Award Number: W81XWH-11-1-0272

TITLE: Evaluating the Efficacy of ERG-Targeted Therapy in Vivo for Prostate Tumors

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REPORT DATE: April 2012

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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Evaluating the Efficacy of ERG-Targeted Therapy in Vivo for Prostate Tumors

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The Johns Hopkins University
Baltimore, MD  21218

The proposed research will examine the suitability of ERG as a target for prostate cancer therapy by using novel modular inducible transgenic mice. Prostate cancer is the most common cancer diagnosed in men in the United States and the second most common cause of cancer deaths in men. Recent efforts to classify distinct molecular subtypes of prostate cancer have shown that >50% of prostate cancers possess a chromosomal translocation involving the ERG oncogene. I hypothesized that ERG can serve as an effective molecular therapeutic target for prostate tumors. I planned to show this with novel autochthonous prostate tumor mouse models. During this first year of support we have been able to adhere to the timeline of our "Statement of Work" - Task#1 - Generate and characterize an inducible ERG prostate specific mouse model (months 1-17). We have obtained the necessary regulatory approval from our resident IACUC for the mouse studies and proceeded with the mating, genotyping and very preliminary characterization of ERG expression from our prostate inducible mouse model. The initial characterization has not demonstrated any detectable prostate specific ERG expression at the protein level using IHC or Western. We need to complete the characterization of the remaining cohorts of animals and specimens using qPCR and other protein based techniques before any firm conclusions can be made.

ERG, prostate cancer, inducible transgenic mouse model
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Body</td>
<td>2</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>4</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>4</td>
</tr>
<tr>
<td>Conclusion</td>
<td>4</td>
</tr>
<tr>
<td>References</td>
<td>5</td>
</tr>
<tr>
<td>Appendices</td>
<td>6</td>
</tr>
</tbody>
</table>
Evaluating the efficacy of ERG targeted therapy in vivo for prostate tumors
PI – Phuoc T. Tran, MD, PhD

INTRODUCTION:
The proposed research program will elucidate the role of ERG in prostate cancer and the suitability of this gene as a target for therapy by using novel modular inducible transgenic mice. Prostate cancer is the most common cancer diagnosed in men in the United States. It has been estimated that 192,000 new cases of prostate cancer were diagnosed in the United States in 2009 and prostate cancer was responsible for 27,000 deaths or the second most common cause of cancer deaths in men (1). Recent efforts to classify distinct molecular subtypes of prostate cancer have led to the novel findings that greater than 50% of prostate cancers possess a chromosomal translocation involving the ETS oncogene family of transcription factors (2, 3). These ETS translocations result in dysregulated overexpression of the ETS oncogene in prostate cancer cells. The most common ETS family member involved in these translocation events is the v-ets erythroblastosis virus E26 oncogene homolog (ERG). Most molecular targeted therapies in other cancers are notable for their lack of serious side-effects and amazing tolerability. I hypothesized that ERG, the most common ETS oncogene found to be mutated in prostate cancer can serve as an effective molecular therapeutic target for prostate tumors. I planned to show this with novel autochthonous prostate tumor mouse models. I also hypothesized that ERG facilitates tumorigenesis alone or in the context of activated AKT1 by dysregulating proliferation, apoptosis and/or senescence programs in vivo. Demonstrating whether prostate tumors in mouse models are dependent for ERG for tumor survival would be the first proof of principle demonstration of molecularly targeted therapy for spontaneously arising prostate tumors in living animals.

The specific aims are below:

Specific Aim#1 - Generate and characterize an inducible ERG prostate specific mouse model.
**Rationale:** I have created a novel prostate TET system mouse model and am interested in the effects of ERG expression alone and in combination with AKT1 in the prostate.
**Study Design:** I will validate inducible expression of both ERG and Luc in vivo using real time-RT-PCR (qPCR), BLI of whole living animals and by organ Western analysis in bi-transgenic ARR2PB-tTA/ERG-tetO-Luc (AE) mice.

Specific Aim#2 – Determine if ERG cooperates with AKT1 for prostate tumorigenesis.
**Rationale:** ERG overexpression in vitro suggests that ERG may facilitate tumorigenesis, but ERG transgenic mouse models vary in the severity of their tumor phenotypes alone and with AKT1 co-overexpression. The mechanism for ERG prostate phenotypes alone or in combination with AKT1 overexpression in vivo are unknown.
**Study Design:** Generate ARR2PB-tTA/ARR2PB-AKT1/ tetO-ERG (AA1E) tri-transgenic mice and compare to single oncogene mice to genetically analyze cooperation in vivo. Investigate using molecular techniques if ERG modulates proliferation, apoptosis and/or senescence programs in vivo.

Specific Aim#3 - Determine if ERG can serve as an effective molecular therapeutic target for prostate tumors in vivo.
**Rationale:** Despite the importance that ERG overexpression is believed to play in prostate tumorigenesis, the therapeutic value of targeting ERG on autochthonous prostate tumors has not been tested in vivo. The mechanism for any autochthonous tumor regression or stasis in vivo upon ERG inactivation is unknown.
**Study Design:** Following development of autochthonous prostate tumors in TET regulated mice I will treat mice with doxycycline to simulate targeted treatment against the ERG oncogene. Investigate using molecular techniques if ERG inactivation modulates proliferation, apoptosis and/or senescence programs in autochthonous prostate tumors in vivo.
**BODY:**

Progress is listed in relation to each specific task in the “Statement of Work” and highlighted by **BOLD** font.

**Task#1 - Generate and characterize an inducible ERG prostate specific mouse model (months 1-17).**

Numbers of mice surviving weaning and for mating: 65  
1a. IACUC and other regulatory approval process for animal work (months 1-4).  
**We applied for and obtained approval from the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center IACUC for the studies described in our DoD grant award (see Appendix for documentation approval).**

1a. Mating mice to characterize (months 4-10).  
The appropriate single transgene *ARR2PB-tTA* (A) and *ERG-tetO-Luc* (E) mice were mated to produce cohorts of (AE) bitransgenic mice. There have been no issues with producing the required numbers of AE mice.

1b. Collecting tissues from AE mice to characterize ERG expression (months 8-14). AE mice will be weaned and placed on water without doxycycline and 5 males for each of the following age time points: 4, 8, 12 and 24 weeks (n=25 mice total, 5 additional for incidentals), will be interrogated using the assays mentioned below in 1d.  
The appropriate numbers of AE bitransgenic mice (n=25) have been placed on doxycycline drinking water (2 mg/ml) changed weekly.

1c. Collecting tissues from AE mice turned OFF to characterize inducible ERG expression (months 8-14). 12 week old males will be followed for the OFF time points: 1, 2 and 4 weeks (n=20 mice total, 5 additional for incidentals) and tissues extracted for interrogation using the assays mentioned below in 1d.  
The appropriate numbers of AE bitransgenic mice have been placed on doxycycline drinking water (2 mg/ml) changed weekly and then had doxycycline drinking water replaced with regular water (n=20).

1d. Performing experiments on tissues from mice (months 14-17). Tissues from 1b and 1c above will be harvested for histology and flash frozen for molecular studies: prostate lobes, other genitourinary (GU) organs, lungs, heart, liver and spleen. These specimens will then be processed for H&E histology and immunohistochemistry (IHC) performed using anti-Myc, anti-FLAG and anti-luciferase antibodies to confirm prostate luminal cell epithelia expression. Whole lobe and organ Western blotting using the same antibodies will also be performed and transcription of ERG confirmed with specimens using qPCR.  
See Table 1 and 2 below for summary of results thus far. We were able to harvest as above for all the “On” time points at least 5 mice: 4, 8, and 12 weeks, but are waiting for the longest time point of 24 weeks to mature before harvesting these 24 week “On” doxycycline mice. Similarly, for the “Off” time points we have been able to collect ≥ 5 mice from the 1 and 2 week time points and are waiting for the 4 week time point to mature before harvesting the tissues from these mice.

We have performed preliminary analysis as summarized below in Table 1 & 2. The AE mice from the “On” time points collected have had no abnormalities on gross or H&E examination of their prostates. The other organs in these mice (lungs, heart, liver and spleen) were also normal on necropsy. Similarly, the AE mice from the “On” and “Off” time course displayed no pathology on gross or histologic exam of the H&E slides. We have attempted IHC and westerns for protein expression of ERG that is tagged by Myc and FLAG epitope tags, but have not been able to see expression using either approach. We are continuing with luc IHC and ERG qPCR with these samples and will also perform all the assays from Tasks# 1b and 1c on the remaining “On” and “Off” samples as they become available.
1e. Analyzing results of experiments on tissues from mice (months 14-17).
See Table 1 below for summary of results thus far and we are awaiting for the longest time point of 24 weeks to mature before fully comparing all the time points and analyzing the data.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>4 wks On DOX</th>
<th>8 wks On DOX</th>
<th>12 wks On DOX</th>
<th>24 wks On DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>6 mice</td>
<td>7 mice</td>
<td>5 mice</td>
<td>Pending</td>
</tr>
<tr>
<td>Gross</td>
<td>WNL</td>
<td>WNL</td>
<td>WNL</td>
<td>Pending</td>
</tr>
<tr>
<td>Histologic</td>
<td>WNL</td>
<td>WNL</td>
<td>WNL</td>
<td>Pending</td>
</tr>
<tr>
<td>Myc IHC</td>
<td>Negative expression</td>
<td>Negative expression</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>FLAG IHC</td>
<td>Negative expression</td>
<td>Negative expression</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>lnc IHC</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>FLAG Western</td>
<td>Negative expression</td>
<td>Negative expression</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>ERG qPCR</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
</tbody>
</table>

A – ARR2PB-tTA; DOX – doxycycline; E – luc-tetO-ERG; IHC – immunohistochemistry; qPCR – quantitative polymerase chain reaction; WNL – within normal limits.

Table 2 – Summary of Task #1c to date.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>1 wks Off DOX</th>
<th>2 wks Off DOX</th>
<th>4 wks Off DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>6 mice</td>
<td>6 mice</td>
<td>Pending</td>
</tr>
<tr>
<td>Gross</td>
<td>WNL</td>
<td>WNL</td>
<td>Pending</td>
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<tr>
<td>Histologic</td>
<td>WNL</td>
<td>WNL</td>
<td>Pending</td>
</tr>
<tr>
<td>Myc IHC</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>FLAG IHC</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>lnc IHC</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>FLAG Western</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
<tr>
<td>ERG qPCR</td>
<td>Pending</td>
<td>Pending</td>
<td>Pending</td>
</tr>
</tbody>
</table>

A – ARR2PB-tTA; DOX – doxycycline; E – luc-tetO-ERG; IHC – immunohistochemistry; qPCR – quantitative polymerase chain reaction; WNL – within normal limits.

Each of the steps/tasks below are dependent on the steps above and have not been initiated.

Task#2 - Determine if ERG cooperates with AKTI for prostate tumorigenesis (months 14-34).
Numbers of mice surviving weaning and for mating: 150
2a. Mating mice for cooperation experiments (months 14-20).
2b. Collecting tissues from cooperation experiments (months 18-30).
2c. Performing experiments on tissues from mice (months 20-32). Tissues from 2b above will be harvested for histology and flash frozen for molecular studies: prostate lobes, other GU organs, lungs, heart, liver and spleen. These specimens will then be processed for H&E histology and IHC performed using anti-Myc, anti-FLAG and anti-luciferase antibodies. Whole lobe and organ Western blotting using the same antibodies will also be performed and transcription of ERG confirmed with specimens using qPCR. IHC for cleaved caspase 3 (CC3) and Ki-67. Senescence markers such as p15, p16, p21 and p27 will be analyzed by IHC and qPCR. In addition, I will perform senescence associated beta-galactosidase (SA-β-gal) staining.

2d. Analyzing results of experiments on tissues from mice (months 22-34).

Task#3 - Determine if ERG can serve as an effective molecular therapeutic target for prostate tumors in vivo (months 34-60)

Numbers of mice surviving weaning and for mating: 120

3a. Mating mice for therapeutic experiments (months 34-40).

3b. Collecting tissues from therapeutic experiments mice ON 6-12 months and then OFF 1-6 months (months 40-56).

3c. Performing experiments on tissues from mice (months 42-58). Tissues from 3b above will be harvested for histology and flash frozen for molecular studies: prostate lobes, other GU organs, lungs, heart, liver and spleen. These specimens will then be processed for H&E histology and IHC performed for Myc, FLAG, luciferase, CC3, Ki-67, p15, p16, p21 and p27. Whole lobe and organ Western blotting using the same antibodies will also be performed and transcription of ERG confirmed with specimens using qPCR. In addition, I will perform SA-β-gal staining.

3d. Analyzing results of experiments on tissues from mice (months 44-60).

KEY RESEARCH ACCOMPLISHMENTS:
• Generation of possibly inducible bitransgenic prostate specific ERG expressing mice.
• Early characterization of inducible regulation of this transgenic ERG model system

REPORTABLE OUTCOMES:
• During this first year of support we have not published any manuscripts, abstracts or presented this work at any venue other then at our own private lab meetings.
• No licenses were applied for.
• No degrees were obtained that are supported by this award.
• We did not develop any cell lines or serum repositories, but tissues from our AS mice were banked for further analysis as described above in the “Body” section.
• No informatics databases were constructed, but a novel animal model was developed that we are trying to characterize as above in the “Body” section.
• No additional funding was applied for based on this work
• No employment or research opportunities applied for and/or received based on experience/training supported by this award.

CONCLUSION:
During this first year of support we have been able to adhere to the timeline of our “Statement of Work” - Task#1 - Generate and characterize an inducible ERG prostate specific mouse model (months 1-17). We have obtained the necessary regulatory approval from our resident IACUC for the mouse studies and proceeded with the mating, genotyping and very preliminary characterization of the ERG expression from our prostate inducible mouse model. The initial characterization has not demonstrated any detectable ERG expression at the protein level using IHC or Western. We need to complete the characterization of the remaining time point specimens and “Off” time course specimens before any firm conclusions can be made. Characterization of the founder lines from the A transgenic mice indicated that expression was feasible using a different tetO-controlled...
reporter line (see original proposal). Similarly, characterization of the founder lines from the E transgenic mice indicated that expression of luciferase was feasible using a different promoter element driving a similar tTA gene in the liver (see Figure 1, Appendix). Thus we are not overly concerned at this time before analyzing all the cohorts in Task#1. If there were ultimately issues with prostate specific inducible expression of ERG we would first attempt to force ERG expression in the prostate using a newly reported TET inducible prostate mouse model, Hoxb13-rtTA (4). If this does not establish an inducible model for prostate specific ERG expression, then we would recreate the luc-tetO-ERG line by re-submitting our transgene construct to our Transgenic Core Facility. We then proceed again as described above for Task#1-3.

“So What”

Despite the importance that ERG overexpression is believed to play in prostate tumorigenesis, the therapeutic value of targeting ERG rearrangements has not been tested in vivo. The ability to interrogate using in vivo model systems whether ERG or other oncogenes are good molecular therapeutic targets could provide a huge leap forward for prostate cancer research and treatment of prostate cancer patients. Demonstrating whether prostate tumors in my inducible transgenic mice are dependent for ERG for tumor maintenance would be the first proof of principle demonstration of molecularly targeted therapy for prostate tumors in vivo and we will be able to determine whether molecularly targeted therapy against ERG in the context of activated AKT1 would be an effective therapy for prostate tumors.

REFERENCES:
**APPENDIX:**

![BLI images]

**Fig 1.** Generation of an inducible luc liver epithelial specific mouse model. Mice containing a liver specific TET driver transgene, LT2-tTA was crossed with a reporter mouse luc-tetO-ERG line to produce bi-transgenic animals (LE). The absence of doxycycline allows the tTA protein to bind and activate the tetO promoter. Addition of doxycycline triggers a conformational change which prevents tetO binding, activation and inhibits ERG and luc transcription. LE animals express luciferase inducibly in the liver as shown by bioluminescence imaging (BLI) (ip injection with luciferin substrate and imaged 10 minutes later on a Xenogen Spectrum machine shows a colored bright region in the right upper abdomen). Dox – doxycycline was given to animals in the drinking water [0.04 mg/ml].
To: Dr. Phuoc Tran  
Department of Oncology  

From: Nancy A. Ator, Ph.D.  
Chair, Animal Care and Use Committee  

Date: 12/10/2010  

Subject: Amendment Approval Memo  

On 12/09/2010, the Johns Hopkins University Animal Care and Use Committee (ACUC) approved the following amendment to your research protocol. A copy of the approved amendment is attached.

Protocol Number: MO09M331  

Title: Transgenic models of oncogene induced tumorigenesis and organ fibrosis  

Expiration Date: 08/21/2011  

Additional modifications to this protocol can be requested by submitting the appropriate amendment form (i.e., Change in Animal Number, Change in Personnel, or Change in Procedures) to the ACUC office for review and approval. Copies of all current forms can be found on our website: www.jhu.edu/animalcare.

For guidance on protocol modifications that require amendments, please refer to the reverse side of this letter. If the locations for outside housing or procedures change, please submit a Change in Location Form, also available on the website.
Please indicate which changes you are requesting by an X next to each category below. Describe the change(s) and reasons on page 2 of this form. Please return a signed copy of this form to the ACUC Office, Reed Hall, room B122 or fax to 443-287-3747 (7-3747).

To add new personnel or change the PI, please complete the Change in Personnel Amendment Request Form or Change in PI Amendment Request Form. To change a location for animal use complete the Change in Location Form. All forms are available on the web at www.jhu.edu/animalcare/forms1.html.

Modify anesthetic or analgesic agents: State the name of the agent, dose or dose range, route of administration and frequency range for any drug to be added. Previously approved agents will remain on the protocol. If you need to withhold analgesia, indicate the reasons why and see "Modify Pain Category" below to see if it applies.

Modify Euthanasia: Describe any changes in the method of euthanasia (be sure proposed method is in compliance with the 2007 AVMA Guideline on Euthanasia, which can be viewed at www.avma.org/resources/euthanasia.pdf)

Modify Procedures: Provide a complete description and rationale for the proposed experimental changes. Indicate if they will change the degree of invasiveness of a procedure or discomfort to the animal. (i.e., the withholding of analgesics; change from non-survival to survival surgery; change in number, duration, or frequency of procedures performed on the animal, etc.). See “Modify Pain Category” below to determine if it applies.

Modify Surgical Procedures: Describe any changes to approved surgical procedures.

Modify Radiation; or Radioactive, Infectious or Biohazardous Agent: Provide rationale for adding this new agent, list all necessary safety precautions, and describe any modifications you plan to make to your currently-approved procedures. Attach pertinent approval letter or copy of application from Health, Safety & Environment as appropriate).

Modify Animal Numbers: Indicate the number of additional animals you are requesting that will fall under each pain category in the chart below. Provide a justification for the change in animal numbers. Each animal should be categorized only once. If adding animals or procedures to category D or E for the first time, please see “Modify Pain Category” below.

<table>
<thead>
<tr>
<th>Number Requested</th>
<th>Pain Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>B Breeder</td>
<td></td>
</tr>
<tr>
<td>C No pain or distress</td>
<td></td>
</tr>
<tr>
<td>D Alleviated Pain or distress</td>
<td></td>
</tr>
<tr>
<td>E Unalleviated Pain or distress</td>
<td></td>
</tr>
</tbody>
</table>

Revised 12/08
Procedure and Animal Numbers Amendment Form, Page 1


**CHANGE IN PROCEDURE(S) AMENDMENT REQUEST FORM**

Describe the requested change(s) following the guidelines for the specific modification as per page 1 of the form (attach additional pages as necessary).

To determine the role of oncogenes, such as ERG, for tumorigenesis and tumor maintenance using the Tet system.

**Justification:** Tumorigenesis is thought to involve multiple steps many of which are determined by changes in specific genes. Studies have demonstrated that oncogenes are causative in tumorigenesis. Oncogenes are also involved during normal developmental processes where cells acquire increased migratory abilities enabling cells to form the many and varied organs of the body. Dysfunctional oncogene expression has been implicated in both tumorigenesis and tissue fibrosis. The Tran laboratory is interested in understanding the role of various oncogenes, including but not limited to Twist1, hSNAl1 and ERG, in the processes of tumorigenesis, tumor maintenance and tissue fibrosis using mice that express oncogenes. In most cases, the expression of these oncogenes will be induced or turned "ON" and "OFF" using the tetracycline (or doxycycline) regulatory system (TET system).

Development of imaging surrogates for use in localization and monitoring treatment of tumors and organ fibrosis in living rodent subjects has been previously described in approved amendments. Many of the animal models we use are transgenic models (knock in, knock out) that recapitulate human disease. There are no computer simulations that serve this purpose.

We hypothesize that serial non-Invasive imaging followed by confirmation with histopathology will allow our group to monitor the development of tumors and track tumor regression in our cohort of transgenic mouse models using the Tet system.

1) To use non-invasive serial imaging studies and standard histo-pathological analysis to monitor tumorigenesis using the Tet system. We will determine if expression of oncogenes alone or in conjunction with previously approved agents and other oncogenes enhance tumorigenesis and/or lung fibrosis in the mice models as a part of our already approved protocol by providing the animals doxycycline in their water or chow as (MO9M331).

Cohorts of weaned, age-matched, control and experimental mice will be devoid of doxycycline or placed on doxycycline (depending on the transgenic model) in their drinking water to activate expression of an oncogene being studied. Mice will be monitored weekly for symptoms of morbidity as stated below. Prior experience with a separate luciferase tagged primary Twist1 tumor model indicates that bioluminescence imaging (BLI) signal correlates with tumor burden. Therefore, cohorts with the Luc reporter will also be followed for tumor development non-invasively by use of serial BLI (using our already approved imaging amendment) and correlated with disease pathology following necropsy at defined periods. Based on prior literature and our experience mice from each cohort will be sacrificed at time points of between 0-18 months of age depending on physical and imaging findings. These animals will be processed at necropsy for prostate lobes, other genitourinary (GU) organs, lungs, heart, liver and spleen and these specimens will be harvested for histology and flash frozen for molecular studies.

Revised 12/08
Procedure Amendment Form, Page 2
2) To use non-invasive serial imaging studies and standard histo-pathological analysis to monitor tumor maintenance using the Tet system.

Following development of autochthonous tumors in TET regulated oncogene mice as determined by serial imaging and from my time course studies above, we will treat mice with doxycycline to simulate targeted treatment against the tetO-regulated oncogenes.

Tumor moribund mice that are known have tumors from imaging or suspected based on time course experiments above will be injected intraperitoneally with 100 micrograms of doxycycline in PBS and then restricted to water containing doxycycline changed weekly (or depending on the system normal water free of doxycycline). Cohorts of tumor morbid mice following oncogene inactivation will be followed by weekly inspection and imaging. At defined periods of between 0-12 month animals will serially imaged and sacrificed and necropsies and tumor analysis performed as above; or before if euthanasia is required for humane reasons.

All animals will be monitored and euthanized immediately if they exhibit the following symptoms:
- Ulceration and bleeding of the tumor
- Anorexia indicated by the absence of feces in cage
- Does not drink water leading to dehydration evidenced by tenting of the skin
- Hunched up, unwilling to move, favoring a limb or guarding the incision site
- Failure to groom reflected in a ruffled or dirty coat
- Excessive licking/scratching, redness and swelling at incision site, and self-mutilation
- Aggressive behavior especially when attempting to pick up the animal
- Squealing, struggling, twitching, tremors, convulsions, weakness
- Panting, labored breathing, reddish-brown nasal/ocular discharge
- Cold or blue extremities (hypothermia) or hot or red extremities (hyperthermia)

I understand that these changes must not be implemented until I receive approval for the changes from the Animal Care and Use Committee.

PI Signature: ___________________________ Date: 11/18/2010

IACUC Chair’s Signature: ___________________________ Date: 12/9/10

Revised 12/08
Procedure Amendment Form, Page 2