre-inducible manner).

Keywords:

c-Myc, prostate cancer, castration resistance, chemoresistance, prostate tumor model, Ai-Myc model, PB-Cre4, p53

Overall Project Summary

In this second year of funding, we have experienced delay due to the unexpected rapid growth of lethal epididymis tumor in our *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* model. However, we have made significant progress in charactering tumor growth in this model and in solving the technical problems as discussed in detail below. Please note that we have renamed our CAG-SMIL (CAG promoter driving LoxP flanked “Stop” cassette, followed by c-Myc, IRES, and Luc2) model as Ai-Myc model (expression of c-Myc in any tissue in a Cre-inducible manner). Three major tasks were proposed in our SOW.

Major Task1: Characterize the tumor development in the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice and the control *PB-Cre4/Ai-Myc* mice. These include Subtasks (1) Generate and expand the population of *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice and *PB-Cre4/Ai-Myc* mice (1-18 months) and (2) Perform full necropsy on mice from each group every two months after the BLI imaging. Collect prostate tissues / tumors for histopathology / immunohistochemistry (IHC), Western blot and/or qRT-PCR analysis (8-24 months).

For Subtask 1, we have continued mating the male *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice with the female *p53^{loxP/loxP}* mice to expand the population of the targeted *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice for conditional knockout of p53 and overexpression of c-Myc in prostate epithelial cells. We have similarly generated a population of *PB-Cre4/Ai-Myc* mice as control.

For Subtask 2, we have observed that the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice rapidly developed large tumors within 3-5 months. Full necropsy revealed that all of these mice developed large epididymis tumors that resulted in early euthanization due to large tumor burden and/or death (Figure 2).

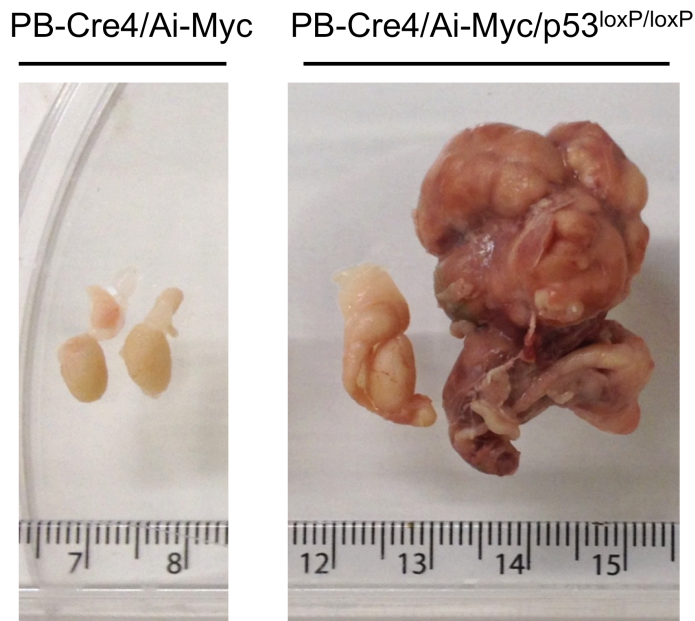


Figure 2. The *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice developed lethal epididymis tumors in 3-5 months. The Right Panel shows epididymis tumors collected from a 4-month old *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mouse. Please note that the testis is mostly embedded in the large epididymis tumor on the right side. The Left Panel shows epididymis and testis tissues collected from an age matched *PB-Cre4/Ai-Myc* mouse.

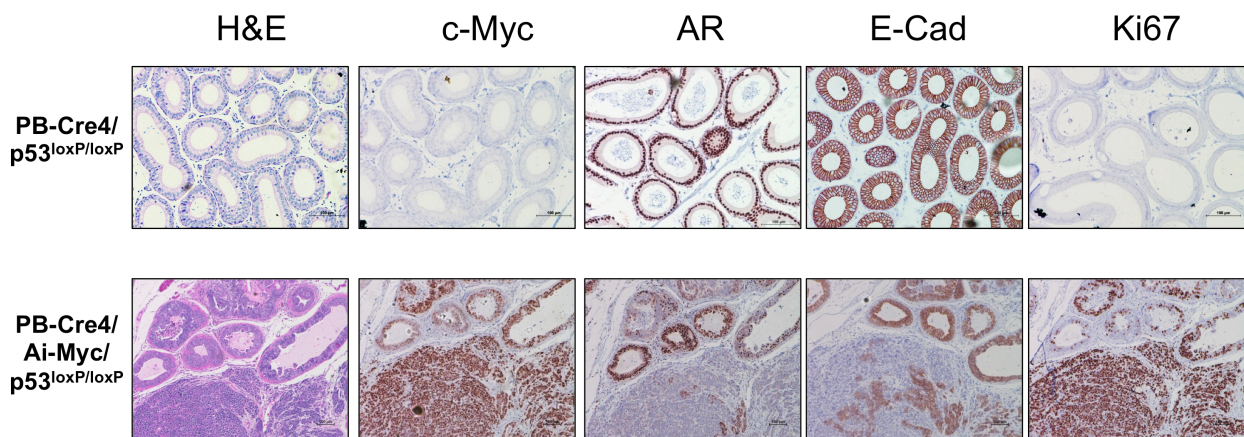


Figure 3. H&E and IHC staining of the epididymis tumor from a 4-month old *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mouse (lower panels) and the epididymis tissue from an age-matched *PB-Cre4/p53^{loxP/loxP}* mouse (upper panels).

We next performed detailed histopathological analysis including H&E staining and IHC staining for AR, c-Myc, E-Cad, and Ki67 on these lethal epididymis tumors as well as the epididymis tissue from the control PB-Cre4/*p53*^{loxP/loxP} mice. Loss of p53 alone did not significantly alter the histology of epididymis, with apparent normal tissue structure and epithelial cells stained positive for AR and E-Cad, and mostly negative for Ki67 and c-Myc (Figure 3). In contrast, overexpression of c-Myc and loss of p53 together induced largely poorly differentiated tumors that strongly overexpress c-Myc transgene, and are largely negative for AR and E-Cad, and highly proliferative as indicated by ubiquitous expression of Ki67.

At the time of euthanization due to large epididymis tumor burden, about 30% of the *PB-Cre4/Ai-Myc/p53*^{loxP/loxP} mice also developed significant prostate tumors, with microscopic prostate tumors and/or mPIN presented in all mice (Figure 4). This is in sharp contrast to the much delayed prostate tumorigenesis in the PB-Cre4/Ai-Myc mice. Detailed histological analysis revealed that these prostate tumor and mPIN foci express high levels of c-Myc transgene, and are positive for AR and E-cad, indicating that they are well differentiated. These prostate tumors and mPIN are also highly proliferative, as indicated by the presence of a large number of Ki67 positive cells. It is interesting that the number of Ki67 positive cells in these prostate tumors (Figure 4) are significantly lower than those in the epididymis tumors (Figure 3), which may partially explain the outgrowth of lethal epididymis tumors over prostate tumors in these *PB-Cre4/Ai-Myc/p53*^{loxP/loxP} mice. Altogether, these data suggest that as we have predicted, knockout of p53 greatly enhanced prostate tumor progression in our *PB-Cre4/Ai-Myc* model, although the outgrowth of lethal epididymis tumors greatly limited our ability to directly use it to study prostate cancer, therapy response and therapy resistance.

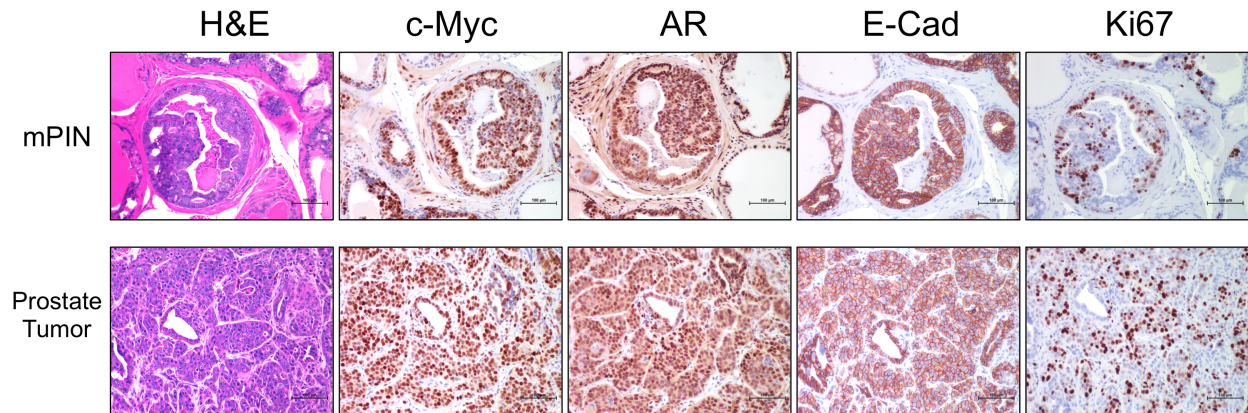


Figure 4. H&E and IHC staining of the prostate tumor from a 4-month old *PB-Cre4/Ai-Myc/p53*^{loxP/loxP} mouse. Both mPIN (upper panels) and invasive prostate tumor loci (lower panels) are presented.

Although PB-Cre4 transgenic mice have been extensively used in prostate tumor modeling and research, we have observed significant off-target Cre activity in the epididymis, which led to overexpression of c-Myc, knockout of p53, extensive cell proliferation, and rapid growth of the lethal epididymis tumors in our *PB-Cre4/Ai-Myc/p53*^{loxP/loxP} model. However, tumor of epididymis is a rare type of cancer in human, and most of them are benign. The

aggressive types of epididymis tumors are extremely rare. Therefore, this may significantly limit the clinical application of our model as a tumor model for epididymis tumors.

The rapid growth of tumors from epididymis has brought a significant technical problem when using our *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice to model prostate cancer, and therapy response and resistance as we have proposed in Major Tasks 2 and 3. Therefore, we proposed to perform castration to remove testis and epididymis altogether in the young *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice at 6-week old. This will eliminate the concerns on epididymis tumors. We also proposed to perform subcutaneous implantation of testosterone pellets to continue supporting prostate tumor growth. We have re-written the animal protocol to include such procedures, and received approvals from the local IACUC and the USAMRMC ACURO. We have initiated performing the early castration and testosterone pellet implantation on the young *PB-Cre4/CAG-SMIL/p53^{loxP/loxP}* mice. Our initial data until the end of this second year supports similar rapid growth of prostate tumors in these surgically operated mice while epididymis tumors were successfully eliminated.

We are in the process of building up the population of these surgically operated mice. We predict to be able to provide the detailed information, including histopathology analysis of the obtained prostate tumors in the next Annual Report. We will perform the proposed studies on CRPC in Major Task 2 and chemoresistance of CRPC in Major Task 3 by simply removing the testosterone pellets from these mice as an alternative "castration" approach.

Major Task 2: Study how the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* prostate tumors respond to castration and the molecular signatures of castration resistance of these tumors. These include Subtask (1) At 6-8 month of age (or time to be optimized), *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice will be performed castration or sham operated. Prostate tumors will be collected for histopathology / IHC, Western blot and/or qRT-PCR analysis for their acute response to castration and the development of castration-resistant prostate tumors (12-30 months). Subtask (2) cDNA Microarray will be performed on the above tumors for the molecular signature of castration-resistant prostate tumors (18-36 months).

Due to the unexpected technical problems as described above in detail, we have not been able to carry out the proposed studies in full scale in the second year. However, as part of the study, we did begin accumulating a population of *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice with early castration and testosterone implantation. Based on our current observation, we may be able to perform "castration" (removal of the implanted testosterone pellet) at 3-5 month of age instead of 6-8 month of age as originally proposed. The results from these studies will be updated in the next Annual Report.

Major Task 3: Study how the castration-resistant *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* prostate tumors respond to chemotherapy (docetaxel) and the molecular signatures of chemoresistance of these tumors. These include Subtask (1) *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice with castration-resistant prostate tumors will receive weekly intravenous administration of docetaxel or solvent control. Prostate tumors will be collected for histopathology / IHC, Western blot and/or qRT-PCR analysis for their acute response to chemotherapy and the development of

chemo-resistant prostate tumors (20-36 months). Subtask (2) cDNA microarray will be performed on the above tumors for the molecular signature of chemo-resistant CRPC tumors (24-36 months).

Due to the unexpected technical problems as described above in detail and due to the nature of the proposed studies in Major Task 3, we will begin these studies in the third year when CRPC tumors are generated. Therefore, no study has been carried out in this Task.

Key Research Accomplishments

- Expanded the population of the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice and *PB-Cre4/Ai-Myc* mice.
- Characterized tumor progression in the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice and identified the unexpected rapid growth of the lethal epididymis tumors along with prostate tumor growth.
- Full necropsy on the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice at death or at the time of euthanization due to large tumor burden following previously approved animal protocol.
- Characterized the histopathology of the epididymis tumors and prostate tumors, including IHC staining for c-Myc, AR, E-Cad, and Ki67 etc.
- Designed new research strategies to overcome the above technical problems, which involved early castration to fully remove testis and epididymis at 6 weeks of age, testosterone pellet implantation, and re-implantation (if necessary).
- Rewrote the animal protocol to cover these additional animal studies, and received local IACUC approval and ACURO approval for the rewrite of animal protocol.
- Initial characterization of the prostate tumors obtained from the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice with early castration and testosterone pellet implantation.

Conclusion

In this second year, we have carried out detailed studies on the tumorigenesis in the *PB-Cre4/Ai-Myc/p53^{loxP/loxP}* mice (lethal epididymis tumors and rapidly growing prostate tumors). Due to the unexpected technical problem, the outgrowth of lethal epididymis tumors greatly limited our ability to directly use our model for prostate cancer study. Please note, our novel Ai-Myc model works as designed (overexpression of c-Myc and Luc2 in a Cre-inducible manner). This technical problem resulted from the Cre expression outside of prostate in the PB-Cre4 model; yet, there is no better model for more accurately targeting prostate epithelial cells. In addition, it is the un-predicted Mother Nature that overexpression of c-Myc and loss of p53 led

to lethal epididymis tumor growth in mice. Accordingly, we have modified our study by performing early castration together with testosterone pellet implantation, and our initial observation suggests rapid prostate tumor growth without interruption from the epididymis tumor. Therefore, we have successfully solved this epididymis tumor problem and our current results supports the studies in the years 3.

Publications, Abstracts, and Presentations:

Wei Wang, Jiangong Ren, Bingning Dong, Michael M. Ittmann, David D. Moore, Feng Yang. A novel c-Myc transgenic model for human cancers. [abstract]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia, PA. Philadelphia (PA): AACR; Cancer Res 2015;75(15 Suppl):Abstract nr 2280. doi:10.1158/1538-7445.AM2015-2280

Inventions, patents and licenses: Nothing to report

Reportable Outcomes: Nothing to report

Other Achievements: Nothing to report

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Appendices: None