Protocol Detail Report

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Protocol Information

Protocol Number: 06-10-028
User Reference Number: 297
Category:

Title:
Functional Analysis of BHD Using Conditional Knockout Strategy

Protocol Type:
Original

Amendment Reason:
N/A

PI: Teh, Bin
Department: Research
Location: Van Andel
Author: Chen, JinDong
State: APPROVED
Emergency Phone: (616) 234-5536

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Stock Protocol: No

Expiration Date: 10/11/2009
Inactive Date:
Tissue Harvest: No
Long Term: No

Protocol Sources

Protocol Associates
Chen, Jin Dong; Williams, Bart; Antio, Sabrina; Wang, PengFei

Protocol Keywords

Description:

Objective:

Rationale:

Segment Information

Segment Number: 1

Segment description

Species: Mus musculus
Enrichment: No

Animal Termination Information:
Reason:
Disposition:
Termination Days:

Strain/Stock/Breed
C57BL/6
TG or KO from a non-vendor

Euthanasia Method(s)
See Segment Questionnaire

Allow Transfer: Yes

Stress Name	Warning Level	Authorized
Category 1 (click yellow question mark)		3525
Category 2 (click yellow question mark)		0
Category 3 (click yellow question mark)		0

Procedure(s):

Segment Questionnaire

Administrative Data

1 Q: 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.
<updated 120304>

A: Yes

2 Q: PI Telephone Number:
<updated 060903>

A: 616-234-5296

3 Q: PI FAX Number:
<updated 060903>

A: 616-234-5297

4 Q: PI Email:
<updated 060903>

A: bin.teh@vai.org

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

A: Yes

6 Q: 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.
<updated 020904>

A: No

Study Objectives

8 Q: 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. <updated 060903>

A: 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 to better 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 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'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, we will cross BHD-flox strain with Villin-Cre mice, which express Cre in intestine.

Animal Requirements

9 Q: 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.
<updated 021104>

A: 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 will be 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.

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.

10 Q: Age of animals:
<updated 060903>

A: Neonatal through Adult

11 Q: Sex of animals:
<updated 120403>

A: Both males and females

12 Q: Vendor source:
<updated 060903>

A: Jackson Labs
Other - 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 will be 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.

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.

13 Q: Duration of the study:
<updated 060903>

A: 3 years.

14 Q: Total number of animals to be used for Year 1:
NOTE: Enter zero if there are no animals planned for use during Year 1.
<updated 060903>

A: 1175

15 Q: Total number of animals to be used for Year 2:
NOTE: Enter zero if there are no animals planned for use during Year 2.
<updated 060903>

A: 1175

16 Q: Total number of animals to be used for Year 3:
NOTE: Enter zero if there are no animals planned for use during Year 3.
<updated 060903>

A: 1175

17 Q: 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).
<updated 060903>

A: 3525

18 Q: Average daily census:
(Definition: The average number of animals on the shelf on any given day during an experiment.)
<updated 060903>

A: 200

19 Q: Will you house the animals in the Vivarium?
<updated 060903>

A: Yes

Transportation

21 Q: Will animals be transported within the Vivarium from Suites 1, 2 or Transgenic to Quarantine?
<updated 060903>

A: No

24 Q: Will animals be transported within the Vivarium from Suites 1, 2 or Transgenic to Imaging (either ultrasound or confocal imaging)?
<updated 081904>

A: No

27 Q: Will animals be transported from Quarantine to Imaging (either ultrasound or confocal imaging)?
<updated 081904>

A: No

30 Q: Will animals be transported outside the Vivarium facility on the 2nd floor (other than confocal imaging)?
<updated 081904>

A: No

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

A: Yes

34 Q: 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?
<updated 081904>

A: Yes

36 Q: Will animals be shipped to locations outside of VARI?
<updated 060903>

A: No

Rationale For Animal Use

45 Q: 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?
<updated 060903>

A: Yes

47 Q: 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?
<updated 060903>

A: Yes

50 Q: 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?
<updated 060903>

A: Yes

52 Q: 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.
<updated 040104>

A: **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.

Therefore, we need to produce 51 BHD-flox/+ mice and 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 double 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 BHD 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 100 pups, 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 (these mice 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 Breeding Step #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 order to 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**

Description Of Experimental Design

53 Q: 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.)
<updated 060903>

A: This protocol includes 6 experiments, 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 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.

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.

Description Of Animal Procedures

55 Q: 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?
<updated 060903>

A: Yes

57 Q: How will the animals be identified?
<updated 060903>

A: Cage cards
Ear notches

58 Q: 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.
<updated 060903>

A: No

60 Q: Will you be administering experimental injections or inoculations?
<updated 060903>

A: Yes

61 Q: 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.
<updated 060903>

A: No

64 Q: 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.
<updated 060903>

A: 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.

65 Q: 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.
<updated 060903>

A: Intraperitoneal

66 Q: 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.
<updated 021104>

A: Abdomen

67 Q: 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.
<updated 060903>

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

68 Q: 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.
<updated 060903>

A: 100 microliters

69 Q: 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.
<updated 060903>

A: For Experiment #3, we will inject the tamoxifen according to the following schedule. The tamoxifen will be administered when the mice reach 24 weeks of age.

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.

70 Q: 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.
<updated 060903>

A: No

105 Q: Will you collect blood?

<updated 060903>

A: No

119 Q: Will tail biopsies be performed?
<updated 060903>

A: Yes

120 Q: 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?
<updated 060903>

A: Yes

122 Q: 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.
<updated 060204>

A: No

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

A: No

126 Q: Will the mice be exposed to any other potential stressors (food or water deprivation, noxious stimuli, environmental stress, etc.)?
<updated 060903>

A: No

128 Q: Does this protocol include the generation or use of any transgenic or knockout animals?
<updated 120304>

A: Yes

129 Q: For transgenic and knockout animals, are you expecting any phenotypic consequences?
<updated 120403>

A: Yes

130 Q: For transgenic and knockout animals, describe any expected phenotypic consequences and any special care or monitoring that these phenotypes may require.
<updated 120304>

A: 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.

131 Q: 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?
<updated 021104>

A: Yes

133 Q: 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".
<updated 040104>

A: None

134 Q: 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.
<updated 021104>

A: This protocol includes 6 experiments, all of which explore the BHD gene conditional 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.

Surgery

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

A: No

Pain Or Distress

149 Q: 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).
<updated 021104>

A: No

Anesthesia, Analgesia, Tranquilization

151 Q: 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.

<updated 021104>

A: No

Method Of Euthanasia

173 Q: 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?
<updated 040104>

A: Yes

174 Q: Do you have any other methods of euthanasia?

<updated 021104>

A: No

Hazardous Agents

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

A: No

190 Q: 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.
<updated 060204>

A: No

194 Q: 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.
<updated 060204>

A: Yes

194 Q: 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.
<updated 060204>

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

196 Q: Does this protocol include the use of radioisotopes?
<updated 060204>

A: No

198 Q: Does this protocol include the use of machine-generated radiation (for example, radiation produced by a bone densitomer, an x-ray machine, etc.)?
<updated 060204>

A: No

200 Q: Will this protocol generate any contaminated animals or materials, such as bedding, that will need special handling for disposal?
<updated 060903>

A: No

202 Q: 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.

<updated 060204>

A: ABSL-1: Level One

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

A: No

Biological Materials

205 Q: Does this protocol include any biological materials or animal products for use in animals?
<updated 060903>

A: No

Special Considerations

214 Q: 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.)?
<updated 060903>

A: No

Certifications

216 Q: I certify that I have attended the institutionally required investigator training course.
<updated 060903>

A: Yes

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

<updated 060903>

A: October 2004

218 Q: Enter the location (institution, city & country) that you completed the institutionally required investigator training course.
<updated 060903>

A: VARI

220 Q: I certify that I have determined that the research proposed herein is not unnecessarily duplicative of previously reported research.
<updated 060903>

A: Yes

222 Q: I certify that all individuals working on this proposal who are at risk are participating in the Institution's Occupational Health and Safety Program.
<updated 060903>

A: Yes

224 Q: 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
<updated 060903>

A: Yes

226 Q: 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.

<updated 021104>

A: No

230 Q: I certify that I will obtain approval from the IACUC before initiating any significant changes in this study.
<updated 060903>

A: Yes

232 Q: 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.
<updated 060903>

A: Yes

234 Q: I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
<updated 060903>

A: Yes

Attachments

236 Q: Does this protocol include any references to supporting materials (articles, abstracts, etc.)?
<updated 060903>

A: Yes

237 Q: 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).
<updated 060903>

A: 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.

238 Q: Does this protocol include the use of biological materials that must be MAP tested?
<updated 060903>

A: No

240 Q: Does this protocol include the use of hazardous materials?
<updated 060903>

A: Yes

241 Q: 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).
<updated 060903>

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

242 Q: Does this protocol include the use of recombinant DNA?
<updated 060903>

A: No

244 Q: Does this protocol reference any Vivarium Standard Operating Procedures (SOPs)?
<updated 060903>

A: No

246 Q: Does this protocol include animal use at facilities external to VARI?
<updated 060903>

A: No

248 Q: Are there any other applicable attachments for this protocol?
<updated 060903>

A: Yes

249 Q: 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).
<updated 060903>

A: Indication of Roles

Signatures

250 All authorizing signatures for this protocol will be gathered at the time of approval and kept on file in the IACUC office.
<updated 060903>

Bin Teh, M.D., Ph.D.

Protocol Author Name

Protocol Author Signature

October 6, 2006

Signature Date

Post-Approval Form Changes

251 Q: THIS QUESTION IS FOR USE BY THE IACUC ADMINISTRATIVE ASSISTANT ONLY!
Please indicate any procedural changes to the questionnaire since the protocol has been approved. Provide full details.
<updated 031103>

Protocol E-Signatures

E-Signature Event: Submit Protocol Request
Description:

Signed Date/Time: 9/1/2006 14:19:49
Signed By: Johnson, Kaye
Submittal Date/Time: 9/1/2006 14:19:41

Signed Date/Time: 10/10/2006 18:21:40
Signed By: Johnson, Kaye
Submittal Date/Time: 10/10/2006 18:21:32