Protocol Information

Reference Number:	297
Protocol Number:	06-10-028
Title:	Functional Analysis of BHD Using Conditional Knockout Strategy
Category:	Standard
Protocol Type:	Amendment

PI:	Teh, Bin	**Approval Date:**	12/18/2008 12:00 AM
Department:	Research	**Effective Date:**	12/18/2008 12:00 AM
Location:	Van Andel	**Renewal Date:**	10/11/2007 12:00 AM
Author:	Noyes, Sabrina	**Next Review Date:**	10/11/2007 12:00 AM
Status:	Approved	**Stock Protocol:**	No
Emergency Phone:	6162345578	**Expiration Date:**	10/11/2009 12:00 AM
Submittal Date:	12/9/2008 3:11 PM	**Inactive Date:**	

Amendment to Protocol Form

1 Amendment to Protocol

Are you amending this protocol**only**to update the associates?

a ☐ Yes

b ☑ No

2 [1b] Amendment Reason

Briefly indicate the reason for this amendment. This should focus on**why**the protocol is being amended.

AMENDMENT SUBMITTED OCTOBER 29. 2008

Since tumorigenesis requires more than one genetic change to progress, knockout of one tumor suppressor gene may not guarantee the formation of tumor. Knockout of two or three tumor suppressor genes may be necessary. The VHL gene is another important kidney cancer -related gene. However, knockout of VHL alone in the mouse kidney has not led to the formation of kidney tumors, implying that additional genetic mutations in other kidney cancer -related genes may be required. To address this issue, we decided to develop a double gene knockout mouse model involving the BHD and VHL genes. Another gene RB is also an important tumor suppressor gene that is associated with tumor initiation. Weexpect that this combination of knockouts in mice can produce kidney cancer. The goal of this project is to exploit our knowledge of the genetic kidney disease and to improve the understanding of tumor suppressor genes and their mechanism in causing tumor formation. Currently, the VHL -, Bhd-, and Rb knockout mice are available in our repository. We are amending this protocol to add two additional experiments to determine whether double or triple knockout mice could

promote tumorigenesis. We are also updating the key associates on this protocol.

3 [1b] Amendment Summary

Briefly outline**what**is changed in the amendment.
We are adding two additional experiments to this protocol to develop a double gene knockout mouse model involving the BHD and VHL genes to determine if these mice could promote tumorigenesis. It is possible that mutations in VHL or RB alone cause cancer. However, so far there is no conditional knockout mouse models developed produce renal cell carcinoma. Actually, VHL-knockout alone and RB-knockout alone mice are expected to produce during the breeding, and we will keep these mice as controls.

We are also adjusting the personnel on this protocol to remove Dan Huang and add Aikseng Ooi and Tristan Kempston. Their roles are outlined under the Protocol Associates section.

Experiment #7:
In this experiment, we will cross the BHD-flox/flox mice with VHL-flox/flox mice to generate double BHD-/VHL-flox heterozygous mice. Heterozygous BHD-flox/+;VHL-flox/+ mice will be intercrossed to produce double homozygous BHD-flox/flox;VHL-flox/flox mice. BHD-flox/flox;VHL-flox/flox mice will be further bred to CMV-Cre mice to produce BHD-flox/+;VHL-flox/+;CMV-Cre heterozygous knockout mice.

Experiment #8:
In this experiment, we will cross the RB-flox/flox mice with VHL-flox/flox mice to generate double RB-flox/+;VHL-flox/+ heterozygous mice. Heterozygous RB-flox/+;VHL-flox/+ mice will be intercrossed to produce double homozygous RB-flox/+;VHL-flox/+ mice. RB-flox/+;VHL-flox/+ mice will be bred to CMV-Cre mice to generate double heterozygous RB-flox/+; VHL-flox/+;CMV-Cre heterozygous knockout mice.

The phenotype will be assessed at eight intervals:
1) Day 1 after birth
2) Day 7 after birth
3) Day 21 after birth
4) 8 weeks old
5) 16 weeks old
6) 24 weeks old
7) 52 weeks old
8) 78 weeks old

We will also use mice for:
9) histological and pathological exams
10) establishment of fibroblast cell lines

For all of these 10 groups, we will use 10 mice per genotype group to assess the phenotype based on our previous experience.

The experimental design for experiments 7 and 8 is similar to the previous experiments. The difference is that experiments 7 & 8 involve two tumor suppressor genes called double knockout.

GRANITE Header Questions

1 Protocol Number

Protocol Number

- 06-10-028 -

2 Reference Number

Reference Number

297

3 Protocol Type

Protocol Type

Amendment

4 Title

Title

Functional Analysis of BHD Using Conditional Knockout Strategy

5 Principal Investigator

Principal Investigator

Teh, Bin	(616) 234-5296	Bin.Teh@vai.org

6 Author

Author

Noyes, Sabrina	(616) 234-5273	sabrina.noyes@vai.org

7 Created By

Created By

Noyes, Sabrina	(616) 234-5273	sabrina.noyes@vai.org

8 Site

Site

Van Andel

9 Department

Department

Research

10 Emergency Phone

Emergency Phone

6162345578

11 Audit Trail

Audit Trail

☑ Yes ☐ No

12 Unrestricted View

Unrestricted View

☐ Yes ☑ No

13 Stock Protocol

Stock Protocol

☐ Yes ☑ No

14 Protocol Associates

Protocol Associates

Noyes, Sabrina

Co-PI ☐ **Key Associate** ☑ **Authorized To Order Animals** ☐

Responsibilities: Administrative support.

Comments:

Standard Procedures: Standard Procedure | Type | Species

Chen, Jin Dong

Co-PI ☐ **Key Associate** ☐ **Authorized To Order Animals** ☑

Responsibilities: Set up matings, administer tamoxifen injections, monitor animals post-tamoxifin injection, perform euthanasia and necropsies

Comments:

Standard Procedures:	Standard Procedure	Type	Species

Williams, Bart

Co-PI ☐ **Key Associate** ☑ **Authorized To Order Animals** ☐

Responsibilities: Advisory capacity only.

Comments:

Standard Procedures:	Standard Procedure	Type	Species

Kempston, Tristan

Co-PI ☐ **Key Associate** ☑ **Authorized To Order Animals** ☐

Responsibilities: Daily observation of general health and watch for criteria for euthanasia.

Comments:

Standard Procedures:	Standard Procedure	Type	Species

Ooi, Aikseng

Co-PI ☐ **Key Associate** ☑ **Authorized To Order Animals** ☐

Responsibilities: Administer tamoxifen injections and monitor animals post-tamoxifin injection.

Comments:

Standard Procedures:	Standard Procedure	Type	Species

Teh, Bin

Co-PI ☐ **Key Associate** ☑ **Authorized To Order Animals** ☐

Responsibilities: Oversee research.

Comments:

Standard Procedures: Standard Procedure | Type | Species

15 Protocol Category

Protocol Category

Standard

16 In Vivo Protocol

In Vivo Protocol

☑ In Vivo ☐ In Vitro

17 Accounts

Accounts

10301-4603
10301-4611
10301-4613

18 Amendment Reason

AMENDMENT SUBMITTED OCTOBER 29, 2008Since tumorigenesis requires more than one genetic change to progress, knockout of one tumor suppressor gene may not guarantee the formation of tumor. Knockout of two or three tumor suppressor genes may be necessary. The VHL gene is another important kidney cancer-related gene. However, knockout of VHL alone in the mouse kidney has not led to the formation of kidney tumors, implying that additional genetic mutations in other kidney cancer-related genes may be required. To address this issue, we decided to develop a double gene knockout mouse model involving the BHD and VHL genes. Another gene RB is also an important tumor suppressor gene that is associated with tumor initiation. We expect that this combination of knockouts in mice can produce kidney cancer. The goal of this project is to exploit our knowledge of the genetic kidney disease and to improve the understanding of tumor suppressor genes and their mechanism in causing tumor formation. Currently,the VHL-, Bhd-, and Rb knockout mice are available in our repository. We are amending this protocol to determine whether double or triple knockout mice could

Vivarium SOP #6.002 - Blood Collection Techniques in Mice. We will be collecting 200µl of blood, although we will draw the maximum available volume to ensure death by exsanguination. The purpose for this collection is to evaluate the levels of blood urea nitrogen, which is an indicator of potential kidney failure or dysfunction. We plan to perform this terminal blood collection on all animals in this protocol which develop kidney-related criteria for euthanasia (kidney tumors or kidney cysts), as well as a matching number of control (healthy) mice of the same ages. Animals which develop criteria for euthanasia which are clearly not kidney-related will be euthanized by CO2. This added procedure is also Category 1, so this does not change the Stress Level numbers. This amendment does not add any animals. This amendment updates the response to questions #53 (experimental design), 105-117 (blood collection), 134 (endpoint), 151-171 (anesthesia), 174 (method of euthanasia), 244 (SOP attachments).

Mus musculus Questionnaire 89 (Mus musculus) #1

1 Allow Transfer

Allow Transfer

☑ Yes ☐ No

2 SSB

SSB

C57BL/6

TG or KO from a non-vendor

3 Authorized Amounts Per Pain/Distress Category

Authorized Amounts Per Pain/Distress Category

Stress Level	Requested	On Order	Received	Available
Category 1	1047	0	217	830

Strains:

Stress Level	Requested	On Order	Received	Available
Category 1 (click yellow question mark)	3525	0	1052	2473

Strains:

Stress Level	Requested	On Order	Received	Available
Category 2 (click yellow question mark)	0	0	0	0

Strains:

Stress Level	Requested	On Order	Received	Available
Category 3 (click yellow question mark)	0	0	0	0

Strains:

	Requested	On Order	Received	Available
Totals	4572	0	1269	3303

4 Euthanasia Methods

Euthanasia Methods
See Segment Questionnaire

☑ **Primary** ☐ **Secondary**

Comments:

5 Administrative Data

6 Protocol Questionnaire Version 1.0 for Mus musculus ========== P

Protocol Questionnaire Version 1.0 for Mus musculus
========== Please click "YES" and continue with the protocol questionnaire. If you have any questions regarding the questionnaire version, please contact the IACUC Administrative Assistant.

a ☑ Yes

b ☐ No

7 PI Telephone Number: <updated 060903>

PI Telephone Number:
234-5296

8 PI FAX Number: <updated 060903>

PI FAX Number:
234-5297

9 PI Email: <updated 060903>

PI Email:
bin.teh@vai.org

10 Is this protocol funded (either partially or wholly) through internal source(s)? <updated 060903>

Is this protocol funded (either partially or wholly) through internal source(s)?

a ☑ Yes

b ☐ No

11 Is this protocol funded (either partially or wholly) through external source(s)? NOTE: This inclu

Is this protocol funded (either partially or wholly) through external source(s)? NOTE: This includes any external funding submissions which are not yet approved or funded, as well as any applications for external funding which have not yet been submitted.

a ☐ Yes

b ☑ No

12 Study Objectives

13 Briefly explain the aim of the study and why the study is important to human or animal health, the

Briefly explain the aim of the study and why the study is important to human or animal health, the advancement of knowledge or the good of society. The response MUST be in language understandable to a lay person.
Birt-Hogg-Dubé syndrome (BHD) is a hereditary cancer syndrome associated with a wide spectrum of

diseases, including kidney tumors, skin tumors, colon tumors and lung diseases. We have mapped the BHD gene in chromosome 17 and recently it was identified as a novel kidney cancer gene. BHD has been considered as a tumor suppressor gene. The goal of this project is to exploit our knowledge of the genetic disease, Birt-Hogg-Dubé (BHD) syndrome, to improve the understanding of tumor suppressor genes and their mechanism in causing tumor formation. By knocking out the BHD gene in mouse or specific mouse tissues such as kidney, intestine, or lung, we will increase our knowledge of the BHD gene and its effects on tumor formation in kidney and other organs, and other biological processes. These studies will provide insight into discovery of drug targets that will hopefully lead tobetter treatment and care for both patients with BHD mutations and other forms of cancer involving the same cancer-causing pathways.

Our specific aims in this research proposal are:
1) to determine the functional role of the BHD gene in kidney cancer, and
2) to confirm that the BHD gene is required for the development of kidney, intestine and lung and that inactivation of the gene will induce related tumorigenesis.

First, we will create a BHD conditional knockout mouse strain, also called a BHD-flox strain. This conditional knockout strain should not manifest any phenotype, since the knockout is not completed until the animal is exposed to Cre.

Once we have established the conditional knockout, we will breed this knockout to various tissue-specific Cre strains to create kidney-specific, intestine-specific, and lung-specific BHD-deficient knockout mice,to confirm the corresponding tumor formation. This work is outlined in several experiments, addressed below.

The following proposed studies will provide important new information concerning the functional biology of the BHD gene in mice. As a consequence, it will lead to better understanding of the gene in humans, and facilitate the development of early identification, intervention and therapeutic strategies for related patients.

Experiment #1
Since the BHD-construct contains a neomycin antibiotic gene that may lead to unknown background, we will cross the BHD-flox chimeric mice to FLPeR mice to remove the neomycin gene in order to reduce potential background noise. The offspring of this cross will then be used in Experiments #2-6.

Experiment #2:
We will firstcross the BHD-flox strain with the CMV-Cre strain, which is a system Cre strain. This experiment will create BHD knockout mice in which the loss of BHD is throughout all tissues. This will allow us to observe both homozygous and heterozygous BHD knockouts. We expect the BHD-deficient mice will develop BHD disease similar to human's. Since homozygous deletion of BHD will lead to embryonic lethality, we will do the following conditional knockout experiments in related organs/tissues.

NOTE: Although we have determined that the conventional BHD knockout is embryonically lethal, the construct for the conditional BHD-flox knockout focuses on a different exon group than the one used for the conditional knockout. Therefore, we expect that we will be able to produce viable homozygous knockouts mice from this conditional knockout strain, as the portion of the gene excised by the conditional knockout constuct is not thought to be as severe a mutation as the portion we used in the conventional knockout construct.

Experiment #3:
Since mating BHD-flox with CMV-Cre strain may result in homologous embryonic lethality, we will cross

BHD-flox mice with ERTM-Cre strain, which is responsive to the application of tamoxifen, a synthetic estrogen. Unlike mice crossed to the CMV-Cre strain, mice crossed to the ERTM-Cre mice will not express Cre until exposed to tamoxifen. This will allow us to test and control freely systemic response to BHD elimination at different life stages. However, this experiment is not suitable for production of heterozygous BHD mice like Experiment #2. So both experiment #2 and experiment #3 are necessary. For Experiment #3, we hypothesize that the systemic loss of BHD, even in the post-developmental stages, may result in mortality. (Please note that we will euthanize the mice as they develop criteria for euthanasia as outlined in Question #131; we will not allow the individual animals to progress to morbidity or mortality.)

Experiment #4:
In this experiment, BHD-flox strain will be crossed with Ksp-Cre mice to give rise to kidney-specific BHD knockout mice. Kidney cancer is one of the important features of BHD syndrome, and Ksp-Cre mice express in Cre in the proximal tubular, the distal tubular, and the collecting duct cells of the kidney. So it is logical to try to further assess the role of BHD in kidneys.

Experiment #5:
In this experiment, we will further cross the BHD-flox mice with Sglt2-Cre mice, which also express Cre kidney cells, but in the proximal tumular cells only. The human disease RCC (Renal Clear Carcinoma) is believed to derive from the proximal tubular cells of the kidney, but there is currently no compelling evidence of this. Since it is not clear which type of kidney cells give rise to kidney cancer (proximal tubular cells, distal tubular cells, or collecting ducts), it is necessary to use different kidney Cre-expressing strains.

Experiment #6:
In patients with confirmed deletion of BHD in the intestine, there is an increased level of colon cancer. To confirm whether BHD deletion in intestine will cause colon cancer, wewill cross BHD-flox strain with Villin -Cre mice, which express Cre in intestine.

AMENDMENT SUBMITTED JANUARY 31, 2007
This work is not duplicative of other research. A search has been performed to verify that.
Search Terms: BHD conditional knockout
Database Searched: PubMed
Date Searched: 1-31-07
NOTE: No date limits were put on this search. PubMed returned all records associated with these search terms.

AMENDMENT SUBMITTED OCTOBER 29, 2008
Since tumorigenesis requires more than one genetic change to progress, knockout of one tumor suppressor gene may not guarantee the formation of tumor. Knockout of two or three tumor suppressor genes may be necessary. The VHL gene is another important kidney cancer-related gene. However, knockout of VHL alone in the mouse kidney has not led to the formation of kidney tumors, implying that additional genetic mutations in other kidney cancer-related genes may be required. To address this issue, we decided to develop a double gene knockout mouse model involving the BHD and VHL genes. Another gene RB is also an important tumor suppressor gene that is associated with tumor initiation. We expect that this combination of knockouts in mice can producekidney cancer. The goal of this project is to exploit our knowledge of the genetic kidney disease and to improve the understanding of tumor suppressor genes and their mechanism in causing tumor formation. Currently, the VHL-, Bhd-, and Rb knockout mice are available in our repository. We have added Specific Aim #3 to the original protocol below and added 2 additional experiments.

Our specific aims in this research proposal are:
1) to determine the functional role of the BHD gene in kidney cancer, and
2) to confirm that the BHD gene is required for the development of kidney, intestine and lung and that inactivation of the gene will induce related tumorigenesis.
3) to determine whether double or triple knockouts could promote tumorigenesis.

Experiment #7:
In this experiment, we will cross the BHD-flox/flox mice with VHL-flox/flox mice to generate double BHD -/VHL-flox heterozygous mice. Heterozygous BHD-flox/+;VHL-flox/+ mice will be intercrossed to produce double homozygous BHD-flox/flox;VHL-flox/flox mice. BHD-flox/flox;VHL-flox/flox mice will be further bred to CMV-Cre mice to produce BHD-flox/+;VHL-flox/+;CMV-Cre heterozygous knockout mice.

Experiment #8:
In this experiment, we will cross the RB-flox/flox mice with VHL-flox/flox mice to generate double RB-flox/+;VHL-flox/+ heterozygous mice. Heterozygous RB-flox/+;VHL-flox/+ mice will be intercrossed to produce double homozygous RB-flox/+;VHL-flox/+ mice. RB-flox/+;VHL-flox/+ mice will be bred to CMV-Cre mice to generate double heterozygous RB-flox/+; VHL-flox/+;CMV-Cre heterozygous knockout mice.

The phenotype will be assessed at eight intervals:
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For all of these 10 groups, we will use 10 mice per genotype group to assess the phenotype based on our previous experience.

The experimental design for experiments 7 and 8 is similar to the previous experiments. The difference is that experiments 7 & 8 involve two tumor suppressor genes called double knockout.

14 Animal Requirements

15 List all strains used in this protocol, including knockout and transgenic lines. NOTE: Please lis

List all strains used in this protocol, including knockout and transgenic lines. NOTE: Please list strains by their common name, if available. For example, the gene-targeted strain 129-Trp53(tm1tyj) should be cited by its common name, which is p53. Another example is the transgenic strain C57BL/6j-TgN(pPGKneobpA) 3Ems, which should be cited by its common name, PGKneo.
The BHD-flox (KOH(R4R3-BHD)046BT4A3) conditional knockout mice will be obtained by crossing the BHD knockout chimeric mice (obtained from Dr. Swiatek's animal protocol #04-08-022, Gene Targeting Service) to C57BL/6 mice.

The FlpeR-Frt transgenic mice will be obtained from the Repository

The CMV-Cre transgenic mouse willbe obtained from the Repository.

The ERTM-Cre transgenic mouse will be obtained from Dr. Bin Teh's protocol #05-09-025, "The Functional Study of HRPT2 in Various Tissues Using Conditional Knockout Models".

The Ksp-Cre knock-in mouse will be obtained from the Repository.

The Sglt2-Cre transgenic mouse will be obtained from Dr. Bin Teh's protocol #06-06-019, "Conditional Knockout of VHL in Proximal Tubular Cells in the Kidney".

The Villin-Cre transgenic mouse will be obtained from the Repository.

The C57BL/6 mice will be obtained from the Vivarium breeding supply.

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AMENDMENT SUBMITTED OCTOBER 29, 2008
The VHL-flox/flox transgenic mousewill be obtained from the Repository.

The RB-flox/flox transgenic mouse will be obtained from the Repository.

The BHD-/VHL-flox mice will be obtained by crossing the BHD-flox/flox mice with VHL-flox/flox mice.

16 Age of animals: <updated 060903>

Age of animals:

a ☐ Adult (over four weeks of age)

b ☑ Neonatal through Adult

c ☐ Embryos

d ☐ Other

17 Sex of animals: <updated 120403>

Sex of animals:

a ☐ Only males

b ☐ Only females

c ☑ Both males and females

18 Vendor source: <updated 060903>

Vendor source:

a ☐ Charles River

b ☐ Jackson Labs

c ☐ Taconic Farms

d ☐ Harlan

e ☑ Other

19 [18e] Other response -2386

Non Standard Option (other) response
The BHD-flox (KOH(R4R3-BHD)046BT4A3) conditional knockout mice will be obtained by crossing the BHD knockout chimeric mice (obtained from Dr. Swiatek's animal protocol #04-08-022, Gene Targeting Service) to C57BL/6 mice.

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AMENDMENT SUBMITTED OCTOBER 29, 2008
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The BHD-/VHL-flox mice will be obtained by crossing the BHD-flox/flox mice with VHL-flox/flox mice.

20 Duration of the study: <updated 060903>

Duration of the study:
3 years.

21 Total number of animals to be used for Year 1: NOTE: Enter zero if there are no animals planned f

Total number of animals to be used for Year 1: NOTE: Enter zero if there are no animals planned for use during Year 1.
1175

22 Total number of animals to be used for Year 2: NOTE: Enter zero if there are no animals planned f

Total number of animals to be used for Year 2: NOTE: Enter zero if there are no animals planned for use during Year 2.

1175

23 Total number of animals to be used for Year 3: NOTE: Enter zero if there are no animals planned f

Total number of animals to be used for Year 3: NOTE: Enter zero if there are no animals planned for use during Year 3.

2222

24 Total number of animals to be used for the duration of the entire study: NOTE: This is the sum of

Total number of animals to be used for the duration of the entire study: NOTE: This is the sum of the totals for Years 1, 2 and 3 (see previous questions).

4572

25 Average daily census: (Definition: The average number of animals on the shelf on any given day duri

Average daily census: (Definition: The average number of animals on the shelf on any given day during an experiment.)
200

26 Will you house the animals in the Vivarium? <updated 060903>

Will you house the animals in the Vivarium?
a ☑ Yes

b ☐ No

27 Transportation

28 Will animals be transported within the Vivarium from Suites 1, 2 or Transgenic to Quarantine? <upd

Will animals be transported within the Vivarium from Suites 1, 2 or Transgenic to Quarantine?
a ☐ Yes

b ☑ No

29 Will animals be transported within the Vivarium from Suites 1, 2 or Transgenic to Imaging (either u

Will animals be transported within the Vivarium from Suites 1, 2 or Transgenic to Imaging (either ultrasound or confocal imaging)?
a ☐ Yes

b ☑ No

30 Will animals be transported from Quarantine to Imaging (either ultrasound or confocal imaging)? <u

Will animals be transported from Quarantine to Imaging (either ultrasound or confocal imaging)?

a ☐ Yes

b ☑ No

31 Will animals be transported outside the Vivarium facility on the 2nd floor (other than confocal ima

Will animals be transported outside the Vivarium facility on the 2nd floor (other than confocal imaging)?

a ☐ Yes

b ☑ No

32 Will animals be transported to the 4th or 5th floor? <updated 060903>

Will animals be transported to the 4th or 5th floor?

a ☑ Yes

b ☐ No

33 [32a] Following is the required process to transport animals to the 4th or 5th floor: It may be necessar

Following is the required process to transport animals to the 4th or 5th floor: It may be necessary, on a few occasions, to transfer live animals to the 4th or 5th floor for experimental purposes. The animals will be transported from the Vivarium in disposable cardboard buckets on carts. The buckets, which can transport up to three animals each, are available throughout the Vivarium. The animals must be placed in the bucket along with sufficient food and water source gel packs as needed. The transport buckets will be carried through the cagewash area, passed through the hall door and placed on a cart that has been placed outside the facility in the corridor. The mice will be transported to the 4th or 5th floor in the freight elevator. These mice will not be returned to the facility and will be euthanized within 12 hours of leaving the facility. The disposable transport buckets will not be returned to the Vivarium, but will be discarded in non-biohazard trash. Do you agree to this transportation process?

a ☑ Yes

b ☐ No

34 Will animals be shipped to locations outside of VARI? <updated 060903>

Will animals be shipped to locations outside of VARI?

a ☐ Yes

b ☑ No

35 Rationale For Animal Use

36 Explain your rationale for animal use in this protocol by answering the next series of questions.

Explain your rationale for animal use in this protocol by answering the next series of questions.

37 REGARDING MATHEMATICAL AND/OR COMPUTER MODELS: The following statement outlines the limitations of

REGARDING MATHEMATICAL AND/OR COMPUTER MODELS: The following statement outlines the limitations of mathematical and/or computer models in research. Please read the statement and indicate if it is applicable to your protocol. ==> Mathematical models and computer simulations rely heavily on predictions based on complete knowledge of a system. Since the interactions involved in complex biological systems and pathways are still largely unknown, mathematical and computer models are extremely limited in their ability represent a complex living system. Is this statement applicable to this protocol?

a ☑ Yes

b ☐ No

38 REGARDING CELL, TISSUE AND ORGAN CULTURE SYSTEMS: The following statement outlines the limitations

REGARDING CELL, TISSUE AND ORGAN CULTURE SYSTEMS: The following statement outlines the limitations of cell, tissue and organ culture systems in research. Please read the statement and indicate if it is applicable to your protocol. ==> These culture systems can be used effectively to screen chemical and physical agents in a rapid manner. The absence of the complex biological system is often advantageous in obtaining information on biological systems. However, cultured cells can lose their original properties and their genetic composition can be variable and unpredictable. Culture systems may not replicate the in vivo genetic response due to loss of the 3 dimensional structure of the animal and the complex biochemical, cell, tissue and organ interactions that occur in the living animal. Is this statement applicable to this protocol?

a ☑ Yes

b ☐ No

39 Justify the appropriateness of the species selected for use in this protocol by answering the next

Justify the appropriateness of the species selected for use in this protocol by answering the next series of questions.

40 REGARDING THE USE OF MICE: The following statement outlines the justification of the use of mice i

REGARDING THE USE OF MICE: The following statement outlines the justification of the use of mice in research. Please read the statement and indicate if it is applicable to your protocol. ==> The use of non-mammals has significant limitations in research. Although many different non-mammalian species (such as frogs, squid, zebrafish, and birds) can model a specific component of a system, there is not one single non-mammalian species that models a complete mammalian system accurately and reliably. In addition, there are many mammalian diseases for which models in non-mammals do not exist. Mice have significant advantages over other mammals as research subjects. Due to their short generation time and prolific breeding, investigators are able to perform studies in a cost-effective, time-efficient manner while using minimal numbers of animals. Due to their small size, they can be easily handled and have relatively minimal housing and care needs. Due to their common use as research subjects, a voluminous body of literature is available encompassing their breeding, housing, development & care. In addition, this widespread use has led to the intensive investigation of all aspects of mice in research (behavioral, genetic, biochemical, etc.) and the development of highly useful cell lines, biochemical products and more. Mice bear their young in utero which mimics human gestation and facilitates studies on early embryonic development. Finally, although mice are low on the phylogenetic scale, the structure and function of genes is very similar between mice and humans. For all these reasons and more, mice are the best small animal model for human disease. Is this statement applicable to this protocol?

a ☑ Yes

b ☐ No

41 Justify the number of animals to be used. The response must demonstrate that the number of animals

Justify the number of animals to be used. The response must demonstrate that the number of animals used in this protocol reflect the minimum number of animals needed for this protocol to be viable and statistically significant. Be sure to account for all experimental, control, breeding, and test pilot populations. For example, it is not sufficient to say that 100 animals are needed for an experiment. It is better to explain that 100 animals are needed because there are 5 injections being studied, and 10 animals are needed for statistical significance in each group. Therefore, 5 injections x 10 animals x 2 (for test and control groups) = 100 animals. SAMPLE TEXT: Two studies are planned/year; each study will last 1-3 months. Each study will contain 20 animals. Literature reports nearly 100% tumor take and low surgical mortality using the two techniques described in the surgery section. Twenty animals are necessary in the initial study to evaluate the two surgical procedures (Ten animals each should allow sufficient evaluation). Subsequent studies will require twenty animals to allow for animal loss due to surgical complications and no/poor tumor growth. While these procedures are fairly simple, complications during and post surgery can arise which require euthanization of the animal; this is a greater concern since this is a new procedure at VARI. The cell line used in this study has not been well characterized in an orthotopic model, so tumor growth rate and metastatic spread have not been fully evaluated. Twenty animals/study will allow for up to a 40% "failure rate" and still provide ~12 animals with similar tumor and metastatic pathogenesis for subsequent genomic

and proteomic evaluation. There are no animals needed for breeding for this protocol. 10 animals per study x 2 (for test and control groups) x 2 studies a year x 3 years = 120 animals

EXPERIMENT #1

Number Breakdown for Experiment #1:

This experiment involves crossing the mouse strain BHD-flox mice to FLPeR-Frt strain to generate a non-neomycin BHD-flox mice.

We will need to use the offspring of Experiment #1 to seed the breeding for Experiments #2-6. Please see

each individual experiment for the details on the use of the BHD flox mice; the numbers are just summarized here to outline what mice of which genotypes are necessary:

For Experiment #2, we will need 12 BHD-flox/+ mice and 20 BHD flox/flox mice.

For Experiment #3, we will need 3 BHD-flox/+ mice and 3 BHD flox/flox mice.

For Experiment #4, we will need 12

BHD-flox/+ mice and 20 BHD flox/flox mice.

For Experiment #5, we will need 12 BHD-flox/+ mice and 20 BHD flox/flox mice.

For Experiment #6, we will need 12 BHD-flox/+ mice and 20 BHD flox/flox mice.

For Experiment #7, we will need 3 VHL-flox/flox mice and 3 BHD-flox/flox mice.

For Experiment #8, we will need 3

RB-flox/flox mice and 3 VHL-flox/flox

Therefore, we need to produce 51 BHD-flox/+ miceand 83 BHD flox/flox mice as a result of Experiment #1.

Experiment #1, Breeding Step #A-Breeding the chimera to produce BHD-flox heterozygous:

3 male chimeras (obtained from Dr.

Pam Swiatek's protocol #04-08-022) will be bred to 3 C57BL/6 females, bred once to produce 30 offspring. These will be set up as paired matings over time.

Of these 30:

50% of the total, or roughly 15 pups, will be heterozygous (BHD flox/+)

Total mice used and produced in

Breeding Step #1A: 36

Experiment #1, Breeding Step #B- Breeding the BHD-flox heterozygous to the FLPeR heterozygous:

7 heterozygous BHD males from Breeding Step #1A will be bred to 7 heterozygous FLPeR females (obtained from the Repository), bred twice to produce 140 offspring. These will be set up as paired matings over time.

Of these 140: 25% of the total, or roughly 35 pups, will be double heterozygous (BHD flox/+; FLPeR flp/+)

Total mice used and produced in Breeding Step #1B: 147

Experiment #1, Breeding Step #C- Breeding to Get BHD Homozygous and BHD Heterozygous:

16 double heterozygous males from

Breeding Step #1B will be intercrossed to 16 double heterozygous female littermates from Breeding Step #1B, bred twice to produce 320 offspring. These will be set up as paired mating over time.

Please note that all of the breeding parents of this cross (the ddouble heterozygous animals which are the result of Breeding Step #1B and which are intercrossed in Breeding Step #1C) have been exposed to

FLPeR (as they are all double heterozygous). Therefore, we do not need to track the FLPeR genotype further, as it has already played its role of removing the neomycin cassette from the gene. For the offspring of this Breeding Step #1C, we only need to consider the BHD genotype.

Of these 320:

25% of the total, or roughly 120 pups, will be BBHD homozygous

(need 83)

50% of the total, or roughly 240 pups, will be BHD heterozygous (need 51)

Total mice used and produced in Breeding Step #1C: 320

Totals for Experiment #1:

Breeding Step 1A: 36

Breeding Step 1B: 147 Breeding Step 1C: 320

Total for Experiment #1: 503

EXPERIMENT #2

Number Breakdown for Experiment #2:

This experiment involves crossing the BHD-Flox strain to CMV-Cre to generate conventional BHD

knockout mice. This cross will take place using BHD-Flox mice which have already been crossed to FLPeR-frt mice in order to remove the neomycin cassette (these mice are the result of Experiment #1). However, since the FLPeR aspect of the genotype is not important for this cross, we only refer to the BHD -flox and the CMV-Cre strains below.

The phenotype will be assessed at eight intervals (for each interval, we

will assess 3 genotypes: 1) BHD homozygous; Cre heterozygous, 2) double heterozygous, and 3) BHD wild type; Cre heterozygous):

1) Day 1 after birth

2) Day 7 after birth

3) Day 21 after birth

4) 8 weeks old

5) 16 weeks old

6) 24 weeks old

7) 52 weeks old

8) 78 weeks old

We will also use mice for (again, we will use three genotypes: 1) BHD homozygous; Cre het, 2) double heterozygous, and

3) BHD wild-type; Cre heterozygous):

9) histological and pathological exams

10) establishment of fibroblast cell lines

For all of these 10 groups, we will use 10 mice per genotype group to assess the phenotype.

Mice needed for analysis:

100 BHD homozygous; Cre heterozygous mice (produced in Breeding Step #2 below)

100 BHD heterozygous; Cre heterozygous mice (produced in Breeding Step #1 below)

100 BHD wild type; Cre heterozygous mice (produced in Breeding Step #1 below)

In addition, we will also evaluate the phenotype at the prenatal stage by breeding double heterozygous males (from Breeding Step #2 below) to double heterozygous female mice. The pregnant females

will be euthanized by CO2 and the embryos collected for analysis.

Prenatal 10 days post coitum 10 BHD heterozygous; Cre heterozygous pregnant female mice

Prenatal 12 days post coitum 10 BHD heterozygous; Cre heterozygous pregnant female mice

Mice needed for analysis:

20 BHD heterozygous ; Cre heterozygous mice (produced in

Breeding Step #2B)

Experiment #2, Breeding Step #A- Breeding to get BHD Heterozygous & BHD wild-type:

Twelve BHD-flox/+ mice (obtained from Experiment #1) will be bred twice to 12 Cre tg/tg mice (obtained from the Repository).

These will be set up as paired matings over time.

Given an approximate litter of 10 pups, that should produce 240 offspring in the F1 generation.

Of these 240:

50%of the total, or roughly 120 pups, will be double heterozygous (BHD flox/+; Cre tg/+).

50%of the total, or roughly 120 pups, will be wildtype/heterozygous (BHD +/+; Cre tg/+)

Total mice used and produced in Breeding Step #2A: 252

Experiment #2, Breeding Step #B-Breeding to Get BHD Homozygous:

20 BHD flox/+; Cre tg/+ mice (obtained from Breeding Step #2A) will be bred twice to 20 BHD flox/flox; Cre +/+ mice(obtained from Experiment #1). These will be set up as paired mating over time.

Given an approximate litter of 10 pups, that should produce 400 offspring in the F2 generation.

Of these 400:

25% of the total, or roughly 100pups, will be BHD homozygous; Cre heterozygous (BHD flox/flox; Cre tg/+).

25% of the total, or roughly 100 pups, will be double heterozygous (BHD flox/+; Cre tg/+).

Total mice used and produced in

Breeding Step#2B: 400

Totals for Experiment #2:

Breeding Step 2A: 252

Breeding Step 2B: 400

Total for Experiment #2: 652

EXPERIMENT #3

Number Breakdown for Experiment #3:

This experiment involves crossing the mouse strain BHD-flox to ERTM-Cre to generate a tamoxifen-sensitive conditional knockout. This cross will take place using BHD-Flox mice which have already been crossed to FLPeR-frt mice in order to remove the neomycin cassette (thesemice are the result of Experiment #1).

This experiment will be performed on 30 adult mice:

Adult (24 weeks) 10 BHD homozygous; ERTM Cre heterozygous mice;

10 BHD heterozygous; ERTM Cre heterozygous mice;

10 BHD wild-type; ERTM Cre heterozygous mice

Mice needed for analysis:

10 BHD homozygous; ERTM heterozygous mice (produced in Breeding Step #2)

10 BHD heterozygous; ERTM heterozygous mice (produced in Breeding Step #1)

10 BHD wild-type; ERTM heterozygous mice (produced in Breeding Step #1)

Experiment #3, Breeding Step #A- Breeding to Get BHD Heterozygous & Wild Type:

Three BHD-flox/+ mice (obtained from Experiment #1) will be bred once to 3 ERTM tg/tg mice (obtained from the Dr. Bin Teh's protocol #05-09-025). These will be set up as paired matings over time.

Given an approximate litter of 10 pups, that should produce 30

offspring in the F1 generation.

Of these 30:

50% of the total, or roughly 15 pups, will be double heterozygous (BHD flox/+; Cre tg/+).

50% of the total, or roughly 15 pups, will be wildtype/heterozygous (BHD +/+; Cre tg/+)

Total mice used and produced in BreedingStep #3A: 33

Experiment #3, Breeding Step #B- Breeding to Get BHD Homozygous:

Three BHD flox/+; Cre tg/+ mice (from Breeding Step #3A) will be bred twice to 3 BHD flox/flox mice (obtained from Experiment #1).

Given an approximate litter of 10 pups, that should produce 60 offspring in the F2 generation.

Of these 60:

25% of the total, or roughly 15

pups, will be BHD homozygous; ERMT Cre heterozygous (BHD flox/flox; Cre tg/+).

Total mice used and produced in Breeding Step #3B: 60

Totals for Experiment #3:

Breeding Step 3A 33

Breeding Step 3B 60

Total for Experiment #3: 93

EXPERIMENT #4

This experiment involves crossing the BHD-Flox strain to Ksp-Cre to generate tissue-specific knockout mice. This cross will take place using BHD-Flox mice which have already been crossed to FLPeR-frt mice in orderto remove the neomycin cassette (these mice are the result of Experiment #1).

Experiment #4 uses the same number of animals and the same breeding scheme as Experiment #2.

Experiment #4 Total: 652 mice (all Category 1)

EXPERIMENT #5

This experiment involves crossing the BHD-Flox strain to Sglt2-Cre to generate tissue-specific knockout mice. This cross will take place

using BHD-Flox mice which have already been crossed to FLPeR-frt mice in order to remove the neomycin cassette (these mice are the result of Experiment #1).

Experiment #5 uses the same number of animals and the same breeding scheme as Experiment #2.

Experiment #5 Total: 652 mice (all Category 1)

EXPERIMENT #6

This experiment involves crossing the BHD-Flox strain to Villin-Cre to generate tissue-specific knockout mice. This cross will take place using BHD-Flox mice which have already been crossed to FLPeR-frt mice in order to remove the neomycin cassette (these mice are the result of Experiment #1).

Experiment #6 uses the same number of animals and the same breeding scheme as Experiment #2.

Experiment #6 Total: 652 mice (all Category 1)

TOTALS FOR PROTOCOL:

Experiment #1 503

Experiment #2 652

Experiment #3 93

Experiment #4 652

Experiment #5 652

Experiment #6 652

Subtotal: 3204

10% increase for death, error, etc. 321

GRAND TOTAL 3525

AMENDMENT SUBMITTED OCTOBER 29, 2008

EXPERIMENT #7

Number Breakdown for Experiment #7:

This experiment involves crossing the mouse strain BHD-flox/flox to VHL-flox/flox to generate a BHD-flox/flox,VHL-flox/flox mouse strain. This cross will take place

using BHD-flox/flox mice from Experiment#1,VHL-flox/flox mice, CMV-Cre mice that are available in our repository.

We need to produce around 100 BHD-flox/+; VHL-flox/+;CMV-Cre mice as a result of Experiment #7.

Experiment #7, Breeding Step #7A- Breeding the BHD-flox/floxand VHL-flox/flox to produce BHD-

flox/+,VHL-flox+ heterozygous:

3 BHD-flox/flox mice (male or female, obtained from Experiment#1 of this protocol) will be bred to 3 VHL-flox/flox (male or female, obtained from VAI repository), bred once to produce 30 offspring. These will be set up as paired matings over time.

Of these 30:

100% of the total, or 30 pups, will

be BHD-flox/+;VHL-flox/+ mice.

We need 20 BHD-flox/+;VHL-flox/+ mice for experiment#7B.

Total mice used and produced in Breeding Step #7A: 36

Experiment #7, Breeding Step #B- Intercrossing the BHD-flox/+;VHL-flox/+ to produce BHD-flox/flox;VHL-flox/flox:

10 BHD-flox/+,VHL-flox/+ males will be bred to 10 BHD-flox/+;VHL-flox/+ females from Breeding Step #7A, bred twice to produce 200 offspring. These will be set up as paired matings over time.

Of these 200:

6% of the total, or roughly 12 pups, will be double homozygous (BHD flox/flox; VHL- flox/flox)

Total mice used andproduced in

Breeding Step #7B: 220

Experiment #7, Breeding Step #C-Breeding to Get BHD-flox/+;VHL-flox/+;CMV-Cre Heterozygotes:

10 double homozygous (BHD flox/flox; VHL- flox/flox) mice from Breeding Step #7B will be bred to 10 CMV-Cre mice from VAI repository, bred twice to produce 200 offspring. These will be set up as paired mating over

time.

Please note that we suppose all the 10 CMV-Cre mice are heterozygotes. Therefore, the offspring of this Breeding Step #7C, 50% will be BHD-flox/flox;VHL-flox/flox;CMV-Cre mice.

Of these 200:

50% of the total, or roughly 100 pups, will beBHD-flox/flox;VHL-

flox/flox;CMV-Cre mice.

Total mice used and produced in Breeding Step #7C: 220

Totals for Experiment #7

Breeding Step 7A: 36

Breeding Step 7B: 220

Breeding Step 7C:220

Total for Experiment #7:476

EXPERIMENT #8

Number Breakdown for Experiment #8:

This experiment involves crossing the mouse strain BHD-flox/flox to VHL-flox/flox to generate a RB-flox/flox;VHL-flox/flox mouse strain. This cross will take place using RB-flox/flox mice and VHL-flox/flox mice that are available in

our repository.

We need to produce around 100 RB -flox/+;VHL-flox/+;CMV-Cre miceas a result of Experiment #8.

Experiment #8, Breeding Step #8A- Breeding the RB-flox/flox and VHL -flox/flox to produce RB-flox/+;VHL-flox/+ heterozygous:

3 RB-flox/flox mice (male or female, obtained from VAI

repository) will be bred to 3 VHL-flox/flox (male or female, obtained from VAI repository), bred once to produce 30 offspring. These will be set up as paired matings over time.

Of these 30:

100% of the total, or 30 pups, will be RB-flox/+;VHL-flox/+ mice.

We need 20 RB-flox/+;VHL-flox/+ mice for experiment#8B.

Total mice used and produced in Breeding Step #8A: 36

Experiment #8, Breeding Step #B-Intercrossing the RB-flox/+;VHL-flox/+ to prduce RB-flox/flox;VHLflox/flox:

10 RB-flox/+;VHL-flox/+ males will be bred to 10 RB-flox/+;VHL-flox/+ females from Breeding Step #8A, bred twice to produce 200

offspring. These will be set up as paired matings over time.

Of these 200:

6% of the total, or roughly12 pups, will be double homozygous (RB-flox/flox; VHL- flox/flox)

Total mice used and produced in Breeding Step #8B: 220

Experiment #8, Breeding Step #C-Breeding to Get RB-flox/+;VHL-flox/+;CMV-Cre Heterozygotes:

10 double double homozygous (RB-flox/flox; VHL- flox/flox) mice from Breeding Step #8B will be bred to 10 CMV-Cre mice from VAI repository, bred twice to produce 200 offspring. These will be set up as paired mating over time.

Please note that we suppose all the

10 CMV-Cre mice are heterozygotes. Therefore, the offspring of this Breeding Step #8C, 50% will be RB-flox/flox;VHL-flox/flox;CMV-cre mice.

Of these 200:

50% of the total, or roughly 100 pups, will be RB-flox/flox;VHL-flox/flox;CMV-Cre mice.

Total mice used and produced in

Breeding Step #8C: 220

Totals for Experiment #8

Breeding Step 8A: 36

Breeding Step 8B: 220

Breeding Step 8C:220

Total for Experiment #7:476

TOTALS FOR PROTOCOL:

Experiment #1 503

Experiment #2 652

Experiment #3 93

Experiment #4 652

Experiment #5 652

Experiment #6 652

Experiment#7 476

Experiment#8 476

Subtotal: 4156

10% increase for death, error.416

GRAND TOTAL4572

42 Description Of Experimental Design

43 Briefly explain the experimental design and specify all animal procedures. This description should

Briefly explain the experimental design and specify all animal procedures. This description should allow the IACUC to understand the experimental course of an animal from its entry into the experiment to the endpoint of the study. (NOTE: Explicit details on the animal procedures themselves will be provided in the next section. This section serves as an overview of the entire experimental course. List the animal procedures involved in throughout the course of the experiment in this section; provide full details on each animal procedure in the next section.)

This protocol includes8experiments, all of which explore the BHD gene conditional knockout.

Experiment #1

Since the BHD-construct contains a neomycin antibiotic gene that may lead to unknown background, we will cross the BHD-flox chimeric mice to FLPeR mice to remove the neomycin gene in order to reduce potential background noise. The offspring of this cross will then be used to begin the breeding in Experiments #2-6; they will not undergo any experimentation themselves

Experiment #2

We will first cross the BHD-flox strain with the CMV-Cre strain, which is a system Cre strain. This experiment will create BHD knockout mice in which the loss of BHD is throughout all tissues. This will allow us to observe both homozygous and heterozygous BHD knockouts. We expect the BHD-deficient mice will develop BHD disease similar to human. Since homozygous deletion of BHD will lead to embryonic lethality, we

will do the following conditional knockout experiments (#4-6) in related organs/tissues.

NOTE: Although we have determined that that the conventional BHD knockout is embryonically lethal, the construct for the conditional BHD-flox knockout focuses on a different exon group than the one used for the conditional knockout. Therefore, we hope that we will be able to produce viable homozygous

knockouts mice from this conditional knockout strain, as the portion of the gene excised by the conditional knockout construct is not thought to be as severe a mutation as the portion we used in the conventional knockout construct.

The mice will be bred and then euthanized by CO2 at the various timepoints outlined in the animal numbers (question #52). If any animal presents criteria for

euthanasia as outlined in question #131, they will be euthanized at that time. We expect that the heterozygous animals will develop tumors, as will the homozygous animals (if they do not turn out to be embryonically lethal). Live-born mice will be checked for kidney malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In addition, anatomical and histological analyses will be performed.

This experiment does not include any imaging or blood collection.

Experiment #3

Because mating BHD-flox with the CMV-cre strain may result in homologous embryonic lethality, we will cross BHD-flox mice with the ERTM-Cre strain, which is responsive to the application of tamoxifen, a synthetic estrogen. Unlike mice crossed to the CMV-Cre strain, mice crossed to the

ERTM-Cre mice will not express Cre until expose to tamoxifen. This will allow us to test and control freely systemic response to BHD elimination at different life stages. However, this experiment is not suitable for production of heterozygous BHD mice like Experiment #2. So both experiment #2 and experiment #3 are necessary.

The BHD conditional knockout mice will be crossed to the ERTM-Cre mice. 30 offspring (10 of each

of the following genotypes: BHD homozygous; ERTM heterozygous, BHD heterozygous; ERTM heterozygous, BHD wild-type; ERTM heterozygous) will be treated with tamoxifen to instigate the conditional BHD knockout. The tamoxifen will be administered when the mice reach 24 weeks of age. The dose is an intraperitoneal administration (full details including injection vehicle, dose, volume, and schedule are cited in questions #64-69).

For Experiment #3, we hypothesize that the systemic loss of BHD, even in the post-developmental stages, will result in mortality. (Please note that we will euthanize the mice as they develop criteria for euthanasia as outlined in Question #131; we will not allow the individual animals to progress to morbidity or mortality.) Therefore, mice will be maintained for up to 45 days after the final dose of tamoxifen. They will be monitored on a daily basis for criteria for euthanasia. If any individual animal presents criteria

for euthanasia, that animal will be euthanized by CO2 at that point. If any animals remain alive at the end of the 45 day period, they will be euthanized by CO2 at that point. All euthanized animals will be necropsied and kidney, intestine, and other tissues collected for analysis.

This experiment does not include any imaging or blood collection.

Experiment #4

In this experiment, BHD-flox strain will be crossed with Ksp-cre mice to give rise to kidney-specific BHD knockout mice. Kidney cancer is one of the important features of BHD syndrome, and Ksp-Cre miceexpress in Cre in the proximal tubular, the distal tubular, and the collecting duct cells of the kidney. So it is logical to try to further assess the role of BHD in kidneys.

The mice will be bred and then euthanized by CO2 at the various timepoints outlined in the animal numbers (question #52). If any animal presents criteria for euthanasia as outlined in question #131, they will be euthanized at that time. We expect that the heterozygous and homozygous animals may develop tumors and kidney disease. Live-born mice will be checked for kidney malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In

addition, anatomical and histological analyses will be performed.

This experiment does not include any imaging or blood collection.

Experiment #5

In this experiment, we will further cross the BHD-flox mice with Sglt2 -cre mice, which also express Cre kidney cells, but in the proximal

tubular cells only. The human disease RCC (Renal Clear Carcinoma) is believed to derive from the proximal tubular cells of the kidney, but there is currently no compelling evidence of this. Since it is not clear that kidney cancer come from which type of kidney cells, it is necessary to use different kidney Cre-expressing strains.

The mice will be bred and then euthanized by CO2 at the various timepoints outlined in the animal

numbers (question #52). If any animal presents criteria for euthanasia as outlined in question #131, they will be euthanized at that time. We expect that the heterozygous and homozygous animals may develop tumors and kidney disease. Live-born mice will be checked for kidney malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In addition, anatomical and histological analyses will be performed.

This experiment does not include any imaging or blood collection.

Experiment #6

In patients with confirmed deletion of BHD in the intestine, there is an increased level of colon cancer. To confirm whether BHD deletion in intestine will cause colon cancer, we will cross BHD-flox strain with Villin-cre mice, which express Cre in intestine.

The mice will be bred and then euthanized by CO2 at the various timepoints outlined in the animal numbers (question #52). If any animal presents criteria for euthanasia as outlined in question #131, they will be euthanized at that time. We expect that the heterozygous and homozygous animals may develop tumors and colon cancer or related intestinal diseases. Live-born mice will be checked for intestinal

malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In addition, anatomical and histological analyses will be performed.

This experiment does not include any imaging or blood collection.

AMENDMENT SUBMITTED JANUARY 31, 2007

We are adding blood collection to this protocol. This is a one-time collection only, we will collect by cardiac puncture according to Vivarium SOP #6.002 - Blood Collection Techniques in Mice. Wewill be collecting 200µl of blood, although we will draw the maximum available volume to ensure death by exsanguination. The purpose for this collection is to evaluate the levels of blood urea nitrogen, which is an indicator of potential kidney failure or dysfunction.

We plan to perform this terminal blood collection on all animals in this protocol which develop kidney-related criteria for euthanasia (kidney tumors or kidney cysts), as well as a matching number of control (healthy) mice of the same ages. Animals which develop criteria for euthanasia which are clearly not kidney-related will be euthanized by CO2.

This added procedure is also Category 1, so this does not change the Stress Level numbers.

This amendment does not add any animals.

AMENDMENT SUBMITTED OCTOBER 29, 2008

Experiment #7

Previous studies showed that knockout of VHL or BHD alone in mice has not developed kidney tumors (except for kidney cysts), indicating two or more genetic changes may be required for kidney tumorigenesis. In this experiment, we are going to double knockout the kidney cancer-related genes in mouse to see whether tumors will form in the kidney. We already have created BHD-flox/flox mice in experiment#1 and VHL-flox/flox mice are available in our VAI repository.

First, the BHD-flox/flox mice will breed to VHL-flox/flox mice to generate heterozygous BHD-flox/+;VHL-flox/+ mice. The generated BHD-flox/+;VHL-flox/+ mice will subject to intercross to produce homozygous BHD-flox/flox;VHL-flox/flox mice. Then, the homozygous BHD-flox/flox;VHL-flox/flox mice will breed to CMV-Cre mice (available in VAI repository) to give rise to heterozygous BHD-+/-;VHL-+/-,

CMV-Cre mice. Since homozygous knockout mice are embryonic lethal, double homozygous knockout mice are not expected in this experiment.

Endpoint

The mice will be bred and then euthanized by CO2 at the various time points outlined in the animal numbers (question #52). If any animal presents criteria for euthanasia as cited in Vivarium

SOP #6.031 ¿ Euthanasia of Mice, they will be euthanized at that time. We expect that the heterozygous and homozygous animals may develop tumors and colon cancer or related intestinal diseases. Live-born mice will be checked for intestinal malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In addition, anatomical and histological analyses will be performed.

This experiment does not include any imaging orblood collection.

Experiment #8

The RB gene is another important tumor suppressor gene. Unpublished data indicated that the RB gene is associated with kidney tumorigenesis. As mentioned in Experiment #7 above, two or more genetic changes may be required for kidney tumorigenesis. In this experiment, we are going to double

knockout the kidney cancer-related genes in mice to see whether tumors will form in the kidney. We already have created RB-flox/flox mice and VHL-flox/flox mice are available in our VAI repository.

First, the RB-flox/flox mice will breed to VHL-flox/flox mice to generate heterozygous RB-flox/+;VHL-flox/+ mice. The generated RB-flox/+;VHL-flox/+ mice will subject to intercross to produce homozygous RB-flox/flox;VHL-flox/flox mice. Then the homozygous RB-flox/flox;VHL

-flox/flox mice will breed to CMV-Cre mice (available in VAI repository) to give rise to heterozygous RB-+/-;VHL-+/-, CMV-Cre mice. Since homozygous knockout mice are embryonic lethal, double homozygous knockout mice are not expected in this experiment.

Endpoint

The mice will be bred and then euthanized by CO2 at the various time points outlined in the animal numbers. If any animal presents

criteria for euthanasia as cited in Vivarium SOP #6.031 ¿ Euthanasia of Mice, they will be euthanized at that time. We expect that the heterozygous and homozygous animals may develop tumors and colon cancer or related intestinal diseases. Live-born mice will be checked for intestinal malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In addition, anatomical and histological analyses will be performed.

This experiment does not include any imaging or blood collection.

The phenotype will be assessed at eight intervals:

1) Day 1 after birth

2) Day 7 after birth

3) Day 21 after birth

4) 8 weeks old

5) 16 weeks old

6) 24 weeks old

7) 52 weeks old

8) 78 weeks old

We will also use mice for:

9) histological and pathological exams

10) establishment of fibroblast cell lines

For all of these 10 groups, we will use 10 mice per genotype group to assess the phenotype based on our previous experience.

44 Description Of Animal Procedures

45 Please give explicit details to each of the following questions. ALL applicable questions must be

Please give explicit details to each of the following questions. ALL applicable questions must be answered in full. NOTE: Although surgical procedures are covered in the Surgery section, any questions in this section that apply to procedures performed within the context of surgery (such as experimental injections or methods of restraint) must be answered here fully. The details in the Surgery section may refer back to these answers.

46 The standard procedure for veterinary care at VARI is as follows: Daily veterinary care will be pr

The standard procedure for veterinary care at VARI is as follows: Daily veterinary care will be provided to all animals by the Vivarium staff. The Vivarium staff will consult with the attending veterinarian Dr. Joan Koelzer (616) 437-6415 or the alternate attending veterinarian Dr. Diane Egedy (616) 827-2950 when necessary. In the case animals are found sick or dead the PI will be notified via email and phone. PI will be notified with symptomology, disposition and animal identifier. In the event the PI cannot be reached,

associates in the PI's lab will be contacted. In the event PI and his/her associates cannot be contacted any sick mice will be treated at the discretion of the Vivarium staff or attending veterinarian. Any animals found dead will be placed in a -20 refrigerator. Do you plan to follow this standard procedure?

a ☑ Yes

b ☐ No

47 How will the animals be identified? <updated 060903>

How will the animals be identified?

a ☑ Cage cards

b ☑ Ear notches

c ☐ Ear tags

d ☐ Transponders

e ☐ Footpad tattoo

f ☐ Tail tattoo

g ☐ Other

48 Will methods of restraint be used? NOTE: The brief placement of a mouse in a "broom-stick" holder

Will methods of restraint be used? NOTE: The brief placement of a mouse in a "broom-stick" holder for tattoo or IV tail injection is not considered restraint at VARI.

a ☐ Yes

b ☑ No

49 Will you be administering experimental injections or inoculations? <updated 060903>

Will you be administering experimental injections or inoculations?

a ☑ Yes

b ☐ No

50 [49a] Do you have more than six experimental injections or inoculations (e.g. cells, infectious agents, a

Do you have more than six experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.)? NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Yes

b ☑ No

51 [50b] Answer the following questions for the 1st experimental injections or inoculations (e.g. cells, inf

Answer the following questions for the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

52 [50b] Enter the name(s) of the 1st experimental injections or inoculations (e.g. cells, infectious agents

Enter the name(s) of the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

For Experiment #3, we will inject 1 microgram of tamoxifen in 100 microliters of sunflower oil intraperitoneally.

Please note that this dosage and the use of sunflower oil as media is based on the following attached paper (p.635): Li et al, Skin abnormalities generated by temporally controlled RXRalpha mutations in mouse epidermis., Nature. 2000 Oct 5;407(6804):633-6.

53 [50b] Select the route of administration for the 1st experimental injections or inoculations (e.g. cells,

Select the route of administration for the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Intramuscular

b ☐ Intravenous

c ☑ Intraperitoneal

d ☐ Subcutaneous

e ☐ Intradermal

f ☐ Orthotopic

g ☐ Arterial

h ☐ Other

54 [50b] Indicate the site of administration for the 1st experimental injections or inoculations (e.g. cells

Indicate the site of administration for the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). For example, right flank, between the shoulder blades, tail vein (for intravenous), etc. NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

Abdomen.

55 [50b] Specify the dosage of the 1st experimental injections or inoculations (e.g. cells, infectious agent

Specify the dosage of the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

For Experiment #3, we will inject 1 milligram of tamoxifen in 100 microliters of sunflower oil.

56 [50b] Specify the volume of the 1st experimental injections or inoculations (e.g. cells, infectious agent

Specify the volume of the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

100 microliters

57 [50b] Specify the schedule of administration of the 1st experimental injections or inoculations (e.g. cel

Specify the schedule of administration of the 1st experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.). NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

For Experiment #3, we will inject the tamoxifen according to the following schedule:

In Week Zero, the animal will be injected once per day for five consecutive days.
In Week Two, the animal will be injected once per day for three consecutive days.
In Week Four, the animal will be injected once per day for three consecutive days.
In Week Six, the animal will be injected once per day for three consecutive days.

58 [50b] Do you have a 2nd group of experimental injections or inoculations (e.g. cells, infectious agents,

Do you have a 2nd group of experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.)? NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Yes

b ☑ No

59 [50b] Do you have a 3rd group of experimental injections or inoculations (e.g. cells, infectious agents,

Do you have a 3rd group of experimental injections or inoculations (e.g. cells, infectious agents, adjuvants,

etc.)? NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Yes

b ☑ No

60 [50b] Do you have a 4th group of experimental injections or inoculations (e.g. cells, infectious agents,

Do you have a 4th group of experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.)? NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Yes

b ☑ No

61 [50b] Do you have a 5th group of experimental injections or inoculations (e.g. cells, infectious agents,

Do you have a 5th group of experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.)? NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Yes

b ☑ No

62 [50b] Do you have a 6th group of experimental injections or inoculations (e.g. cells, infectious agents,

Do you have a 6th group of experimental injections or inoculations (e.g. cells, infectious agents, adjuvants, etc.)? NOTE: Combine agents that have the same route, dose, volume and schedule of administration.

a ☐ Yes

b ☑ No

63 Will you collect blood? <updated 060903>

Will you collect blood?

a ☑ Yes

b ☐ No

64 [63a] What is the frequency of blood withdrawal? <updated 060903>

What is the frequency of blood withdrawal?

This is a one-time terminal collection.

65 [63a] What is the volume of blood to be collected? <updated 060903>

What is the volume of blood to be collected?
We will be collecting 200µl of blood, although we will draw the maximum available volume to ensure death by exsanguination.

66 [63a] Will you use a retroorbital bleed procedure? <updated 060903>

Will you use a retroorbital bleed procedure?

a ☐ Yes

b ☑ No

67 [63a] Will you use a saphenous vein blood withdrawal procedure? <updated 060903>

Will you use a saphenous vein blood withdrawal procedure?

a ☐ Yes

b ☑ No

68 [63a] Will you use a cardiac puncture (terminal) blood withdrawal procedure? <updated 060903>

Will you use a cardiac puncture (terminal) blood withdrawal procedure?

a ☑ Yes

b ☐ No

69 [68a] The standard procedure for performing a cardiac puncture (terminal) blood withdrawal at VARI is as

The standard procedure for performing a cardiac puncture (terminal) blood withdrawal at VARI is as follows: The animal will be anesthetized with Avertin or Isoflurane. The mouse will be held by the scruff of the skin about the shoulders so that its head is up and its rear legs are down. A 22-gauge needle attached to a 1mL syringe will be inserted 5mm from the center of the thorax toward the animal's chin, 5-10mm deep, holding the syringe 25-30 degrees away from the chest. If blood doesn't appear immediately, the syringe will be aspirated slightly. When blood appears the plunger will be gently pulled back to obtain the maximum amount of blood available. After performing the withdrawal the animal's breathing and heartbeat will be checked to ensure death. The carcass will be frozen. Do you plan to follow this standard procedure?

a ☑ Yes

b ☐ No

70 [63a] Will you use a blood withdrawal procedure other than retroorbital bleed, saphenous vein, or cardiac

Will you use a blood withdrawal procedure other than retroorbital bleed, saphenous vein, or cardiac puncture (terminal)?

a ☐ Yes

b ☑ No

71 Will tail biopsies be performed? <updated 060903>

Will tail biopsies be performed?

a ☑ Yes

b ☐ No

72 [71a] The standard procedure for tail biopsies at VARI is as follows: The tail of a mouse contains a var

The standard procedure for tail biopsies at VARI is as follows: The tail of a mouse contains a variety of tissues including bone, cartilage, blood vessels and nervous tissues. In a young mouse, the tissue near the tip of the tail is soft and the bones have not yet mineralized completely. Therefore, biopsy of the tail tip of a young mouse probably amounts to momentary pain for the animal. As the animal ages, tissue maturation includes mineralization of the bone and increased vascularity. Tail tip biopsy performed on an older animal is

likely to involve more than momentary pain and distress as well as the potential for significant hemorrhage. Therefore, the tail biopsy will be performed without general anesthesia in mice less than 3.5 weeks (25 days). The total amount of tail clipped and removed will be the minimum required and will not exceed 1.0 centimeters of tail. The amount of blood loss is usually small when tails are clipped at the recommended age; hemostatis is achieved by direct pressure on the end of the tail, electrocauterization, or silver nitrate. Repeated tail amputation on a single mouse will not be performed. An alternative to tail clipping will be performed if an additional sample is needed. An ear punch will serve as an alternative tissue sample to tail biopsy. Do you plan to follow this standard procedure?

a ☑ Yes

b ☐ No

73 Will you use radiation? NOTE: This includes the use of radioisotopes, machine-produced radiation

Will you use radiation? NOTE: This includes the use of radioisotopes, machine-produced radiation (the bone densitometer, x-ray machine, etc.), and all other types of radiation.

a ☐ Yes

b ☑ No

74 Do you intend to perform any other procedures not already addressed? <updated 060903>

Do you intend to perform any other procedures not already addressed?

a ☐ Yes

b ☑ No

75 Will the mice be exposed to any other potential stressors (food or water deprivation, noxious stimu

Will the mice be exposed to any other potential stressors (food or water deprivation, noxious stimuli, environmental stress, etc.)?

a ☐ Yes

b ☑ No

76 Does this protocol include the generation or use of any transgenic or knockout animals? <updated 1

Does this protocol include the generation or use of any transgenic or knockout animals?

a ☑ Yes

b ☐ No

77 [76a] For transgenic and knockout animals, are you expecting any phenotypic consequences? <updated 12040

For transgenic and knockout animals, are you expecting any phenotypic consequences?

a ☑ Yes

b ☐ No

78 [77a] For transgenic and knockout animals, describe any expected phenotypic consequences and any special

For transgenic and knockout animals, describe any expected phenotypic consequences and any special care or monitoring that these phenotypes may require.

We expect that these animals may have kidney, colorectal, and lung cysts. If mice present criteria for euthanasia as stated in Question #131, they will be euthanized.

79 According to standard Vivarium practice, the following symptoms are considered cause for euthanasia

According to standard Vivarium practice, the following symptoms are considered cause for euthanasia: > Tumor size of 2500 cubic millimeter or greater > 20 percent loss of body weight in one week > Inability to eat or drink > Behavior abnormality > Slow, shallow, labored breathing > Hunched posture > Ruffled fur (for 3 days), failure to groom > Hypo- or hyper- thermia > Diarrhea or constipation (3 days) > Skin sores, infections, necrotic tissues and tumors > Lethargy (for 3 days) > Impaired mobility > Persistent bleeding > Paralysis > CNS signs (persistent seizures, spasticity, weakness) > Self-segregation from other animals
Please read the list carefully, as some of the symptoms listed above may be expected and allowable under certain experimental circumstances. Do you agree to euthanize all animals meeting any of the above criteria?

a ☑ Yes

b ☐ No

80 List any other symptoms which should be considered cause for euthanasia

(other than the standard Vi

List any other symptoms which should be considered cause for euthanasia (other than the standard Vivarium criteria, listed below): > Tumor size of 2500 cubic millimeter or greater > 20 percent loss of body weight in one week > Inability to eat or drink > Behavior abnormality > Slow, shallow, labored breathing > Hunched posture > Ruffled fur (for 3 days), failure to groom > Hypo- or hyper- thermia > Diarrhea or constipation (3 days) > Skin sores, infections, necrotic tissues and tumors > Lethargy (for 3 days) > Impaired mobility > Persistent bleeding > Paralysis > CNS signs (persistent seizures, spasticity, weakness) > Self-segregation from other animals If there are no other symptoms to consider cause for euthanasia, simply state "None".

None

81 Indicate the expected endpoint of the experiment. Please be aware that death as an endpoint (in pl

Indicate the expected endpoint of the experiment. Please be aware that death as an endpoint (in place of euthanasia) must always be scientifically justified. Some examples of experimental endpoint are: > The study will continue until the mice develop tumors no greater than 2500 cubic millimeters. At that time, they will be euthanized and dissected. > The study will end six weeks after injection, when all the mice will be sacrificed and necropsies performed. > The mice will be maintained as part of an aging study (aging studies maintain mice beyond 12 months of age). They will be sacrificed as they develop any symptoms meeting the criteria for euthanasia cited earlier in this protocol.

This protocol includes8experiments, all of which explore the BHD geneand related genes VHL, RBconditional knockout.

Experiment #1

The offspring of this cross will only be used to begin the breeding in Experiments #2-6; they will not undergo any experimentation themselves. These mice will be euthanized by CO2 when their breeding usage is at an end. Mice produced which do not meet the target genotypes for the breeding will be euthanized by CO2 when the genotyping results have been received.

Experiment #2:

We will first cross the BHD-flox strain with the CMV-Cre strain, which is a system Cre strain. Mice produced which do not meet the target genotypes for the breeding will be euthanized by CO2 when the genotyping results have been received.

The offspring of the target genotypes will be euthanized by CO2 at the various timepoints outlined in the animal numbers (question #52). If any animal presents criteria for euthanasia as outlined in question #131, they will be euthanized at that time. We expect that the heterozygous animals will develop tumors, as will the homozygous animals (if they do not turn out to be embryonically lethal). Live-born mice will be checked for kidney malformations as well as for physiological or behavioral defects such as weakness, seizures etc. In addition, anatomical and histological analyses will be performed.

Experiment #3:
Because mating BHD-flox with the CMV-cre strain may result in homologous embryonic lethality, we will cross BHD -flox mice with the ERTM-Cre strain, which is responsive to the application of tamoxifen, a synthetic estrogen.

The BHD conditional knockout mice will be crossed to the ERTM-Cre mice. Mice produced which do not meet the target genotypes for the breeding will be euthanized by CO2 when the genotyping results have been received.

For Experiment #3, we hypothesize that the systemic loss of BHD, even in the post-developmental stages, will result in mortality. (Please note that we will euthanize the mice as they develop criteria for euthanasia as outlined in Question #131; we will not allow the individual animals to progress to morbidity or mortality.) Therefore, mice will be maintained for up to 45 days after the final dose of tamoxifen. They will be monitored on a daily basis for criteria for euthanasia. If any individual animal presents criteria for euthanasia, that animal will be euthanized by CO2 at that point. If any animals remain alive at the end of the 45 day period, they will be euthanized by CO2 at that point. All euthanized animals will be necropsied and kidney, intestine, and other tissues collected for analysis.

Experiment #4:
The experimental endpoint (both timing and method) for this experiment is exactly the same as for experiment #2.

Experiment #5:
The experimental endpoint (both timing and method) for this experiment is exactly the same as for experiment #2.

Experiment #6:
The experimental endpoint (both timing and method) for this experiment is exactly the same as for experiment #2.

AMENDMENT SUBMITTED JANUARY 31, 2007
<PALIGN>We plan to perform terminal blood collection on all animals in this protocol which develop kidney-related criteria for euthanasia (kidney tumors or kidney cysts), as well as a matching number of control (healthy) mice of the same ages. Animals which develop criteria for euthanasia which are clearly not kidney-related will be euthanized by CO2. We will be collecting 200µl of blood, although we will draw the maximum available volume to ensure death byexsanguination.AMENDMENT SUBMITTED OCTOBER 29, 2008Experiment #7:The experimental endpoint (both timing and method) for this experiment is exactly the same as for experiment #2.

Experiment #8:
The experimental endpoint (both timing and method) for this experiment is exactly the same as for experiment#2

82 Surgery

83 Will you be performing surgery in the course of this protocol? <updated 060903>

Will you be performing surgery in the course of this protocol?

a ☐ Yes

b ☑ No

84 Pain Or Distress

85 All animals must be classified according to the highest pain or distress stress level they are expe

All animals must be classified according to the highest pain or distress stress level they are expected to encounter in the course of the protocol (this information is entered on the Segment Profile tab). The three stress levels are: ==> Category 1 is defined as: minimal, transient, or no pain or distress. ==> Category 2 is defined as: pain or distress relieved by appropriate measures. ==> Category 3 is defined as: unrelieved pain or distress. Do you have any animals that are classified as Stress Level Category 3? NOTE: If animals are indicated in Category 3, a scientific justification will be required (see next question).

a ☐ Yes

b ☑ No

86 Anesthesia, Analgesia, Tranquilization

87 Will you be using any anesthesia, analgesia or tranquilization during the course of this protocol?

Will you be using any anesthesia, analgesia or tranquilization during the course of this protocol? NOTE: All animals indicated as pain or distress Stress Level Category 2 (as entered on the Segment Profile tab) must receive some form of anesthesia, analgesia or tranquilization. The three stress levels are: ==> Category 1 is defined as: minimal, transient, or no pain or distress. ==> Category 2 is defined as: pain or distress relieved by appropriate measures. ==> Category 3 is defined as: unrelieved pain or distress.

a ☑ Yes

b ☐ No

88 [87a] Will you be using Isoflurane during the course of this protocol? <updated 060903>

Will you be using Isoflurane during the course of this protocol?

a ☑ Yes

b ☐ No

89 [88a] The standard administration of Isoflurane at VARI is as follows: Isoflurane is administered via in

The standard administration of Isoflurane at VARI is as follows: Isoflurane is administered via inhalation through the use of an Isoflurane anesthesia machine. Personnel must receive training for this machine. The anesthetic agent is 2-2.5% of Isoflurane in oxygen at one liter per minute. Do you plan to follow this standard administration?

a ☑ Yes

b ☐ No

90 [87a] Will you be using Avertin during the course of this protocol? <updated 060903>

Will you be using Avertin during the course of this protocol?

a ☐ Yes

b ☑ No

91 [87a] Will you be using a Ketamine/Xylazine cocktail during the course of this protocol? <updated 060903

Will you be using a Ketamine/Xylazine cocktail during the course of this protocol?

a ☐ Yes

b ☑ No

92 [87a] Will you be using some other form of anesthesia, analgesia, or tranquilization (other than Isoflura

Will you be using some other form of anesthesia, analgesia, or tranquilization (other than Isoflurane, Avertin or a Ketamine/Xylazine cocktail) during the course of this protocol?

a ☐ Yes

b ☑ No

93 [87a] For all forms anesthesia, the animal must be monitored to ensure that it is in Stage 3 (surgical an

For all forms anesthesia, the animal must be monitored to ensure that it is in Stage 3 (surgical anesthesia) and not Stage 2 (non-surgical) or Stage 4 (medullary paralysis). Several criteria can be used for determining the depth of anesthesia. The most common method used at VARI to determine depth of anesthesia is to test using several of the following criteria: > Ocular nystagmus, the side-to-side movement of the eyeball, is absent. To

check, evaluate visually. This movement occurs in Stage 2, light anesthesia. > Palpebral (blink) reflex is absent. To check, lightly touch the corner of the eye with a fragment of suture material. This reflex is absent in Stage 3. > Corneal reflex is present. To check, lightly touch the cornea with a fragment of suture. This reflex is present in Stage 3 and absent in Stage 4. > Skeletal muscles are relaxed. To check, evaluate visually. Muscle tone is usually absent in Stage 3 and present in Stage 2. > Toe and tail pinch response is absent. To check, simply pinch toe or tail with fingernail or forceps. These responses are absent in Stage 3 and present in Stage 2. Will you be using this method to determine depth of anesthesia?

a ☑ Yes

b ☐ No

94 [87a] Are any paralytic agents used during the course of this protocol? <updated 060903>

Are any paralytic agents used during the course of this protocol?

a ☐ Yes

b ☑ No

95 Method Of Euthanasia

96 The most common method of euthanasia used at VARI is: > Mice will be euthanized by CO2 inhalatio

The most common method of euthanasia used at VARI is: > Mice will be euthanized by CO2 inhalation (from compressed gas) until respiration and heartbeat have ceased. However, mice under 7 days will be euthanized by decapitation with surgical scissors and mice under anesthesia will be euthanized by cervical dislocation. NOTE: Dry ice is not an acceptable source of CO2. Do you plan to use CO2 inhalation (as stated above) as one of your methods of euthanasia?

a ☑ Yes

b ☐ No

97 [96a] Do you have any other methods of euthanasia? <updated 021104>

Do you have any other methods of euthanasia?

a ☑ Yes

b ☐ No

98 [97a] Indicate the proposed method(s) of euthanasia and give full details:

<updated 060903>

Indicate the proposed method(s) of euthanasia and give full details:
We are adding blood collection to this protocol. This is a one-time collection only, we will collect by cardiac puncture according to Vivarium SOP #6.002 - Blood Collection Techniques in Mice. We will be collecting 200µl of blood, although we will draw the maximum available volume to ensure death by exsanguination.

99 [97a] Will a chemical agent be used for euthanasia? <updated 060903>

Will a chemical agent be used for euthanasia?

a ☐ Yes

b ☑ No

100 [99b] Do the method(s) of euthanasia used include ANY methods which are NOT recommended by the AVMA Panel

Do the method(s) of euthanasia used include ANY methods which are NOT recommended by the AVMA Panel Report on Euthanasia (e.g. decapitation or cervical dislocation without anesthesia)?

a ☐ Yes

b ☑ No

101 At the conclusion of the study, all remaining animals will be euthanized. Carcass disposal will be

At the conclusion of the study, all remaining animals will be euthanized. Carcass disposal will be handled by the Vivarium staff according to standard procedures.

102 Hazardous Agents

103 Does this protocol include the use of Recombinant DNA (rDNA)? <updated 060903>

Does this protocol include the use of Recombinant DNA (rDNA)?

a ☐ Yes

b ☑ No

104 Does this protocol include the use of biological agents? NOTE: This includes all tissues, tissue

Does this protocol include the use of biological agents? NOTE: This includes all tissues, tissue cultures, cell lines, proteins, etc. of both human and non-human origin.

a ☐ Yes

b ☑ No

105 Does this protocol include the use of hazardous chemicals or drugs? NOTE: This includes all contr

Does this protocol include the use of hazardous chemicals or drugs? NOTE: This includes all controlled substances, new drugs with unknown properties, chemicals that require special handling, etc.

a ☑ Yes

b ☐ No

106 [105a] List the hazardous chemicals or drugs that will be used in this protocol: NOTE: An MSDS must be s

List the hazardous chemicals or drugs that will be used in this protocol: NOTE: An MSDS must be submitted for each hazardous chemical or drug. List all MSDS under the attachments heading of this protocol form. Submit a copy (paper or electronic) of each of the MSDS per the directions in the attachments section. All MSDS must be received prior to the commencement of animal work in this protocol.

Tamoxifin will be used as an injectate in Experiment #3.

107 Does this protocol include the use of radioisotopes? <updated 060204>

Does this protocol include the use of radioisotopes?

a ☐ Yes

b ☑ No

10 8 Does this protocol include the use of machine-generated radiation (for example, radiation produced

Does this protocol include the use of machine-generated radiation (for example, radiation produced by a bone densitomer, an x-ray machine, etc.)?

a ☐ Yes

b ☑ No

10 9 Will this protocol generate any contaminated animals or materials, such as bedding, that will need

Will this protocol generate any contaminated animals or materials, such as bedding, that will need special handling for disposal?

a ☐ Yes

b ☑ No

11 0 Indicate the Animal Biosafety Level at which the study will be conducted. NOTE: The Animal Biosaf

Indicate the Animal Biosafety Level at which the study will be conducted. NOTE: The Animal Biosafety Level is determined by the risk associated with activities involving vertebrate animals which may be either experimentally or naturally infected. The risk is assessed based on the danger to humans, not the danger to the animals. The details on the four levels are below. Definition: (ABSL-1) Animal Biosafety Level One ========== Is suitable for work involving well characterized agents that are not known to cause disease in healthy adult humans, and that are of minimal potential hazard to laboratory personnel and the environment. Definition: (ABSL-2) Animal Biosafety Level Two ========== Involves practices for work with those agents associated with human disease. It addresses hazards from ingestion as well as from percutaneous and mucous membrane exposure. Definition: (ABSL-3) Animal Biosafety Level Three ========== Involves practices suitable for work with animals infected with indigenous or exotic agents that present the potential of aerosol transmission and of causing serious or potentially lethal disease. Definition: (ABSL-4) Animal Biosafety Level Four ========== Involves practices suitable for addressing dangerous agents that pose high risk of life threatening disease, aerosol transmission, or related agents with unknown risk of transmission.

a ☑ ABSL-1: Level One

b ☐ ABSL-2: Level Two

c ☐ ABSL-3: Level Three

d ☐ ABSL-4: Level Four

11 Are there any other issues involving hazards to humans that need to be addressed? <updated 060204>

Are there any other issues involving hazards to humans that need to be addressed?

a ☐ Yes

b ☑ No

11 Biological Materials

11 Does this protocol include any biological materials or animal products for use in animals? <update

Does this protocol include any biological materials or animal products for use in animals?

a ☐ Yes

b ☑ No

11 Special Considerations

11 Does this protocol call for any special considerations such as special housing, equipment or animal

Does this protocol call for any special considerations such as special housing, equipment or animal care (e.g. special caging, water, feed or waste disposal, environmental enhancement, etc.)?

a ☐ Yes

b ☑ No

11 Certifications

11 I certify that I have attended the institutionally required investigator training course. <updated

I certify that I have attended the institutionally required investigator training course.

a ☑ Yes

b ☐ No

11 [117a] Enter the date (month & year) that you completed the institutionally required investigator training

Enter the date (month & year) that you completed the institutionally required investigator training course.

October 2004

11 [117a] Enter the location (institution, city & country) that you completed the institutionally required in

Enter the location (institution, city & country) that you completed the institutionally required investigator training course.

VARI

12 I certify that I have determined that the research proposed herein is not unnecessarily duplicative

I certify that I have determined that the research proposed herein is not unnecessarily duplicative of previously reported research.

a ☑ Yes

b ☐ No

12 I certify that all individuals working on this proposal who are at risk are 1 participating in the In

I certify that all individuals working on this proposal who are at risk are participating in the Institution's Occupational Health and Safety Program.

a ☑ Yes

b ☐ No

12 I certify that the individuals listed as ASSOCIATES on this protocol are 2 authorized to conduct the

I certify that the individuals listed as ASSOCIATES on this protocol are authorized to conduct the procedures involving animals that are detailed in this protocol and that they have attended the institutionally required investigator training course and received training in the following: > The biology, handling and care of this species > Aseptic surgical methods and techniques (if necessary) > The concept, availability and use of research or testing methods that limit use of animals or minimize distress > The proper use of anesthetics, analgesics, and tranquilizers (if necessary) > Procedures for reporting animal welfare concerns > Appropriate methods of humane euthanasia

a ☑ Yes

b ☐ No

12 Does this protocol include any Stress Level Category 3 proposals? NOTE: All 3 animals must be class

Does this protocol include any Stress Level Category 3 proposals? NOTE: All animals must be classified according to the highest pain or distress stress level they are expected to encounter in the course of the protocol (this information is entered on the Segment Profile tab). The three stress levels are: ==> Category 1 is defined as: minimal, transient, or no pain or distress. ==> Category 2 is defined as: pain or distress relieved by appropriate measures. ==> Category 3 is defined as: unrelieved pain or distress.

a ☐ Yes

b ☑ No

12 I certify that I will obtain approval from the IACUC before initiating any 4 significant changes in t

I certify that I will obtain approval from the IACUC before initiating any significant changes in this study.

a ☑ Yes

b ☐ No

12 I certify that I will notify the IACUC regarding any unexpected study results 5 that impact the anima

I certify that I will notify the IACUC regarding any unexpected study results that impact the animals and that any unanticipated pain or distress, morbidity or mortality will be reported to the attending veterinarian and the IACUC.

a ☑ Yes

b ☐ No

12 I certify that I am familiar with and will comply with all pertinent 6 institutional, state and feder

I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.

a ☑ Yes

b ☐ No

12 Attachments 7

12 Does this protocol include any references to supporting materials (articles, 8 abstracts, etc.)? <up

Does this protocol include any references to supporting materials (articles, abstracts, etc.)?

a ☑ Yes

b ☐ No

12 [128a] List supporting materials cited within the protocol. All supporting 9 materials must be submitted as

List supporting materials cited within the protocol. All supporting materials must be submitted as attachments to the protocol. NOTE: If any documents are not yet available for attachment, cite them as "PENDING RECEIPT". Forward any attachments (electronic or physical) to Kaye Johnson, IACUC Administrative Assistant (kaye.johnson@vai.org, 4th floor, x5702).

For Experiment #3, we will inject 1 microgram of tamoxifen in 100 microliters of sunflower oil intraperitoneally.

Please note that this dosage and the use of sunflower oil as media is based on the following paper (attached):
Li et al, Skin abnormalities generated by temporally controlled RXRalpha mutations in mouse epidermis., Nature. 2000 Oct 5;407(6804):633-6.

13 0 Does this protocol include the use of biological materials that must be MAP tested? <updated 06090

Does this protocol include the use of biological materials that must be MAP tested?

a ☐ Yes

b ☑ No

13 1 Does this protocol include the use of hazardous materials? <updated 060903>

Does this protocol include the use of hazardous materials?

a ☑ Yes

b ☐ No

13 2 [131a] List all hazardous materials used in the course of the protocol. A Material Safety Data Sheets (MS

List all hazardous materials used in the course of the protocol. A Material Safety Data Sheets (MSDS) must be submitted as an attachment for each of the hazardous materials. NOTE: If any documents are not yet available for attachment, cite them as "PENDING RECEIPT". Forward any attachments (electronic or physical) to Kaye Johnson, IACUC Administrative Assistant (kaye.johnson@vai.org, 4th floor, x5702).
Tamoxifin will be used as an injectate in Experiment #3.

13 Does this protocol include the use of recombinant DNA? <updated 060903>

3

Does this protocol include the use of recombinant DNA?

a ☐ Yes

b ☑ No

13 4 Does this protocol reference any Vivarium Standard Operating Procedures (SOPs)? <updated 060903>

Does this protocol reference any Vivarium Standard Operating Procedures (SOPs)?

a ☑ Yes

b ☐ No

13 5 [134a] List all SOPs (title and number) referenced in this protocol and submit as attachments. NOTE: If

List all SOPs (title and number) referenced in this protocol and submit as attachments. NOTE: If any documents are not yet available for attachment, cite them as "PENDING RECEIPT". Forward any attachments (electronic or physical) to Kaye Johnson, IACUC Administrative Assistant (kaye.johnson@vai.org, 4th floor, x5702).

Vivarium SOP #6.002 - Blood Collection Techniques in Mice
Vivarium SOP #6.005 - Isoflurane Anesthesia in Mice

13 6 Does this protocol include animal use at facilities external to VARI? <updated 060903>

Does this protocol include animal use at facilities external to VARI?

a ☐ Yes

b ☑ No

13 7 Are there any other applicable attachments for this protocol? <updated 060903>

Are there any other applicable attachments for this protocol?

a ☑ Yes

b ☐ No

138 [137a] List all other attachments: NOTE: If any documents are not yet available for attachment, cite the

List all other attachments: NOTE: If any documents are not yet available for attachment, cite them as "PENDING RECEIPT". Forward any attachments (electronic or physical) to Kaye Johnson, IACUC Administrative Assistant (kaye.johnson@vai.org, 4th floor, x5702).

139 Signatures

140 All authorizing signatures for this protocol will be gathered at the time of approval and kept on f

All authorizing signatures for this protocol will be gathered at the time of approval and kept on file in the IACUC office.

141 Post-Approval Form Changes