SingHealth

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

Research Project Proposal Application

Instructions/Checklist for Principal Investigator

- Complete all sections in (a) the application form and (b) Declaration of Ethics/Biosafety Considerations form by typing in clearly and attaching additional information and relevant documents where necessary. Indicate 'NA' where not applicable.
- **Consult SEMC/NCC vets before submission for IACUC review to ensure the protocol is professionally put up without technical hitches to delay IACUC approval.**
- **Only one PI is allowed per application and the PI and team collaborators must have attended the 'Responsible Care & Use of Laboratory Animals' course.** Project team members including research assistants who are expected to handle animals must be included in the list of team collaborators.

Documents for submission:

- ❑ 1 hardcopy application form (print 2-sided) bearing original PI's signature, name-stamp and date.
- ❑ 2 sets photocopies of the above endorsed application (photocopy 2-sided).
- ❑ 1 set photocopies of the project team's 'Responsible Care & Use of Laboratory Animals' course certificates.
- ❑ Softcopies of the application and the project team's short CVs with recent publication references to be emailed to SingHealth IACUC Secretariat.
- ❑ 1 hardcopy and 1 softcopy of the Vet's Check List.

SingHealth IACUC Secretariat
@ SingHealth Office of Research
7 Hospital Drive #04-02 Block A
SingHealth Research Facilities
(former School of Nursing)
Singapore 169611
Email: iacuc@singhealth.com.sg
SingHealth IACUC website sited on SingHealth Research Intranet & Internet

Guidance Document on Oversight of Research by SingHealth Institutional Research Committees

Responsible Conduct of Research

All research projects undertaken by any SingHealth Principal Investigator and/or performed within any SingHealth Institution shall obtain the necessary approvals from each or all the following research committees, depending on the nature of the research projects.

SingHealth Centralised Institutional Review Board (CIRB) for the Protection of Human Subjects in Research reviews all research involving human subjects in order to protect the rights and welfare of human research subjects that are recruited to participate in research activities. CIRB complies with the MOH regulations governing human subjects' research according to the statement of principles contained in the Singapore Guidelines for Good Clinical Practice, the various Reports of the Bioethics Advisory Committee, Singapore and the applicable regulations in force.

SingHealth Institutional Animal Care and Use Committee (IACUC) overseeing activities involving any live, vertebrate animal used or intended for use in research, research training, teaching, experimentation or biological testing or for related purposes. All such research projects shall obtain the consent of the SingHealth IACUC before commencement.

SingHealth Institutional Biosafety Committee (IBC) is constituted to provide an overarching scheme and to develop a framework for oversight of biosafety in research by guiding SingHealth Member Institutions to achieve a common protocol review process for risk assessments of research protocols involving the use of biohazardous materials (e.g. infectious microorganisms, laboratory animals, biological agents or toxins, cells, tissues and fluids, recombinant DNA, and GMOs) which may represent hazards to individuals and/or the environment.

SingHealth Member Institutions that handle biological agents and toxins covered by the Biological Agents and Toxins Act 2005 (BATA), would form its own BATA Sixth Schedule Biosafety Committee to meet the statutory requirements and the Singapore Biosafety Guidelines for Research on Genetically Modified Organisms.

For detailed information, please refer to SingHealth Research Intranet & Internet.

Declaration of Ethics/Biosafety Considerations

Please note that approval of this IACUC application is subject to approval by the Institutional Review Board (IRB) and/or Institutional Biosafety Committee (IBC) if your project involves human tissues or using materials requiring biosafety approval.

For more information on IBC and application form, see the following web link: http://mysinghealth/singhealth/corporateoffice/ibc

For more information on IRB and application form, see the following web links:
http://mysinghealth/singhealth/corporateoffice/research/support/irb.htm
http://research.singhealth.com.sg/Ethics/CIRB/Pages/Default.aspx

Please type either **"Y"** (Yes) or **"N"** (No), where appropriate, if your study involves the following

Description	**Y or N**
Human Subject	N
Use of Human Tissues or Cells	N
Requirement for Containment Class 2 and Above	N
Use of Material Requiring Biosafety Approval http://mysinghealth/Singhealth/CorporateOffice/IBC/Training/	N

Application requires approval from:

Ethics	**Y or N**
IRB	N
IBC	N

If any of the above is applicable to your IACUC application, please complete the respective application forms for approval from the respective committees. Queries on IBC or IRB matters, pls contact the following:

For IBC matters, contact Ms Cindy GOH at cindy.goh.j.e@sgh.com.sg
For IRB matters, contact CIRB at irb@singhealth.com.sg

If your IRB and/or IBC applications have been approved, please indicate the protocol /approval number and date of approval below.

SingHealth

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

Research Project Proposal Application

For office use only

Proposal #: _____
Application Date: _____
Approval Date: _____
Expiration Date: _____

A. Administrative Data:

Project Title: Functional analysis of *PBRM1* using conditional knockout strategy

Proposed Project Commencement Date: April 2011

Expected Duration of the Project: 3 years

Project Team:

(a) Principal Investigator: Teh Bin Tean

RCULA Certificate Number/Date: 14th December 2006

Designation: Director, NCCS-VARI Translational Cancer Research Laboratory

Qualifications: MD., Ph.D.

Dept/Organisation: Division of Medical Sciences, National Cancer Centre (S)

Tel No: +65 6436 8309

Fax No: +65 6372 0161

Email Address: bin.teh@vai.org/Teh.B.T@nccs.com.sg

(b) Team Collaborators:

Name	**Designation/Dept/Organisation**	**Qualifications**	**RCULA Certificate Number/Date**
Jindong Chen	Senior Scientist	Ph.D.	27 January 2011
Huang Dachuan	Research Fellow/DMS/NCC	Ph.D.	015/05 2 March 20052007

Congrong Liu	Research Officer/DMS/NCC	B.Sc	27 January 2011
Bernice Wong	Research Officer/DMS/NCC	B.Sc.	13 September
Siew Ee Yan	Research Officer/DMS/NCC	B.Sc.	13 July 2006

It is recommended that someone from the team collaborator (table above) be identified to assist the PI on queries that may arise from his/her IACUC submission.

Name:	Jindong Chen/**Dachuan Huang**
Contact Number:	65161785 / **63266196**
Email address:	jindong_chen@hotmail.com; **huangdachuan@gmail.com**

B. Objectives of the Study:

1. To determine the functional role of the *PBRM1* gene in kidney cancer

2. To confirm that the *PBRM1* gene is required for the development of kidney cancer and that inactivation of the gene will induce related tumorigenesis

3. To determine whether double knockout with *Vhl* gene (*Pbrm1*/*Vhl*) and *Bhd* (*Pbrm1*/*Bhd*) in mice could promote tumorigenesis if *Pbrm1* knockout alone does not induce tumorigenesis (*VHL* knockout alone did not generate kidney tumors)

C. Abstract of the Study:
(Use additional sheets if necessary.)

Recently, we have identified a new kidney cancer-related gene *PBRM1* through exome sequencing in a series of primary clear cell renal carcinoma (ccRCC)[1]. We found that 41% (92/227) examined cases carried *PBRM1* gene mutations, indicating *PBRM1* is the second most important kidney cancer-related gene following *VHL*. The previous research[2] and our *in vitro* data suggested that *PBRM1* play a role of suppressing tumor growth in ccRCC, implying that *PBRM1* is a candidate of tumor suppressor gene (TSG). To determine its role of tumor suppressor, we decide to knockout the mouse *Pbrm1* gene in mouse kidney and expect the affected mice will develop kidney tumors. This study will increase our knowledge of the *PBRM1* gene and its effects on tumor formation in kidney. Further investigations will provide insight into the discovery of drug targets that will hopefully lead to better treatment and care for both patients with *PBRM1* mutations and other forms of cancer involving the same cancer-causing pathways.

Since tumorigenesis requires more than one genetic change to progress, knockout of one TSG may not guarantee the formation of tumors. Knockout of two or three TSG may be necessary. The von Hippel-Lindau (*VHL)* gene is another important kidney cancer-related gene associated with VHL syndrome. *VHL* mutations have been identified in approximately 70% of kidney cancers[3]. Interestingly, mutations in both *VHL* and *PBRM1* genes lead to ccRCC subtypes. 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 *Pbrm1* and *Vhl* genes. In addition, another kidney cancer-related gene *BHD* (identified from Birt-Hog-Dube cancer syndrome) plays a critical role in kidney tumorigenesis. Unlike *VHL* and *PBRM1*, *BHD* mutations cause different subtypes of renal carcinomas (clear cell RCC, papillary RCC, chromophobe RCC, etc.), implying that *BHD* could be an upstream gene of *VHL* and *PBRM1*. Since we have already created the *Bhd* knockout mice[4], which develop low rate of ccRCC, we intend to create a *Pbrm1-Bhd* double knockout mice to check whether generated double knockout mice could increase the incidence of ccRCC, Since mutations in these genes have been identified in kidney cancers, we know that these genes are associated with kidney cancers. Using the kidney-specific combination knockout strategy, we expect that these mice can produce kidney cancer since the disruption of these genes is only restricted to the kidneys. 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.

First, we will create a *Pbrm1* conditional knockout mouse strain, also called a *Pbrm1*-flox strain. This conditional knockout strain should not manifest any phenotype, since the knockout is not completed until the animal is exposed to Cre. Cre is the abbreviation of Cre recombinase, which is used as a tool to modify genes and chromosomes. Cre deletes a segment of DNA (Cre sequence) in an experimental animal and has been used to generate animals with mutations limited to certain cell types. Flox refer to a gene that has been marked by flanking two special Cre sequences. The gene marked with Cre sequences still function normally until it is exposed to Cre recombinase. The floxed gene will be disrupted once it is exposed to Cre recombinase that is supposed to cut the gene following the two special Cre sequences flanking it.

Once we have established the conditional knockout, we will breed this knockout to kidney-specific Cre strains to create kidney-specific *Pbrm1*-deficient knockout mice, to confirm the corresponding tumor formation.

However, there is a high possibility that *Pbrm1*-deficient knockout mice do not develop any tumors like *Vhl*-knockout mice or generate tumors at very low rate. In this case, we will generate *PBRM1-Vhl* and *Pbrm1-Bhd* double deficient knockout mouse model to test that the hypothesis that multiple genetic alterations are required to facilitate tumorigenesis.

The following proposed studies would provide important new information concerning the functional biology of the *Pbrm1* 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.

D. Animal Requirements:

Species: Mice

Strains or Stocks: (1) Sglt2-Cre transgenic mouse
(2) *Vhl*-flox/flox transgenic mice
(3) Kidney-specific *Vhl* knockout mice (*Vhl*-flox/flox; Sglt2-Cre)
(4) *Pbrm1*-flox/flox
(5) *Pbrm1*; Sglt2-Cre knockout mice
(6) *Bhd*-flox/flox transgenetic mice
(7) Kidney-specific *Bhd* knockout mice (*Bhd*-flox/flox; Sglt2-Cre)
(8) Kidney-specific *Pbrm1*/*Vhl* double knockout mice
(9) Kidney-specific *Pbrm1*/*Bhd* double knockout mice

(10) C57B/6

Age: 0-2 years

Weight or Size: 0-30g

Sex: Male and Female

Source(s): (1) and (4) are generated by Cancer Science Institute (CSI) Singapore
(2) and (3) are obtained from Jackson Laboratory, USA
(5) and (6) are generated by this IACUC protocol
(7) is obtained from Animal Resource Centre, Australia

Holding Location: Duke-NUS Vivarium

Animal Procedure Location(s): Duke-NUS Vivarium

Proposed Number of Animals Used:

Year 1	Year 2	Year 3	Total
602	826	446	1874

E. Rationale for Animal Use:
(Use additional sheets if necessary.)

1. Explain your rationale for animal use, rather than performing *in vitro* study, computer modelling, tissue culture, etc. Justify the 3Rs – *Replacement* of animals with other methods; *Reduction* in the number of animals used; *Refinement* of project and the techniques used to minimise impact on animals.

Limitations of Mathematical and/or Computer Models
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, thus necessitating the use of animal models in biomedical research. Reference: The Johns Hopkins Center for Alternatives to Animal Testing (CAAT)

Limitations of Culture systems
The culture of cells, tissues, and organs of animal and human origin in an environment outside the body, collectively known as in vitro systems, has reached a high level of sophistication and allows scientists to study the effects of substances on cellular events in isolation from other biological phenomena. Such methods often provide reliable data that may be difficult or impossible to obtain in whole animals. The fact that the above tests are conducted in isolated systems, independent of other complex biological systems, creates limitations in their interpretation. In the end, the validity of such tests must be verified by testing in appropriate mammalian model systems and possibly in later human clinical trials. Reference: The Johns Hopkins Center for Alternatives to Animal Testing (CAAT)

2. Justify the appropriateness of the species selected as the animal model.

Microorganisms (e.g., yeasts, bacteria), invertebrates, and lower vertebrates are used to provide simple, manageable systems to gain insight into fundamental processes that are relevant to understanding the nature of human diseases and disorders. In particular, the wide array of marine and freshwater invertebrates represent a great potential for biomedical research. These lower organisms are excellent models for the study of certain basic life processes because they permit manipulation and reduce complexity that can obscure understanding of a basic biological process. Although the fundamental knowledge obtained using these diverse species is generally applicable to humans, interspecies transfer of information must be approached with caution and requires validation in higher animals

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 and care. In addition, this widespread use has led to their 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 in 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 a suitable small animal model for biomedical research.

3. Justification of the number of animals to be used.

   (a) Will any statistical analysis (e.g. χ^2 test, Fisher's Exact test, Student's t-test and ANOVA) be used?

   - Yes (proceed to b)

   1. No (proceed to c)

   (b) Indicate the statistical method(s) and provide the following parameters in order to justify the number of animals to be used.

   i. The effect size of biological interest (δ): The effect size stands for how large a biological effect would be of scientific interest.

   ii. The standard deviation (σ):

   iii. The significant level (α):

   iv. The desired power of the experiment $(1-\beta)$:

   v. The alternative hypothesis (i.e. a one- or two-sided test):

   (c) Explain the rationale and basis for choosing the number of animals to be used.

Mice obtained from various sources will be used for i) testing for germline transmission, ii)generating *Pbrm1*-floxed mice, iii) generating *Pbrm1* knockout mice, iv) producing double *Pbrm1*/*Vhl* double floxed mice, v) developing *Pbrm1*/*Vhl* double knockout mice. The detailed experimental design is discussed in section (G). All the estimations listed below are calculated based on the average litter size is 8 mice.

IMPORTED MICE (year 1):

Mice will be imported from United States:

(1) *Sglt2*-Cre transgenic mouse
(2) *Vhl*-flox/flox transgenic mice
(3) *Bhd*-flox/flox transgenetic mice
(4) Kidney-specific *Vhl* knockout mice (*Vhl*-flox/flox; Sglt2-Cre)
(5) Kidney-specific *Bhd* knockout mice (*Bhd*-flox/flox; Sglt2-Cre)

We intend to import 10 mice (5 mice each sex) for each strain. In total, 50 transgenetic mice will be transferred to our mouse room in Duke-NUS vivarium.

Total mice imported: 50 (5x10) (year 1)

EXPERIMENT #1 (year 1)

Number of mice is needed in this experiment: For generation of chimeric mice and test of germline transmission, **12** chimeric founder mice are expected to generate for us in the Center of Life Science (CeLS) in the National University of Singapore (NUS). Each chimeric founder mouse will be mated with 1 wild-type C57/B6 mice to test for germline transmission. We intend to test each chimeric founder mice for 3-5 litters (on average 8 mice/litter, 4 litters/chimera). Therefore, number of mice will be generated ≅ 12x4x8 ≅ 400. Chimeras showing germline transmission will be crossed with WT 129Ola mice to generate *Pbrm1*-flox mice in pure 129Ola strain. We plan to use 2 chimeras which give germline transmission to generate *Pbrm1*-flox mice. Assuming we can obtain germline transmission within 3 litters, we will obtain about 50 pups (no. of pups born ≅ 50). Therefore, we estimate approximately **500** mice will be generated. We will only keep the germline-transmitted heterozygous mice for further breeding in **EXPERIMENT #2** and mice of unwanted genotype will be sacrificed after genotype is determined. This part of the project will take about 5 months to finish.

Total mice used and produced in EXPERIMENT #1: 512 (year 1)

EXPERIMENT #2 (year 1)

Number of mice is needed in this experiment to generation of homozygous *Pbrm1*-flox mice:

Two male and two female heterozygous *Pbrm1*-flox mice from EXPERIMENT #2 are needed to set up two mating cages. We need four litters. In each litter, about one quarter of the offspring will be *Pbrm1*-folx/flox mice. We estimate

that approximate 32 pups will be produced and eight of them will be homozygous *Pbrm1*-flox mice.

Total mice produced in EXPERIMENT #2 : 40 (year 1)

EXPERIMENT #3 (year 2)

Number of mice needed in this experiment to generate kidney proximal tubule-specific *Pbrm1* knockout mice (*Pbrm1*-flox/+; Sglt2-Cre-tg/+). Below are the calculations:

Mice are needed at Step #3A: to create kidney proximal tubule-specific *Pbrm1* heterozygous knockout mice (*Pbrm1*-flox/+; Sglt2-Cre-tg/+) in this step, two homozygous *Pbrm1*-Flox mice (one each sex) generated in experiment #2 will be crossed to two Sglt2-Cre (tg/+) transgenic mice (one each sex) to produce knockout *Pbrm1* heterozygotes. The mice will breed at least twice. Of their offspring, 50% will be heterozygotes. Given an approximate litter of 10 pups, that should produce around 40 offspring and approximate 20 of them should be heterozygous knockout mice. Assuming the error rate is 10%, 44 mice are needed in this step to meet our requirement.

Total mice generated in Step #3A: 4x10+10%(4x10)=**44**

Mice are needed at Step #3B: to produce kidney proximal tubule-specific *Pbrm1* homozygous knockout mice (*Pbrm1*-flox/flox; Sglt2-Cre-tg/+) two female and two male heterozygous *Pbrm1*-flox mice generated in experiment #3A will be inbred to produce homozygous knockout *Pbrm1* mice. The mice will breed three times. Of their offspring, approximately 20% will be homozygotes. Given an approximate litter of 10 pups, that should produce 60 offspring and approximate 12 of them should be homozygous knockout mice. Assuming the error rate is 10%, 66 mice will be produced in this step.

Total mice generated at Step #3A: 6x10+10% (6x10)=**66**

Mice are needed at Step #3C: to quickly obtain desired number of kidney proximal tubule-specific *Pbrm1* homozygous knockout mice (*Pbrm1*-flox/flox; Sglt2-Cre-tg/+), two female and two male heterozygous *Pbrm1*-Flox mice generated in experiment #3B will be inbred. The number of mice will meet the following phenotype analysis at eight intervals (for each interval, we will assess 3 genotypes:
1) *Pbrm1*-floxhomozygous; Cre heterozygous, 2) double heterozygous, and 3) *Pbrm1* 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 need mice for (again, we will use three genotypes: 1) *Pbrm1* homozygous; Cre heterozygous, 2) double heterozygous, and 3) *Pbrm1* wild-type; Cre heterozygous):

9) histological and pathological exams
10) establishment of fibroblast cell lines

For the first eight groups of these 10 groups, we will use 20 homozygous knockout mice per genotype group to assess the phenotype. For the last two groups (groups 9 and 10), 10 mice each are needed. In total, we need 180 homozygous knockout mice (8x20 + 2x10). In this experiment, three female and three male homozygous *Pbrm1*-Flox knockout mice (*Pbrm1*-flox/flox; Sglt2-Cre-tg+) generated in experiment #3B will be inbred to produce homozygous knockout *Pbrm1* mice. Of their offspring, approximately 75% will be homozygotes. Thus, we need generate 240 pups to give rise to 180 homozygous knockout mice. Considering 10% error rate, we need to produce approximate 264 pups to meet our requirement. Given an approximate litter of 10 pups, the three pairs of mating mice need to breed 9 times.

Total mice will be produced in Step #3C: **270** (9x10x3)

Totals for Experiment #3 (year 2):

Breeding Step 3A:	44
Breeding Step 3B:	66
Breeding Step 3C:	270
Total:	**380 (year 2)**

EXPERIMENT #4 (year 2)

Mice are needed at Step #4A: to generate *Pbrm1-Vhl* double heterozygous mice (*Pbrm1*-flox/+; *Vhl*-flox/+; Cre-tg/+), two females (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Vhl*-flox/flox; Cre-tg/+) and two males (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Vhl*-flox/flox; Cre-tg/+) will be used to set up two mating cages. Each mating pair will be inbred at least twice. Given an approximate litter of 10 pups, that should produce 40 mice.

Approximately 75% of the total 40 mice will be *Pbrm1-Vhl* double heterozygous mice (*Pbrm1*-flox/+; *Vhl*-flox/+; Cre-tg/+). Thus, roughly 30 mice will be *Pbrm1-Vhl* double heterozygous. Considering 10% error rate, 44 mice should be produced in this step.

In total, mice needed at **Step 4A**: **44**

Mice are needed at Step #4B: to produce *Pbrm1-Vhl* double knockout mice, *Pbrm1-Vhl* double heterozygous (*Pbrm1*-flox/+; *Vhl*-flox/+; Cre-tg/+) mice will be inbred (3 mating cages). We will expect to obtain approximate six *Pbrm1-Vhl* double knockout mice at this step. From this cross, around 5% of the offspring will be *Pbrm1-Vhl* double knockout mice. Given an approximate litter of 10 pups, we need 120 pups (6/5%) (12 litters) to give rise to 6 *Pbrm1-Vhl* double knockout mice.

Considering 10% error rate, 132 pups are needed at this step #4B.

In total, mice needed in **Step 4B**: **132** (120+120/10%)

Mice are needed at Step #4C: to produce enough number of *Pbrm1-Vhl* double knockout mice (*Pbrm1*-flox/flox; *Vhl*-flox/flox; Cre-tg/+) for phenotype analysis, *Pbrm1-Vhl* double knockout mice from **Step #4B** will be further inbred. The phenotype will be also 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

As calculated as **at Step #3C,** total mice will be produced in Step #4C: **270** (9x10x3)

Totals for Experiment #4 (year 2):	
Breeding Step 4A:	44
Breeding Step 4B:	132
Breeding Step 4C:	270
Total:	**446 (year 2)**

EXPERIMENT #5

Mice are needed at Step #5A: to generate *Pbrm1-Bhd* double heterozygous mice (*Pbrm1*-flox/+; *Bhd*-flox/+; Cre-tg/+), two females (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Bhd*-flox/flox; Cre-tg/+) and two males (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Bhd*-flox/flox; Cre-tg/+) will be used to set up two mating cages. Similar to Step #4A, 44 mice will be used at this step.

In total, mice needed in **Step 5A**: **44**

Mice are needed at Step #5B: to produce *Pbrm1-Bhd* double knockout mice, *Pbrm1-Bhd* double heterozygous (*Pbrm1*-flox/+; *Bhd*-flox/+; Cre-tg/+) mice will be inbred (3 mating cages). Similar to Step #4B, 132 mice will be used in this step.

In total, mice needed in **Step 5B**: **132** (120+120/10%)

Mice are needed at Step #5C: to produce enough number of *Pbrm1-Bhd* double knockout mice (*Pbrm1*-flox/flox; *Bhd*-flox/flox; Cre-tg/+) for phenotype analysis, *Pbrm1-Bhd* double knockout mice from **Step #5B** will be further inbred. The phenotype will be also assessed at eight intervals, as **Step #3C (EXPERIMENT #3)**, the same number of mice (270) will be used at this step.

Total mice will be produced in Step #5C: **270** (9x10x3)

Totals for Experiment #5 (year 3):

Breeding Step 5A:	44
Breeding Step 5B:	132
Breeding Step 5C:	270
Total:	**446 (year 3)**

TOTALS FOR PROTOCOL:

Mice imported	**50**
Experiment #1	512
Experiment #2	40
Experiment #3	380
Experiment #4	446
Experiment #5	446
GRAND TOTAL	**1874**

Estimated no. of mice required / year:

1st year

- Imported mice: 50
- EXPERIMENT #1: 512 mice
- EXPERIMENT #2: 40 mice
- Total no. of mice needed for this year: 602

2nd year

- EXPERIMENT 3: 380
- EXPERIMENT #4: 446
- Total no. of mice needed for this year: 826

3rd year

- EXPERIMENT #5 : 446
- Total no. of mice needed for this year: 446

4. Are you using pregnant animals? If yes, please discuss how you will take care of the offspring.

Yes. We will expand the mouse colonies by breeding the mice with desired genotypes. Tail biopsies (approximately 3-5mm long, ref: http://www.jax.org/imr/tail_phenol.html) will be taken to extract DNA for genotyping and identification codes will be punched on their ears at 14 days of age. Pups at 21 days of age will be weaned, males and females will be separated. They will be kept for long term experiments with free access to water and food until 1-2 years old in the SPF animal unit.

F. Transport of Animals:

Briefly discuss transport of animals in between facilities if required.

When required, these mice are transported in between rooms in microisolators, on a clean trolley that has been swapped with 70% ethanol. This is carried out by two individuals, and extra care is taken when entering and exiting lifts to minimise any possible impact on the mice.

Describe the method of containment to be utilised.

These mice would be contained in microisolators, which has a hepa filter installed to minimize the risk of contamination. As long as the microisolators remain closed at all times during the transport of animals between facilities, the air in the microisolator is still relatively clean.

G. Description of Experimental Design & Animal Procedures:

Explain the experimental design and specify all animal procedures. Your 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. *A complete copy of the research protocol is required and a study flowchart must be included for clarity and to facilitate IACUC evaluation. (Use additional sheets or separate attachment if necessary.)*

Our recent study indicated that approximate 40% of the renal cell carcinomas are attributed to mutations in *PBRM1* gene. In order to determine its physiological function and better understand its role in tumorigenesis, we have decided to knock out the mouse *Pbrm1* gene. Since *Pbrm1* knockout vector has been constructed through EUCOMM project by The International Knockout Mouse Consortium (IKMC) and corresponding knockout ES clones are available. We purchased one of the ES clones to inject it into blastocysts. The treated blastocysts will be tranfered into foster female mouse to produce chimeric offspring. The chimeric founder mice were generated in our collaborator's laboratory in International Agency of Research on Cancer, WHO. These mice will be used for i) testing for germline transmittion (EXPERIMENT #1), ii) tumour incidence and tumour spectrum studies (EXPERIMENT #2 and #3). Since many genes do not lead to tumorigenesis after knockout alone (e.g. *Vhl*), it is possible that knockout of *Pbrm1* alone will not cause kidney tumors, Since increasing evidences have shown that multi-genetic changes are required to facilitate tumorigenesis, we designed to develop double kidney cancer-related gene knockout mouse models, The knock-out mice will be bred into other knock-out mice as indicated in the flowchart to generate experimental mice with desired genotype. Pups of 21 days old will be weaned, males and females will be separated, tail biopsies will be taken for genotyping purpose. Mice will be housed in a cage of maximum 5 animals with free access to food and water until euthanasia for experiments.

EXPERIMENT #1:

We have purchased a *Pbrm1*-floxed ES clone from the International Knockout Mouse Consortium. Injection of the targeted ES cells into blastocysts and subsequent implantation of blastocysts into uteruses of pseudopregnant foster mice will be carried out by Center of Life Science (CeLS) facility in National University of Singapore (NUS). Once we obtain the chimeric mice from CeLS, we will perform test of

germline transmission. First, the chimeric founder mice obtained from CeLS will be bred with wild-type C57/B6 mice to give rise to 3-5 litters of pups. DNA will be extracted from collected tail biopsy for genotyping. Successful germline transmission will be determined by the brown coat colour of the pups and standard PCR genotyping at 2 weeks old. Only the heterozygous *Pbrm1* knock-out mice will be kept and used for further breeding.

EXPERIMENT #2:

In this experiment, we intend to set up *Pbrm1*-flox/flox strain by inbreeding the above heterozygous *Pbrm1* knockout mice from EXPERIMENT #1. The establishment of the *Pbrm1*-flox/flox strain is critical to the future experiments and will act as a founder strain. We will set up two the mating cages when the heterozygous *Pbrm1* knockout mice are two months old. The mating mice will breed twice and are expected to produce 40 pups (4 litters, 10 pups/litter). Surviving pups will be weaned at 3 week of age. Tail biopsy will be collected for genotyping. Non-*Pbrm1*-flox homozygous pups will be euthanized by CO_2 inhalation and followed by cervical dislocation after genotype has been confirmed, as experiment ends at this point after determining the genotypes.

EXPERIMENT #3:

In this experiment, we will cross the *Pbrm1*-flox strain generated in EXPERIMENT #2 to Sglt2-Cre (tg/+) transgenic mice to produce kidney proximal tubule-specific *Pbrm1* knockout mice.

Step #3A: Generation of kidney proximal tubule-specific *Pbrm1* heterozygous knockout mice (*Pbrm1*-flox/+; Sglt2-Cre-tg/+). Two homozygous *Pbrm1*-Flox mice (one each sex) generated in experiment #2 will be crossed to two Sglt2-Cre-tg/+ transgenic mice (one each sex) to produce knockout *Pbrm1* heterozygotes. The mice will breed at least twice. Of their offspring, 50% will be heterozygotes. Given an approximate litter of 10 pups, that should produce around 40 offspring and approximate 20 of them should be heterozygous knockout mice. Four of the heterozygotes will be used in Step #3B to produce homozygous *Pbrm1* mice. The other heterozygotes will be used as further phenotype analysis and controls to homozygotes.

Step #3B: Generation of kidney proximal tubule-specific *Pbrm1* homozygous knockout mice (*Pbrm1*-flox/flox; Sglt2-Cre-tg/+). Two female and two male heterozygous *Pbrm1*-Flox mice generated in experiment #3A will be inbred to produce homozygous knockout *Pbrm1* mice. The mice will breed three times. Of their offspring, approximately 20% will be homozygotes. Given an approximate litter of 10 pups, that should produce 60 offspring and approximate 12 of them should be homozygous knockout mice. Assuming the error rate is 10%, 66 mice will be produced in this step. About 4 of the 12 homozygotes will be used in Step #3C to produce more homozygotes. The other homozygotes and 20 heterozygotes produced in this step will be kept for further phenotype analysis and controls to homozygotes. The remaining heterozygotes will be sacrificed.

Step #3C: Generation of desired kidney proximal tubule-specific *Pbrm1* homozygous knockout mice (*Pbrm1*-flox/flox; Sglt2-Cre-tg/+). Two female and two male heterozygous *Pbrm1*-Flox mice generated in experiment #3B will be inbred to produce desired number of homozygous knockout *Pbrm1* mice. The phenotype will be assessed at eight intervals (for each interval, we will assess 3 genotypes:
1) *Pbrm1*-floxhomozygous; Cre heterozygous, 2) double heterozygous, and 3) *Pbrm1* 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) *Pbrm1* homozygous; Cre heterozygous, 2) double heterozygous, and 3) *Pbrm1* wild-type; Cre heterozygous):

9) histological and pathological exams
10) establishment of fibroblast cell lines

For the first eight groups of these 10 groups, we will use 20 homozygous knockout mice per genotype group to assess the phenotype. For the last two groups (groups 9 and 10), 10 mice each are needed. In total, we need 180 homozygous knockout mice (8x20 + 2x10). In this experiment, three female and three male homozygous *Pbrm1*-Flox knockout mice (*Pbrm1*-flox/flox; Sglt2-Cre-tg+) generated in experiment #3B will be inbred to produce homozygous knockout *Pbrm1* mice. Of their offspring, approximately 75% will be homozygotes. Thus, we need generate 240 pups to give rise to 180 homozygous knockout mice, Considering 10% error rate, we need to produce approximate 264 pups to meet our requirement. Given an approximate litter of 10 pups, the three pairs of mating mice need to breed 9 times. The other *Pbrm1*-floxed concurrently generated in this step will be used as normal controls.

EXPERIMENT #4

Step #4A: this step involves crossing the *Pbrm1*-flox/flox; Cre-tg/+ strain to *Vhl*-flox/flox; Cre-tg/+ strain to generate *Pbrm1*-*Vhl* double heterozygous mice (*Pbrm1*-flox/+; *Vhl*-flox/+; Cre-tg/+). Two females (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Vhl*-flox/flox; Cre-tg/+) and two males (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Vhl*-flox/flox; Cre-tg/+) will be used to set up two mating cages. Each mating pair will be inbred at least twice. Given an approximate litter of 10 pups, that should produce 40 mice.

Approximately 75% of the total 40 mice will be *Pbrm1*-*Vhl* double heterozygous mice (*Pbrm1*-flox/+; *Vhl*-flox/+; Cre-tg/+). Thus, roughly 30 mice will be *Pbrm1*-*Vhl* double heterozygous. Considering 10% error rate, 44 mice should be produced in this

step.

Step #4B: *Pbrm1-Vhl* double heterozygous (*Pbrm1*-flox/+; *Vhl*-flox/+; Cre-tg/+) mice will inbreed to produce *Pbrm1-Vhl* double knockout mice. We need approximate 6 *Pbrm1-Vhl* double knockout mice at this step. From this cross, around 5% of the offspring will be *Pbrm1-Vhl* double knockout mice. Given an approximate litter of 10 pups, we need 120 pups (6/5%) (12 litters) to give rise to 6 *Pbrm1-Vhl* double knockout mice.

Considering 10% error rate, 132 pups are needed at this step #4B.

Step #4C: *Pbrm1-Vhl* double knockout mice (*Pbrm1*-flox/flox; *Vhl*-flox/flox; Cre-tg/+) will be further inbreed to produce enough number of mice for phenotype analysis. Concurrently generated *Pbrm1-Vhl* double floxed mice (*Pbrm1*-flox/flox; *Vhl*-flox/flox; Cre +/+) will be used as controls. 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 the first eight groups of these 10 groups, we will use 20 homozygous double knockout mice mice (*Pbrm1*-flox/flox; *Vhl*-flox/flox; Cre-tg/+) per genotype group to assess the phenotype. For the last two groups (groups 9 and 10), 10 mice each are needed. In total, we need 180 homozygous knockout mice (8x20 + 2x10). In this experiment, three female and three male double homozygous *Pbrm1-Vhl*-Flox knockout mice (*Pbrm1*-flox/flox; *Vhl*-flox/flox; Cre-tg/+) generated in experiment #4A will be inbred to produce homozygous double knockout *Pbrm1-Vhl* mice. Of their offspring, approximately 75% will be double knockout homozygotes. Thus, we need generate 240 pups to give rise to 180 homozygous double knockout mice, Considering 10% error rate, we need to produce approximate 264 pups to meet our requirement. Given an approximate litter of 10 pups, the three pairs of mating mice need to breed 9 times to give that number. The other *Pbrm1-Vhl*-floxed concurrently generated in this step will be used as normal controls.

EXPERIMENT #5

Step #5A: this step involves crossing the *Pbrm1*-flox/flox; Cre-tg/+ strain to *Bhd*-flox/flox; Cre-tg/+ strain to generate *Pbrm1-Bhd* double heterozygous mice (*Pbrm1*-flox/+; *Bhd*-flox/+; Cre-tg/+). Two females (one *Pbrm1*-flox/flox; Cre-tg/+ and one *Bhd*-flox/flox; Cre-tg/+) and two males (one *Pbrm1*-flox/flox; Cre-tg/+ and one

Bhd-flox/flox; Cre-tg/+) will be used to set up two mating cages. Each mating pair will be inbred twice. Given an approximate litter of 10 pups, that should produce 40 mice.

Approximately 75% of the total 40 mice will be *Pbrm1-Bhd* double heterozygous mice (*Pbrm1*-flox/+; *Bhd*-flox/+; Cre-tg/+). Thus, roughly 30 mice will be *Pbrm1-Bhd* double heterozygous. Considering 10% error rate, 44 mice should be produced in this step.

Step #5B: *Pbrm1-Bhd* double heterozygous (*Pbrm1*-flox/+; *Bhd*-flox/+; Cre-tg/+) mice will inbreed to produce *Pbrm1-Bhd* double knockout mice. We need approximate six *Pbrm1-Bhd* double knockout mice at this step. From this cross, around 5% of the offspring will be *Pbrm1-Bhd* double knockout mice. Given an approximate litter of 10 pups, we need 120 pups (6/5%) (12 litters) to give rise to six *Pbrm1-Bhd* double knockout mice. Considering 10% error rate, 132 pups are needed at this step #4B.

Step #5C: *Pbrm1-Bhd* double knockout mice (*Pbrm1*-flox/flox; *Bhd*-flox/flox; Cre-tg/+) will be further inbreed to produce enough number of mice for phenotype analysis. Concurrently generated *Pbrm1-Bhd* double floxed mice (*Pbrm1*-flox/flox; *Bhd*-flox/flox; Cre +/+) will be used as controls. 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 the first eight groups of these 10 groups, we will use 20 homozygous double knockout mice mice (*Pbrm1*-flox/flox; *Bhd*-flox/flox; Cre-tg/+) per genotype group to assess the phenotype. For the last two groups (groups 9 and 10), 10 mice each are needed. In total, we need 180 homozygous knockout mice (8x20 + 2x10). In this experiment, three female and three male double homozygous *Pbrm1-Bhd*-Flox knockout mice (*Pbrm1*-flox/flox; *Bhd*-flox/flox; Cre-tg/+) generated in experiment #4A will be inbred to produce homozygous double knockout *Pbrm1-Bhd* mice. Of their offspring, approximately 75% will be double knockout homozygotes. Thus, we need generate 240 pups to give rise to 180 homozygous double knockout mice, Considering 10% error rate, we need to produce approximate 264 pups to meet our requirement. Given an approximate litter of 10 pups, the three pairs of mating mice need to breed 9 times to give that number. The other *Pbrm1-Bhd*-floxed concurrently generated in this step will be used as normal controls.

For EXPERIMENT #3-5, considering tumour incidence and spectrum studies, mice (males and females) of desired genotypes will be kept for up to 2 years to allow spontaneous tumour development. Mice will be monitored at least once a week for signs of illness and obvious tumour burden. Moribund or distressed mice will be sacrificed by CO_2 inhalation and cervical dislocation. Autopsy will perform and major organs including kidneys, along with tumors, will be collected and dissected for pathological and histological analysis.

In addition, for mouse embryonic fibroblasts (MEFs), adult female mice will be mated with male mice of desired genotype. Pregnancy will be confirmed the next morning by the presence of "plug" on the virginal opening. At embryonic day 12.5, pregnant females will be euthanized with CO_2 inhalation and followed by cervical dislocation and the embryos will be collected for harvesting fibroblasts. For harvesting mouse ES cells, embryos at day 3.5 will be used.

Animal Procedure Schedule:

Time	Animal/Cage Number	Procedure	Resultant Effects	Remedy	Qualified Person Performing
One day old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
One-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
Three-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
Eight-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
16-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
24-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
52-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen
78-week old	20 KO mice and 20 control mice	Sacrifice for phenotype inspection	N.A.	N.A.	Jindong Chen

- Injections or Inoculations: (substances, dose, sites, volume, route and schedules)

N.A.

- Blood Withdrawals: (volume, frequency, withdrawal sites and methodology)

  N.A.

- √ Non-Survival Procedures:

  1. When mice are over one year old, adult tumour baring mice and control normal mice will be sacrificed to collect biopsies for studying tumour spectrum.

  2. Pregnant females with embryos at E12.5 days will be sacrificed to collect embryonic fibroblast and E3.5 for ES cells.

- Radiation: (dosage and schedule)

  N.A.

- √ Methods of Restraint: (chemical, restraint chairs, collars, vests, harnesses, slings, etc.)

  For general inspection and injection, method of hand restraint (e.g. Dorsum Scruff Method) will be used. In the dorsum scruff method, the mouse's back skin is grasped (scruffed) from the occiput to the lumbar area. Performer's thumb and 2nd, 3rd, and 4th fingers hold the scruff. Take enough skin to firmly immobilize the mouse. Since no surgeries are expected, other restraints will not be used.

- √ Animal Identification Methods: (ear-tags, tattoos, collar, cage card, etc.)

  Ear punch for numbering individual mice, their number will also be written on cage cards. The mouse colonies information will be maintained using a database called "The Jackson Laboratory's Colony Management System (JAX-CMS)" provided by Jackson Laboratory (ref: http://www.jax.org/jcms/index.html).

- √ Other Procedures: (survival studies, tail biopsies, etc.)

Tail biopsies will be taken according to the recommendations by NIH at 2 weeks of age to extract genomic DNA for genotyping. Mouse tail and scissors will be disinfected by swabbing with 70% alcohol. Mouse will be restrained manually and 3-5mm long of tail from the tip will be cut and collected with a microcentrifuge tube. Following the biopsy procedure, bleeding will be controlled using local pressure before returning the mouse to the cage.(ref: http://www.ncifcrf.gov/rtp/lasp/intra/acuc/fred/guidelines/Tail_Biopsy.pdf)

- ❑ √ Resultant Effects: (pain, distress, ascites production, etc.)

  Some degree of cancer related chronic pain and distress are expected at later stage of cancer development. We can minimize the pain and distress by administrating analgesia, Ibuprofen (30mg/kg in water).

H. Humane Endpoint Criteria:

At any given time during the research study, animals that suffer from severe or chronic pain and distress that cannot be relieved with therapeutic intervention, must be painlessly euthanised. List the criteria used to determine when therapeutic intervention or euthanasia needs to be administered (if it is called for), and what treatments are allowable without interfering with expected results. Complete in "Comments" section below. Death as an endpoint must always be scientifically justified.

- ❑ √ Tumour size (specify growth in cm)

  Tumour size with more than 2cm diameter, approximately equivalent to 15% of the body weight (if visible) or the tumour burden affects normal body function including eating and drinking.

- ❑ √ More than 20% bodyweight loss over 1 week or more OR more than 10% over 24 hours

  Percentage body weight gain or loss (decreased more than 20% compared the control animals, or decreased by more then 25% over a period of 7 days).

- ❑ √ Inability to eat or drink
  Yes. It will be painlessly euthanized.
- ❑ √ Behavioural abnormalities such as CNS signs, vocalisation, hunched posture, shivering, decreased activity, immobility
  Yes. It will be painlessly euthanized.
- ❑ √ Clinical symptomatology such as ruffled fur-coat, lameness, paralysis, dyspnea, vomiting, edema, not eating or drinking, abnormal discharge
  Yes. It will be painlessly euthanized.

- ❑ Signs of toxicity
  N.A.

- ❑ Wound infection or dehiscence

- ❑ Haematemesis (vomiting of blood)

- ❑ √ Severe or chronic pain
  Yes. It will be painlessly euthanized.

- ❑ Severe bleeding

Others: __

√ Hypothermia of more than 10% from normal body temperature, i.e. lower than 33°C.

Yes. It will be painlessly euthanized.

Loss of reflexes including righting reflex, pinna reflex and papillary reflex
Apathy and ataxia

√ Cyanosis and pale mucous membranes
Yes. It will be painlessly euthanized.

Piloerection
Kyphosis

- Comments:

The main objective of this study is to evaluate if spontaneous tumours arise in these mice. The mice will be constantly monitored from any external changes (i.e. tumours) as well as changes in their behavioural pattern when the mice grow up to expected time of tumor formation and manifest stage (around 1 year old). Since this is a classical study to evaluate the effect of the introduced mutation on mice survival, groups of mice will be aged to observe survival. These mice, if they do show symptoms of tumour-bearing, weight loss, etc., will be sacrificed appropriately.

I. Anaesthesia/Analgesia/Tranquillisation:

Specify the anaesthetics, analgesics, sedatives or tranquillisers that are to be used. Include the name of the agent(s), the dosage, route and schedule of administration.

Schedule	Agent	Dose	Route
Tranquillisation			
Induction			
Maintenance			
Analgesia	Ibuprofen	30 mg/kg—4.7 ml Children's Motrin in 500 ml water	in drinking water
Antibiotics			

J. Survival Surgery:

1. Identify and describe the surgical procedure(s) to be performed. Include the aseptic methods to be utilised, suture materials used and when sutures will be removed.

   No survival surgery involved.

2. Who will perform the surgery and what are their qualifications and/or experience?

(Attach record of formal training in animal surgery if any and publication references.)

N.A.

3. Where will surgery be performed?

   N.A.

4. Describe post-operative care and monitoring required, highlight and identify the individual responsible. State parameters to be monitored.
   Attach post-operative care checklist.

   N.A.

5. Has major surgery been performed on any animal(s) prior to being placed in this study? If yes, please explain.

   N.A.

6. Will more than one major survival surgery be performed on an animal while in this study? If yes, please justify.

   N.A.

K. Pain & Distress Category:

The proper use of animals is imperative. This includes avoidance or minimisation of discomfort, distress and pain consistent with sound scientific practice. Unless evidence to the contrary is established, investigators should consider procedures causing pain and/or distress to human beings will also cause pain and/or distress to animals. For in-depth information on Anaesthetic Management, Euthanasia and dosage of drugs, see the following web links:

http://dcminfo.wustl.edu/pdf/PDF/Welcome.pdf
http://www.uab.edu/uabra/arp/drugandsupply/LabAnimalFormulary
http://www.dar.emory.edu/forms/Vet/Analgesic_drugs_05.pdf
http://www.dar.emory.edu/vet_drug_anesthetic.htm
http://www.iacuc.ucsf.edu/Proc/awDosages.asp
http://www.avma.org/issues/animal_welfare/euthanasia.pdf

Formulatory for primates.xls will be added as web link in the SHS Intranet.

We anticipate the animals will experience some degree of cancer related chronic pain at later stage of cancer development. Unfortunately, we cannot avoid such distress because one of our experimental aims is to study the progression of the carcinogenesis. On the other hand, we can minimize the pain and distress by administrating analgesia. There are mainly two categories of analgesia being used commonly in laboratory, opioids and non-steroidal anti-inflammatory drugs (NSAIDs) like Ibuprofen. Opioids is a potent pain-killer with relatively short effective time, ranging from 4-8hrs while NSAIDs showed longer effective time up to 24hrs. Therefore,

opioids have to be given repeatedly every 2-4 hrs by injection for relieving chronic pain. Moreover, there is potential risk of misusing opioids thus it is used under tight prescription control and regulation. On the other hand, it has been shown that some of the NSAIDs, like Ibuprofen can be given as a routine pain-killer in drinking water to effectively relief mild to moderate pain). Ibuprofen has been recommended for use as a pain reliever with a wide ranging dose of 7.5 to 30 mg/kg (Jenkins, 1987, "Pharmacological Aspects of Analgesic Drugs in Animals: An Overview", JAVMA 191 (10) pp. 1231; Liles, JH and Flecknell, P, 1992 "Use of NSAID for Relief of Pain in Rodents and Rabbits" Lab Animal 26: p. 241-255). Accordingly, we will begin using this drug more routinely as an additive to water to treat mild to moderately painful conditions such as skin lesions, fight wounds and eye abscesses. Considering the effectiveness of the analgesia, the ease of administration of the drug and controlling the drug, we decided to use Ibuprofen to the mice bearing visible tumor of larger than 1cm diameter until euthanasia.

Some points on Ibuprofen: Ibuprofen has anti-inflammatory, analgesic and an anti-pyretic (fever) activity. Ibuprofen is a non-specific COX inhibitor resulting in decreased prostaglandin formation. It is well absorbed orally and the majority is excreted in the urine within 24 hours with a small amount also excreted through the stool. Excretion is virtually 100% within 24 hours of the last dose. Possible side effects may include GI ulceration, blood thinning effects, decrease in efficacy of blood pressure lowering drugs, and an interference with secretion of lithium and aminoglycosides that can result in increased blood levels of those drugs, especially at higher doses (40mg/kg). Therefore, we propose to limit usage up to 30mg/kg.

Number of **NEW** animals used per year

Index of Severity	**Year 1**	**Year 2**	**Year 3**
❑ Minimal, Transient, or No Pain or Distress	602	286	176
❑ Pain or Distress Relieved by Appropriate Measures		540	270
❑ Unrelieved Pain*	N.A.	N.A.	N.A.

** For this category, scientific justification is required to explain why the use of anaesthetics, analgesics, sedatives or tranquillisers during and/or following painful or distressful procedure is contraindicated.*

L. Method of Euthanasia/Animal Disposal at the End of the Study (see above websites in Section K of this application):

Indicate the proposed method, and if chemical agent is used, specify the dosage and route of administration. If the method(s) of euthanasia include those not recommended by the AVA like decapitation or cervical dislocation without anaesthesia, provide scientific justification why such methods be used. Indicate the method of carcass disposal.

Method	**Description**
CO_2 Inhalation	Animals will be put into a cage (with air circulation) with only one gas inlet for CO_2, they will be given excess amount of CO_2 at maximum flow rate until they are euthanized, cervical dislocation will be carried out after euthanasia.
Injectable Agents	**N.A.**

Physical Method	N.A.
Others	N.A.

How will death be verified or assured?

Before animals are removed, all visible movement (including breathing) should have stopped. After removal, check again to confirm respiratory arrest. Verify by touch or by using a stethoscope that there is no heart beat.

M. Hazardous Agents:

Registration documents for the use of recombinant DNA or potential human pathogens must be attached in this application.

	Yes	**No**	**List Agents & Registration Document (if applicable)**
Radionuclides		√	
Biological Agents		√	
Hazardous Chemicals or Drugs		√	
Recombinant DNA		√	

If the above table is checked "Yes" then describe the practices and procedures required for the safe handling and disposal of contaminated animal and/or material associated with this study. Also describe the methods for the removal of radioactive waste and, if applicable, the monitoring of radioactivity.

N. Biological Material/Animal Products for Use in Animals: N.A.

(e.g. types of cell lines (human cell lines, human primary tumours, antiserum, etc.))

1. Specify Material

2. Source:_____ Material Sterile or Attenuated _____Yes _____No

3. If derived from human, has it been tested for HIV, mycoplasma, hepatitis, others? "Check" the appropriate box below. If not tested, ABSL2 level of containment need will be applied. Attach Material Safety Data Sheet (MSDS), when required.

	Type	**Comments (if any)**
	HIV	
	Mycoplasma	
	Hepatitis	
	Others	

4. If derived from rodents, has the material been MAP-Mouse Antibody Production/ RAP-Rat Antibody Production/HAP-Hamster Antibody Production tested?
_____Yes *(attach copy of results)* _____No

5. I certify that the MAP/RAP/HAP tested materials to be used have not been passed through rodent species outside of the animal facility in question and/or the material is derived from the original MAP tested sample. To the best of my knowledge, the material remains uncontaminated with rodent pathogens.

_____
Signed by Principal Investigator

O. Special Concerns/or Requirements of Study:

List any special housing, equipment, animal care (i.e. special caging, water, feed, or waste disposal, etc.)
If applicable, list the staff (research or animal holding) involved in ensuring the special needs are met.

N.A.

P. Project Funding:

How do you expect to support your project?
(Please state if your project is supported by a grant, fund or any other means.)

[ ] NMRC

[ ] SGH Research Fund

[ ] BMRC

[ ] SingHealth

[ ] Others: please specify:

Amount of Fund: $ _____ Account No: _____

Date received: _____

Q. Declaration by Principal Investigator:

I _____, accept full responsibility for assuring that the project will be conducted in accordance with the code of practice for the care and use of laboratory animals and the procedures indicated in the approved protocol.

I assure that all personnel who will use this protocol and work with animals have received appropriate training/instructions in procedural and handling techniques, and/or animal welfare considerations.

I confirm that this protocol bears no duplication of previous studies and have conducted due diligence literature search to confirm as such.

Unnecessary Duplication in Research: [ ] N/A *(e.g., Regulatory Study)*

Has this study been previously conducted? [ ] Yes [√] No *(If "No", provide information about literature search conducted to verify this study has not been conducted previously.)* **Date of Search: Database Name: PubMed Years Covered in Search: 15** **Keywords: PBRM1, knockout, mouse model**
If the study has been conducted previously, explain why it is scientifically necessary to duplicate the experiment.

_____
Signature of Principal Investigator

Date signed: _____

R. Final Approval of the Research Project by SingHealth Institutional Animal Care and Use Committee (IACUC):

This is to certify that the research project:

_____

_____

has been reviewed and now approved by SingHealth Institutional Animal Care and Use Committee (IACUC).

Signed this _____ day of _____, Year _____.

_____
Prof Kanaga Sabapathy
Chairman
SingHealth IACUC

References:

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