CMTT # 5051-18

RIH IACUC
Approved Protocol
12/19/2018

Lifespan

Animal Care and Use Protocol (ACUP)

Principal Investigator: Chiung-Kuei Huang

Principal Researcher: Chiung-Kuei Huang

Project Title: The molecular pathogenesis of cholangiocarcinoma

Veterinarian Consultation (You must consult the veterinarian during ACUP development and must **submit your application to the Veterinarian for pre-review at least 2 weeks before the submission deadline**)**:**

- ☒ Tiffany Borjeson, DVM (444-6842) **Date: 9.23.18**
- ☐ Jessica Johnston, DVM (444-6842) **Date:**

Notification of Central Research Facilities (CRF) Director (must notify prior to submission)**:**

- ☒ Paula Bains-Vallee (444-5788) (pbainsvallee@Lifespan.org) **Date: 09.26.18**

IACUC Orientation

Is this your first submission to the IACUC as Principal Investigator? ☐ yes ☒ no

If yes, *call the IACUC Coordinator at 444-2093 to schedule an appointment for the required Orientation.*

Type of animal experimentation protocol:

- ☐ New
- ☒ De novo for CMTT# 0233-15
- ☐ Significant change to an approved project

This document has been locked to facilitate completion and data entry into the form. Please contact the IACUC Coordinator (Kate Brilliant, 444-2093) or the IACUC Manager (Jacqui Poore 444-5843) if you need to unlock the form to expand a table or section of the form.

1. Nature and Purpose of Proposed Studies

a. Lay Summary

Describe the specific aims and details of animal use in non-scientific terms (e.g. Providence Journal level). The lay summary is used by community representatives on the IACUC and also may be used by Public Relations in the event of an external inquiry into the project. Define all acronyms. Do not copy and paste from a grant proposal. Do not include information that is not relevant to the use of live animals (e.g., details of in vitro experiments).

Background and Significance (1-4 sentences)

Cholangiocarcinoma (CCA) is a liver cancer originating from bile ducts either inside or outside the liver. CCA accounts for 5-10 % of liver cancers in the United States. The possible risk factors associated with CCA include infection with parasites, liver cirrhosis, obesity, alcoholic liver disease, and chronic inflammation of bile ducts. The treatment options for CCA include surgery, radiation, and chemotherapy. Although surgery might alleviate the CCA progression, it should be performed at early stage of CCA to be effective. However, most of CCA patients are diagnosed at late stage and the 5 year survival rate is only 2%, indicating that there is a need to develop effective therapy for this devastating disease. Clarifying the molecular pathogenesis of CCA may identify a potential therapeutic approach toward it.

Question being addressed (1-4 sentences)

The current proposal aims to clarify the molecular pathogenesis of CCA. Accomplishments of the current proposal are expected to yield results which may lead to a potential therapy in treating CCA patients.

How will the results of the study be used? (1-4 sentences)

Completion of these studies may help gastroenterologist and oncologist develop effective treatment regimens for patients with CCA. The results will also be published in peer-reviewed journals and distributed to the hepatology scientific community by oral or poster presentation in the American Association for the Study of Liver Diseases as well as American Association for Cancer Research.

Summarize the Specific Aims (derived from the grant proposal/research plan)

1. **Determine whether targeting ASPH with pharmaceutical approaches may inhibit CCA tumor progression in rodent CCA models.**
2. **Clarify the molecular mechanisms by which ASPH modulates CCA progression by control of RB1 expression.**
3. **Evaluate if activation of ASPH promotes CCA initiation.**
4. **Investigate how ASPH is involved in liver fibrosis associated CCA**
5. **Determine how TET1 is involved in CCA progression.**

b. Technical Abstract

Use the following outline to create a structured technical abstract that provides a clear and concise overview of the proposed work. It must include enough detail to allow the reviewers to understand the rational for the project, the specific objectives of the work, and the animal-related experiments that will be performed. It is not necessary to include excessive detail about the ex vivo analysis of tissues.

- Background: *Present the ideas and reasoning behind the proposed work.*
- Objective/Hypothesis: *State the objective/hypothesis to be tested. Provide evidence or rationale that supports the objective/hypothesis.*

- Specific Aims: *State the specific aims of the study.*
- Study Design: *Briefly describe the study design including appropriate controls.*

Background: Cholangiocarcinoma (CCA) is a devastating disease in the United States. There is no effective therapeutic approach to CCA treatment. Several whole genome sequence studies using human CCA samples have identified around 20% of CCA patients as having IDH1/2 mutations. Mutant IDH1/2 generates an oncometabolite 2-hydroxyglutarate (2-HG) rather than 2-oxoglutarate (2-OG) and this metabolic switch has been suggested to be the possible cause for CCA development and progression through epigenetic modification. Due to this breakthrough finding, several small molecule inhibitors (SMIs) have been developed aimed at IDH1/2 mutations to suppress 2-HG production. These SMIs have been used in clinical trials treating CCA and other cancers with such mutations. Nevertheless, for 80% of CCA patients with wild-type (WT) IDH1/2 there is no effective treatment. Additionally, clinical studies have shown that CCA patients with IDH1/2 mutations survive longer than those with WT IDH1/2, suggesting that other 2-OG dependent enzymes, rather than the enzymes involved in epigenetic modification, may be involved in the malignant progression of CCA with WT IDH1/2. In our preliminary data, we found that the 2-OG-dependent enzymes including aspartate beta-hydroxylase (ASPH) and TET1 play oncogeneic roles in promoting CCA progression. Thus, it is very likely that ASPH and TET1 are the potential therapeutic targets in CCA patients with WT IDH1/2. Additionally, they are most likely the underlying mechanisms contributing to the clinical observations that IDH1/2 mutant CCA patients have better prognosis than those with WT ones.

Objective/Hypothesis: Thus, we hypothesize that ASPH and TET1 promote CCA progression, that 2-HG suppresses CCA progression by inhibiting ASPH and TET1 functions, and that ASPH and TET1 are the potential therapeutic targets in CCA patients with WT IDH1/2. Our long-term objectives are to clarify the underlying molecular pathogenesis of CCA and identify the potential therapeutic targets toward this disease.

Specific Aims:
1) Determine whether targeting ASPH with pharmaceutical approaches inhibits CCA tumor progression in rodent CCA models.
2) Clarify the molecular mechanisms by which ASPH modulates CCA progression by control of RB1 expression.
3) Evaluate if activation of ASPH promotes CCA initiation.
4) Investigate how ASPH is involved in liver fibrosis associated CCA
5) Determine how TET1 is involved in CCA progression.

Scientific justification:

Aim 1:

Objective: In this aim, we try to clarify whether overexpression of ASPH promotes CCA progression and the small molecular inhibitor (SMI) inhibits this.

We have found that the first generation of ASPH inhibitor suppresses CCA progression in a subcutaneous xenograft model (ref1). We propose to use orthotopic xenograft mouse and rat CCA models to further validate our original findings. We want to validate the findings in a mouse orthotopic xenograft model, as the liver tumor microenvironment has been demonstrated to modulate CCA progression (ref2). Additionally, we propose to use a rat synergic CCA model based on the rationale that immune cells play a critical role in tumor progression (ref 4) and the rat CCA model has an intact immune system. The use of ASPH overexpression system is to further validate our hypothesis that ASPH and TET1 promote CCA progression and to evaluate if ASPH SMI has therapeutic potential in those CCA patients with ASPH overexpresion.

Accomplishments of aim 1 will yield the results that may validate our hypothesis and establish ASPH as a potential target in CCA patients.

Aim 2:

Objective: To clarify if ASPH modulates CCA progression by modulating RB1 phosphorylation.

We propose to clarify the molecular mechanism by which ASPH promotes CCA progression through enhancing RB1 phosphorylation which leads to tumor malignant progression. We will evaluate this by using the orthotopic xenograft model. We have found that overexpression of CDK2 and CDK4 could not reverse the impact of ASPH knockdown on CCA progression. We propose to validate the findings in preclinical models which recapitulate the human condition. We include Crispr/Cas9 in the current proposal, since the system could do ASPH knockout which can rigorously evaluate our hypothesis that ASPH and TET1 promotes CCA progression.

Accomplishment of aim 2 will yield the results which will help use complete our long-term objective in clarifying the underlying molecular pathogenesis of CCA development.

Aim 3:

Objective: Determine if ASPH is involved in CCA initiation.

We propose to determine if overexpression of ASPH may elicit CCA tumorigenesis. ASPH is an alpha-ketoglutarate dependent dioxygenase and its enzymatic activity is controlled by the availability of co-substrate, such as alpha-ketoglutarate. Interestingly, recent studies demonstrated that elevation of alpha-ketoglutarte is highly associated obesity and nonalcoholic fatty liver disease (ref 5) as well as that obesity is one of the risk factors for CCA development (ref 6). More importantly, we have found that ASPH promotes CCA progression by inactivating functions of the tumor suppressor, RB1, through promoting its phosphorylation. As RB1 is a tumor suppressor and its mutations are associated with several types of cancers, it is highly possible that inactivation of RB1 by ASPH will result in CCA initiation. Thus, we propose to evaluate if overexpression of ASPH promotes CCA carcinogenesis by challenging ASPH transgenic mice with high-fat diet and alpha-ketoglutarate chronically which mimics the clinical scenario of CCA development.

Accomplishments of this aim will yield the results which may clarify if ASPH is involved in CCA carcinogenesis and identify ASPH as an early diagnosis marker in CCA.

Aim 4:

Objective: We aim to evaluate how ASPH is involved in liver fibrosis associated CCA progression.

In clinical study of CCA tumors, liver fibrosis has been strongly linked to CCA development (ref7). We have found that ASPH promotes CCA progression. We propose to further validate our hypothesis in preclinical animal models. We propose to use the triple transgenic murine model, Albumin$^{Cre+/-}$, p53$^{lox/lox}$, KRa$^{sG12D+/-}$ (ref8), because p53 and KRas mutations have been found to be the most common mutations occurred in CCA patients (ref9). In addition, the triple transgenic mouse has been well characterized and studied in the field of CCA translational research (ref10). The use of Albumin Cre will restrict p53 and KRas mutations in the target organ that we will study. Thus, by inducing liver fibrosis in a CCA transgenic mouse model with alcohol, carbon tetrachloride, and thioacetamide, we will recapitulate the human CCA development in our study. The addition of human transgene in these animal models will further validate our hypothesis that ASPH promotes CCA progression.

Accomplishments of this aim will yield the results that may help establish ASPH as a potential therapeutic target in CCA patients.

Aim 5:

Objective: To clarify the role of TET1 in CCA development.

This aim is to evaluate the impact of TET1 in CCA development in a CCA transgenic animal model. We have demonstrated knockdown of TET1 substantially inhibited CCA progression *in vitro*. We propose to extend the finding to the CCA transgenic mouse model generated by knocking out p53 and knocking KRas mutation since these are two major mutations identified in human CCA patients (ref10). In addition, these experiments will validate our hypothesis that ASPH and TET1 promote CCA progression.

Accomplishment of this aim will yield the results which may establish TET1 as a potential target in CCA patients.

Ref1: Huang CK*, Iwagami Y*, Aihara A*, Chung W, de la Monte S, Thomas JM, Olsen M, Carlson R, Yu T, Dong X, Wands JR. Anti-tumor effects of second generation β-hydroxylase inhibitors on cholangiocarcinoma development and progression. 2014 Oct;60(4):1302-13 PLOS One 2016 Mar 8;11(3):e0150336. doi: 10.1371/journal.pone.0150336.

Ref2: Leyva-Illades D, McMillin M, Quinn M, Demorrow S. Cholangiocarcinoma pathogenesis: Role of the tumor microenvironment. Transl Gastrointest Cancer. 2012;1(1):71-80.

Ref3: Luo J*, Lee SO*, Liang L, Huang CK, Li L, Wen S, and Chang C, "Infiltrating bone marrow mesenchymal stem cells increase prostate cancer stem cell population and metastatic ability via secreting cytokines to suppress androgen receptor signaling." Oncogene 2014 May 22;33(21):2768-78

Ref4: Shiao SL, Ganesan AP, Rugo HS, Coussens LM. Immune microenvironments in solid tumors: new targets for therapy. Genes Dev. 2011 Dec 15;25(24):2559-72. doi: 10.1101/gad.169029.111. Review.

Ref5: Rodríguez-Gallego E, Guirro M, Riera-Borrull M, Hernández-Aguilera A, Mariné-Casadó R, Fernández-Arroyo S, Beltrán-Debón R, Sabench F, Hernández M, del Castillo D, Menendez JA, Camps J, Ras R, Arola L, Joven J. Mapping of the circulating metabolome reveals α-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease. Int J Obes (Lond). 2015 Feb;39(2):279-87. doi: 10.1038/ijo.2014.53. Epub 2014 Mar 28.

Ref6: Chaiteerakij R1, Yang JD, Harmsen WS, Slettedahl SW, Mettler TA, Fredericksen ZS, Kim WR, Gores GJ, Roberts RO, Olson JE, Therneau TM, Roberts LR. Risk factors for intrahepatic cholangiocarcinoma: association between metformin use and reduced cancer risk. Hepatology. 2013 Feb;57(2):648-55. doi: 10.1002/hep.26092. Epub 2012 Dec 12.

Ref7: Tyson, GL and El-Serag HB, Risk Factors of Cholangiocarcinoma. Hepatology. 2011 Jul; 54(1): 173–184.doi: [10.1002/hep.24351]

Ref8: O'Dell MR, Huang JL, Whitney-Miller CL, Deshpande V, Rothberg P, Grose V, Rossi RM, Zhu AX, Land H, Bardeesy N, Hezel AF. Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma. Cancer Res. 2012 Mar 15;72(6):1557-67. doi: 10.1158/0008-5472.CAN-11-3596. Epub 2012 Jan 20.

Ref9: Zou S, Li J, Zhou H, Frech C, Jiang X, Chu JS, Zhao X, Li Y, Li Q, Wang H, Hu J, Kong G, Wu M, Ding C, Chen N, Hu H. Mutational landscape of intrahepatic cholangiocarcinoma. Nat Commun. 2014 Dec 15;5:5696. doi: 10.1038/ncomms6696.

Ref10: Saha SK, Parachoniak CA, Ghanta KS, Fitamant J, Ross KN, Najem MS, Gurumurthy S, Akbay EA, Sia D, Cornella H, Miltiadous O, Walesky C, Deshpande V, Zhu AX, Hezel AF, Yen KE, Straley KS, Travins J, Popovici-Muller J, Gliser C, Ferrone CR, Apte U, Llovet JM, Wong KK, Ramaswamy S, Bardeesy N. Mutant IDH inhibits HNF-4α to block hepatocyte differentiation and promote biliary cancer. Nature. 2014 Sep 4;513(7516):110-4. doi: 10.1038/nature13441. Epub 2014 Jul 2. Erratum in: Nature. 2015 Dec 3;528(7580):152.

c. Experimental Design

If applicable, please include a flowchart(s) or diagram(s) to explain the proposed animal experiments, including the study groups, treatment time points, and euthanasia time points. Begin with the arrival of animals in the facility and/or the first procedure. End with euthanasia. Note when individual animals will be used for more than one procedure

Insert diagram below

Aim 1) Experiment A: Determine whether targeting ASPH with pharmaceutical approaches may inhibit CCA tumor progression in rodent CCA models.

Objective: In this experiment, we try to clarify whether overexpression of ASPH promotes CCA progression and the small molecular inhibitor (SMI) for ASPH, MO-I-1151 can suppress it. The below experiments are proposed to answer this question.

MOUSE

Orthotopic xenograft model:

1. Intrahepatic injection with 5x10^6 human H1 CCA cells manipulated with EV or ASPH. We will treat the mice with vehicle or MO-I-1151, n=15. (15 x 4 treatments=60 mice).
2. Intrahepatic injection with 10^7 human HuCCT1 CCA cells manipulated with EV or ASPH. We will treat the mice with vehicle or MO-I-1151, n=15. (15 x 4 treatments=60 mice).

RAT
1. Intrahepatic injection with 3x10^6 Rat CCA cells manipulated with EV or ASPH. The rats will be treated with vehicle (DMSO+saline) or MO-I-1151 (25mg/kg) every other day till end of experiments, n=15 (15X4 treatments=60 rats).

Figure 1 is the flow chart of xenograft experiments.

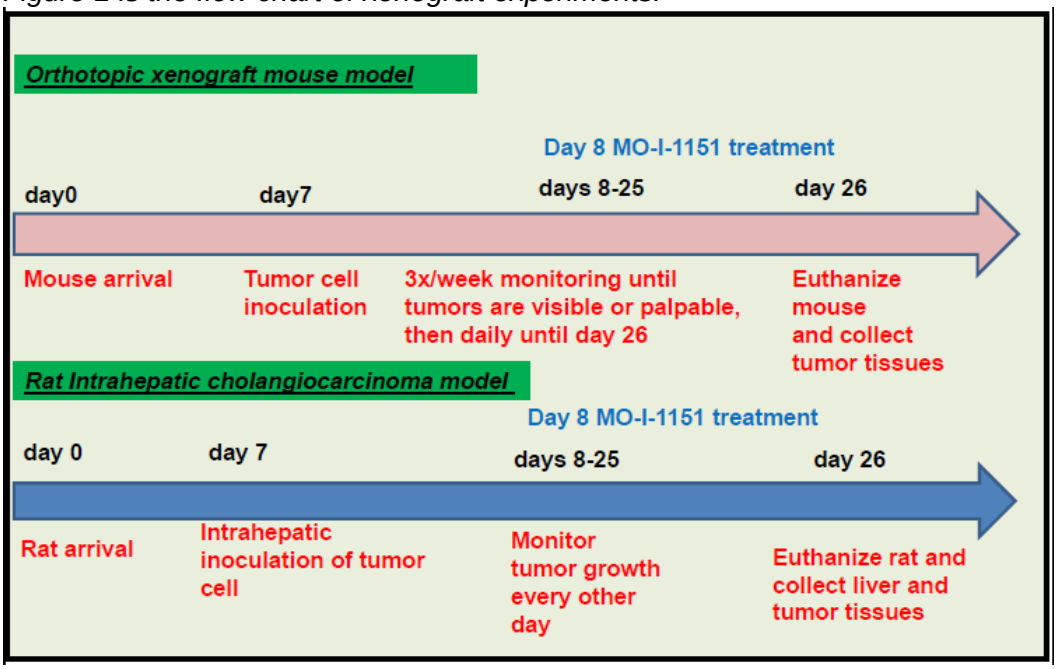


Table 1 summarizes the treatment groups of experiment A.

Animal species	Cell lines	Gene manipulation	Treatment	Tumor model	Place of injection
Mouse	Human H1	EV, ASPH,	Vehicle, MO-I-1151 (25mg/kg)	Orthotopic	liver
Mouse	Human HuCCT1	EV, ASPH,	Vehicle, MO-I-1151 (25mg/kg)	Orthotopic	liver
Rat	Rat BDE-Neu	EV, ASPH	Vehicle, MO-I-1151 (25mg/kg)	intrahepatic injection	liver

Scientific justification of animal numbers requested in aim 1:

We have found that the first generation of ASPH inhibitor suppresses CCA progression in a subcutaneous xenograft model (ref1). We propose to use orthotopic xenograft mouse and rat CCA models to further validate our original findings. We want to validate the findings in a mouse orthotopic xenograft model, as the liver tumor microenvironment has been demonstrated to modulate CCA progression (ref2). Additionally, we propose to use a rat synergic CCA model based on the rationale that immune cells play a critical role in tumor progression (ref 4) and the rat CCA model has an intact immune system. The use of ASPH overexpression system is to further validate our hypothesis that ASPH and TET1 promote CCA progression and to evaluate if ASPH SMI has therapeutic potential in those CCA patients with ASPH overexpresion. Based on our preliminary data, we calculated the effect size and obtained the rough d value 0.75. We then used this to calculate minimum total sample size (two-tailed hypothesis) needed for at least 80% power for reaching a p-value less than 0.05. The minimal mouse number will be 30 in each group. To minimize the use of animals, we propose to use 15

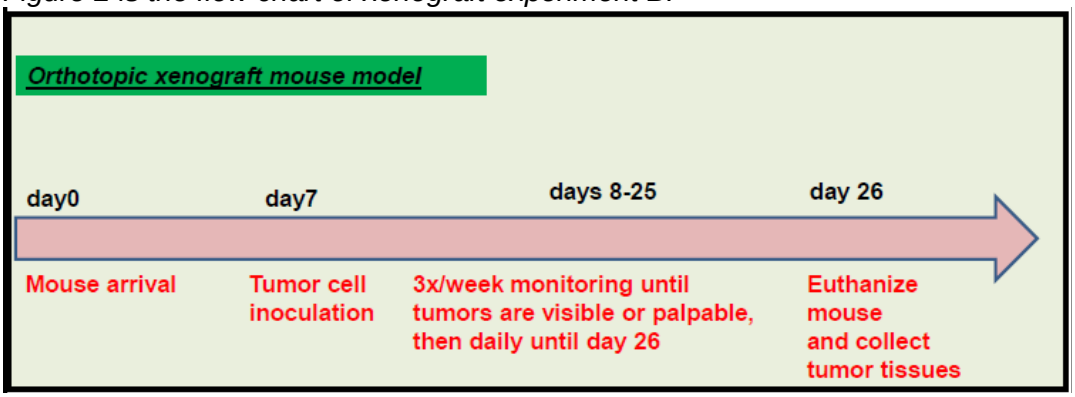
mice in each group as pilot studies. If, unfortunately, we do not get significant difference, we will submit an amendment to request the increase of using 30 mice in each group. **Thus, we request 120 mice (8 groups x 15 mice=120 mice) and 60 rats (4 groups X 15 rats = 60 rats) for this aim.**

Accomplishments of aim 1 will yield the results that may validate our hypothesis and establish ASPH as a potential target in CCA patients.

AIM 2) Experiment B: Clarify the molecular mechanisms by which ASPH modulates CCA progression by control of RB1 expression.

Objective: To clarify if ASPH modulates CCA progression by modulating RB1 phosphorylation. The below experiments are proposed to answer this question.

Figure 2 is the flow chart of xenograft experiment B.



Orthotopic xenograft model:

1. Intrahepatic injection with 5x10^6 human H1 CCA cells manipulated with shLuc control, shRNA-ASPH, shASPH+EV, shASPH+CDK2, shASPH+CDK4, or shASPH+CDK6, n=15 for each treatment (15 x 6 treatments = 90 mice). The schedule of cancer cell injection and tumor collection will be as Fig. 2 illustrated.
2. Intrahepatic injection with 10^7 human HuCCT1 CCA cells manipulated with shLuc control, shRNA-ASPH, shASPH+EV, shASPH+CDK2, shASPH+CDK4, or shASPH+CDK6, n=15 for each treatment (15 x 6 treatments = 90 mice). The schedule of cancer cell injection and tumor collection will be as Fig. 2 illustrated.
3. Intrahepatic injection with 5x10^6 human H1 CCA cells manipulated with Cas9-CTRL, Cas9-ASPH, Cas9-ASPH+EV, Cas9-ASPH +CDK2, Cas9-ASPH+CDK4, or Cas9-ASPH+CDK6, n=15 for each treatment (15 x 6 treatments = 90 mice). The schedule of cancer cell injection and tumor collection will be as Fig. 2 illustrated.
4. Intrahepatic injection with 10^7 human HuCCT1 CCA cells manipulated with Cas9-CTRL, Cas9-ASPH, Cas9-ASPH+EV, Cas9-ASPH +CDK2, Cas9-ASPH+CDK4, or Cas9-ASPH+CDK6, n=15 for each treatment (15 x 6 treatments = 90 mice). The schedule of cancer cell injection and tumor collection will be as Fig. 2 illustrated.

Table 2 summarizes the treatment groups.

Cell lines	Treatment	Tumor model
H1	shLuc shASPH shASPH+EV shASPH+CDK2 shASPH+CDK4 shASPH+CDK6 Crispr/Cas9-control Crispr/Cas9-ASPH Crispr/Cas9-ASPH+EV	orthotopic

	Crispr/Cas9-ASPH+CDK2 Crispr/Cas9-ASPH+CDK4 Crispr/Cas9-ASPH+CDK6	
HuCCT1	shLuc shASPH shASPH+EV shASPH+CDK2 shASPH+CDK4 shASPH+CDK6 Crispr/Cas9-control Crispr/Cas9-ASPH Crispr/Cas9-ASPH+EV Crispr/Cas9-ASPH+CDK2 Crispr/Cas9-ASPH+CDK4 Crispr/Cas9-ASPH+CDK6	orthotopic

Scientific justification of animal numbers requested in aim 2:

We propose to clarify the molecular mechanism by which ASPH promotes CCA progression through enhancing RB1 phosphorylation which leads to tumor malignant progression. We will evaluate this by using the orthotopic xenograft model. We have found that overexpression of CDK2 and CDK4 could not reverse the impact of ASPH knockdown on CCA progression. We propose to validate the findings in preclinical models which recapitulate the human condition. We include Crispr/Cas9 in the current proposal, since the system could do ASPH knockout which can rigorously evaluate our hypothesis that ASPH and TET1 promotes CCA progression. Based on our preliminary data, we calculated the effect size and obtained the rough d value 0.75. We then used this to calculate minimum total sample size (two-tailed hypothesis) needed for at least 80% power for reaching a p-value less than 0.05. The minimal mouse number will be 30 in each group. To minimize the use of animals, we propose to use 15 mice in each group as pilot studies. If, unfortunately, we do not get significant difference, we will submit an amendment to request the increase of using 30 mice in each group. **Thus, we request 360 mice (2 cell lines X 12 groups X 15 mice= 360 mice) for this aim. However, we will not do the proposed Crispr/Cas9 animal studies if we obtain negative results in shRNA-ASPH experiments. This will reduce the use of 180 mice in our proposal.**

Accomplishment of aim 2 will yield the results which will help use complete our long-term objective in clarifying the underlying molecular pathogenesis of CCA development.

Experiment C: Evaluate if activation of ASPH promotes CCA initiation.

Objective: Determine if ASPH is involved in CCA initiation.
To determine if ASPH is involved in CCA initiation, we will generate ASPH knock-in mice and challenge them with:

- alpha-ketoglutarate/2oxoglutarate (2-OG, a substrate of ASPH enzyme) OR
- High fat diet which has been demonstrated to promote 2-OG production to determine if activation of ASPH promotes CCA initiation.

High fat diet:

- Mice will be fed with high fat diet or control diet starting from week 8 until their experimental endpoint at weeks 20, 32, 48, and 72. For high fat diet feeding, High Fat Calories (60%) diet (Soft Pellets, F3282) will be purchased from Bio-Serv. The control diet (F4031, Bio-Serv) will be used as control group. High fat diet has been demonstrated to promote 2-OG production (6μM in normal patient vs. 17μM in nonalcoholic steatosis patients). Reference: Functional diversity of 2-oxoglutarate/Fe(II)-dependent dioxygenases in plant metabolism. Front Plant Sci 2014, 5:524); Mapping of the circulating metabolome reveals alpha-ketoglutarate as a predictor of morbid obesity-associated non-alcoholic fatty liver disease. Int J Obes (Lond) 2015, 39:279-87.).

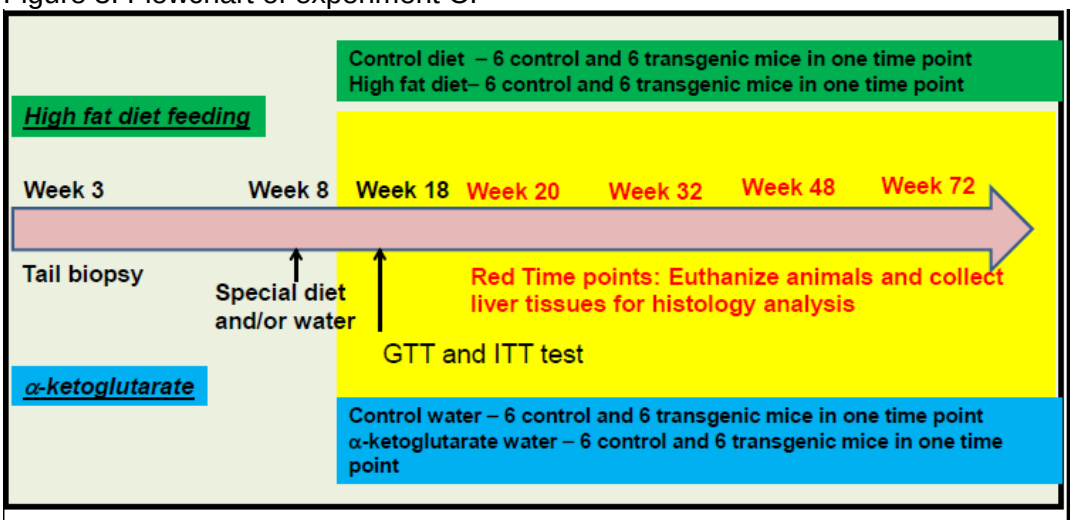
Since we have control and high fat diet treatments and plan to collect at 4 time points (Fig. 3), we will need to have 48 control and 48 transgenic mice. 6 (mice each group) x 2 (treatments) x 4 (time points) x 2 strains = 96.

2-OG:
Mice will have free access to the drinking water containing 50μM 2-OG starting from week 8 until the mice are euthanized at weeks 20, 32, 48, and 72 as Fig.3 indicated. We will need to have 48 control and 48 transgenic mice.
As we proposed to challenge mice with HFD and 2-OG which both are involved in metabolism, we have to consider how the altered metabolism may impact cancer progression. Thus, we will do assays for metabolism as below listed:
a) Insulin tolerance test (ITT)
b) Glucose tolerance test (GTT)

We request 192 mice as below listed for these experiments in aim 3.

Strain	number
fASPH/fASPH EIIaCre+/- (control)	96
fASPH/fASPH, EIIaCre-/-	96

Figure 3. Flowchart of experiment C.



To generate the proposed 192 mice, we have to breed 442 mice. Please see our Appendix 5_C for the detail mouse breeding.

Scientific justification of animal numbers requested in aim 3:
We propose to determine if overexpression of ASPH elicits CCA tumorigenesis. ASPH is an alpha-ketoglutarate dependent dioxygenase and its enzymatic activity is controlled by the availability of co-substrate, such as alpha-ketoglutarate. Interestingly, recent studies demonstrated that elevation of alpha-ketoglutarte is highly associated obesity and nonalcoholic fatty liver disease as well as that obesity is one of the risk factors for CCA development. More importantly, we have found that ASPH promotes CCA progression by inactivating functions of the tumor suppressor, RB1, through promoting its phosphorylation. As RB1 is a tumor suppressor and its mutations are associated with several types of cancers, it is highly possible that inactivation of RB1 by ASPH will result in CCA initiation. Thus, we propose to evaluate if overexpression of ASPH promotes CCA carcinogenesis by chronically challenging ASPH transgenic mice with high-fat diet and alpha-ketoglutarate which mimics the clinical scenario of CCA development. We propose to use 6 mice in each group as a pilot study since this is a cancer initiation study in determining if there is any tumor developed in the experimental mice. If we cannot make a conclusion based on the animal number proposed, we will re-calculate the sample size based on the results of the pilot studies and make appropriate amendment for our animal protocol.

Thus, we request 192 mice (6 mice each groups X 2 treatments X 4 time points X 2 strains X 2 HFD or 2-OG = 192 mice.

Accomplishments of this aim will yield the results which may clarify if ASPH is involved in CCA carcinogenesis and identify ASPH as an early diagnosis marker in CCA.

Aim 4) Experiment D: Investigate how ASPH is involved in liver fibrosis associated CCA
Objective: To determine the role of ASPH in liver fibrosis associated CCA progression. We propose to knockin human ASPH specifically in the liver of triple transgenic CCA model.

MOUSE STUDIES

Obtaining and generation of Albumin Cre (AlbCre)$^{+/-}$, KRas$^{G12D+/-}$, floxed p53/ floxed p53 (p53$^{flox/flox}$) mouse which is a transgenic mouse model for de novo cholangiocarcinoma development. We have the above respective strains from Jax and we have obtained the triple mutant (see below and appendix_5 for breeding strategy). We have AlbCre$^{+/-}$, KRas$^{G12D+/-}$, p53$^{flox/flox}$ mice. We will then proceed to breed into an hASPH knockin mouse. Note that the tumors should be restricted to the liver in this model. This will allow us to generate a "two hit" model, facilitating tumors in the presence of a fibrosis and cirrhosis model.

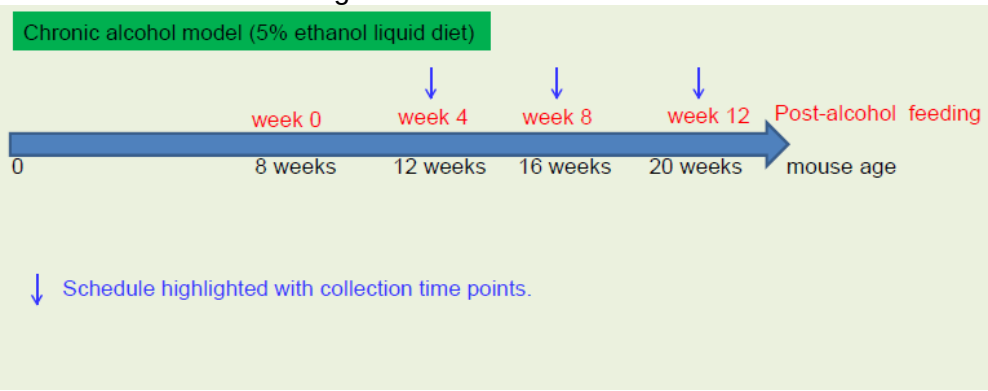
Reference: Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma. Cancer research 2012, 72:1557-67.

Once the above strain is generated we propose:

- **Chronic alcohol feeding in a mouse liver fibrosis/cirrhosis model** (Fig. 4)
- **Carbon tetrachloride in a mouse Liver fibrosis/cirrhosis model** (Fig. 5)
- **Thioacetamide in a Liver mouse fibrosis/cirrhosis model** (Fig. 6)

Chronic alcohol feeding mouse model: Challenge AlbCre$^{-/-}$, KRas$^{G12D+/-}$, p53$^{flox/flox}$, ASPH$^{flox/flox}$ and AlbCre$^{+/-}$, KRas$^{G12D+/-}$, p53$^{flox/flox,}$ ASPH$^{flox/flox}$ mice with 5% alcohol diet (or control) for 4, 8, and 12 weeks. We need 6 mice per group x 2 treatments x 3 time points = 36 x 2 strains = 72 mice

Figure 4 is the chronic alcohol feeding mouse model.

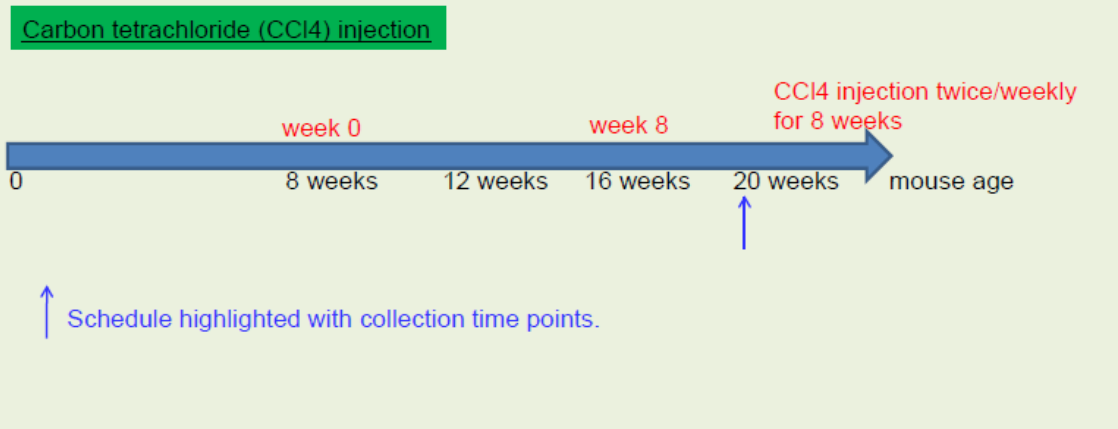


Carbon tetrachloride in a Liver fibrosis/cirrhosis model:

CCl4 or vehicle (olive-oil) will be administered by intraperitoneal (IP) injection at a dose of 1 ml/kg of body weight twice per week for 8 weeks to induce liver cirrhosis. The mice will be challenged with CCl4 starting from 8-weeks old.

We plan to have 9 ASPH$^{flox/flox,}$P53$^{flox/flox}$, KRas$^{G12D+/-,}$ AlbCre$^{-/-}$ mice and 9 ASPH$^{flox/flox,}$P53$^{flox/flox}$, KRas$^{G12D+/-,}$ AlbCre$^{+/-}$ mice. Although it is possible that we may get significant difference by using 6 mice, we have to consider CCl4-induced acute liver toxicity that may lead to sudden death of the experimental mice. Thus, we have to add 3 mice to each group, getting 9 mice in each group. We will collect liver samples at week 20. The experimental schedule will be as indicated in Figure 5. Therefore 9 mice x 2 strains x 1 time point x 2 treatments = 36 mice

Figure 5 is the CCl4 induced liver fibrosis mouse CCA model.

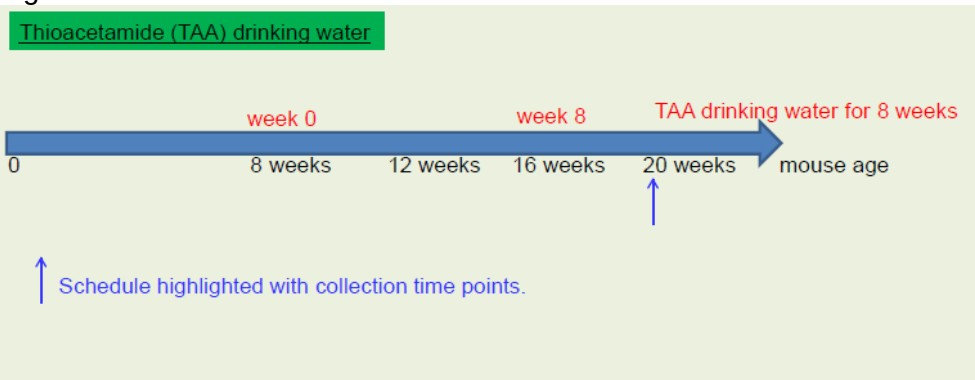


Thioacetamide in a Liver fibrosis/cirrhosis model:

Challenging AlbCre$^{-/-}$, KRas$^{G12D+/-}$, p53$^{flox/flox}$, ASPH$^{flox/flox}$ and AlbCre$^{+/-}$, KRas$^{G12D+/-}$, p53$^{flox/flox,}$ ASPH$^{flox/flox}$ mice with 300 mg/L thioacetamide (TAA) in drinking water, starting at 8 wks of age, to induce liver cirrhosis. Mice will then be euthanized at 20 weeks of age

We plan to have 6 ASPH$^{flox/flox,}$P53$^{flox/flox}$, KRas$^{G12D+/-,}$ AlbCre$^{-/-}$ mice and 6 ASPH$^{flox/flox,}$P53$^{flox/flox}$, KRas$^{G12D+/-,}$ AlbCre$^{+/-}$ mice. We will collect liver samples at week 20 as indicated in Figure 8; Therefore: 6 mice x 2 strains x 1 time point x 2 treatments = 24 mice .

Figure 6 is the flow chart of TAA induced liver fibrosis mouse CCA model.



A note on tansgenic mouse number for Alcohol studies, CCL4 and TAA studies:

The above mouse studies within Experiment D (alcohol, CCL4 and TAA) require a total of 66 ASPH$^{flox/flox,}$P53$^{flox/flox}$, KRas$^{G12D+/-,}$ AlbCre$^{-/-}$ and 66 ASPH$^{flox/flox,}$P53$^{flox/flox}$, KRas$^{G12D+/-,}$ AlbCre$^{+/-}$ mice. Since the genetics of obtaining these mice is 1/16 (the chance of generating both genotypes will be 1/8), we estimate that we will need to generate 1056 mice to obtain the desired genetics. In keeping with the 3R's and being mindful of generating extraneous mice, we will use both the AlbCre heterozygotes and AlbCre homozygotes for experiments. See Appendix 5_D for more details. We request to breed 1056 mice for the proposed experiments.

Scientific justification of animal numbers requested in aim 4:

In clinical study of CCA tumors, liver fibrosis has been strongly linked to CCA development. We have found that ASPH promotes CCA progression. We propose to further validate our hypothesis in preclinical animal models. We propose to use the triple transgenic murine model, Albumin$^{Cre+/-}$, p53$^{lox/lox}$, KRa$^{sG12D+/-}$, because p53 and KRas mutations have been found to be the most common mutations occurred in CCA patients. In addition, the triple transgenic mouse has been well characterized and studied in the field of CCA translational research. The use of Albumin Cre will restrict p53 and KRas mutations in the target organ that we will study. Thus, by inducing liver fibrosis in a CCA transgenic mouse model with alcohol, CCL4, and TAA, we will recapitulate the human CCA development in our study. We propose to use multiple mouse liver fibrosis models because they represent drug-induced and alcoholic liver fibrosis. Although CCL4 and TAA both are drug-induced liver fibrosis models, they are not perfect liver fibrosis models Thus, translational scientists often used both models to validate the important finding in liver fibrosis research. The addition of human transgene in these animal models will further validate our hypothesis that ASPH promotes CCA progression. Based on the preliminary data published (Nature. 2014 Sep 4;513(7516):110-4.), we calculated the effect size, *Cohen's d*, and obtained the rough *d* value, 4. We then used this value to calculate minimum total sample size (two-tailed hypothesis) needed in order to get 80 % of power for reaching p-value less than 0.05. The minimal animal number will be 6 in each proposed treatment. We propose to use 9 mice in CCL4 treated groups as CCL4 has been suggested to cause 20% sudden death. **Thus, we request to breed 1056 mice as proposed above (alcohol, 6 mice per group x 2 treatments x 3 time points = 36 x 2 strains = 72 mice; CCL4, 9 mice x 2 strains x 1 time point x 2 treatments = 36 mice; TAA, 6 mice x 2 strains x 1 time point x 2 treatments = 24 mice; we need 132 mice in total and the chance of getting these mice is 1/8). However, we will not do the proposed CCL4 animal studies if we obtain negative results in TAA experiments. It is because they are both drug-induced mouse fibrotic liver models. This will reduce the use of 576 mice (72 mice in CCL4 X 8 = 576 mice) in our proposal.**

Accomplishments of this aim will yield the results that may help establish ASPH as a potential therapeutic target in CCA patients.

Aim 5) Experiment E: Determine how TET1 is involved in CCA progression.

- We plan to further explore the function of TET1 in CCA malignant progression by using transgenic CCA mouse model (Alb$^{Cre+/-}$ p53$^{flox/flox}$ KRas$^{G12D+/-}$).
- We plan to clarify the role of TET1 in CCA progression by knocking out TET1 in whole body or specifically in the liver in this mouse model.
- We will sacrifice Alb$^{Cre+/-}$ p53$^{flox/flox}$ Kras$^{G12D+/-}$ mice at 18 weeks and Alb$^{Cre+/-}$ p53$^{flox/-}$ Kras$^{G12D+/-}$ mice at 50 weeks as the median survival of the mice are 19 week and 52 weeks, respectively. There is an estimated 20% mortality rate prior to 20 weeks;

We will submit a report back after the first 5-10 KO mice are produced and the percent mortality, percent liver effected and length of survival are known.

5a) We will generate in the proposed new experiments. We will have to use 20 TET1$^{-/-}$ Alb$^{Cre+/-}$ p53$^{flox/flox}$ Kras$^{G12D+/-}$, 20 Alb$^{Cre+/-}$ p53$^{flox/flox}$ Kras$^{G12D+/-}$, 20 TET1$^{-/-}$ Alb$^{Cre+/-}$ p53$^{flox/-}$ Kras$^{G12D+/-}$, and 20 Alb$^{Cre+/-}$ p53$^{flox/-}$, Kras$^{G12D+/-}$ mice. Our breeding strategy is as described in the enclosed appendix 5.

5b) Furthermore, we will generate hepatic specific TET1 knockout mice in CCA mouse model. We will use TET1$^{flox/flox}$ Alb$^{Cre+/-}$ p53$^{flox/flox}$ Kras$^{G12D+/-}$, TET1$^{flox/flox}$ Alb$^{Cre/+}$ p53$^{flox/wt}$ Kras$^{G12D+/-}$, Alb$^{Cre+/-}$ p53$^{flox/flox}$ Kras$^{G12D+/-}$, and Alb$^{Cre+/-}$ p53$^{flox/-}$ Kras$^{G12D+/-}$ mice. 20 mice for each group will be used.
Our detail breeding strategy is listed in the appendix5_ExpE.

New imported mouse strain	number	Resource
TET1 knockout	2	Jackson Lab
TET1$^{flox/flox}$	2	La Jolla Institute for Allergy and Immunology
Mouse number		

TET1 knockout	*658*
Hepatic TET1 knockout	*330*
total	*988*

We request 988 mice for Experiment E.

Scientific justification of animal numbers requested in aim 5:

This aim is to evaluate the impact of TET1 in CCA development in a CCA transgenic animal model. We have demonstrated knockdown of TET1 substantially inhibited CCA progression *in vitro*. We propose to extend the finding to the CCA transgenic mouse model generated by knocking out p53 and knocking KRas mutation since these are two major mutations identified in human CCA patients. In addition, these experiments will validate our hypothesis that ASPH and TET1 promote CCA progression. Based on our preliminary data, we calculated the effect size and obtained the rough d value 1.96. We then used this to calculate minimum total sample size (two-tailed hypothesis) needed for at least 80% power for reaching a p-value less than 0.05. The minimal mouse number will be 20 in each group. **Thus, we request to breed 988 mice for obtaining the proposed animals**. **Our detail breeding strategy is listed in the appendix5_ExpE.**

Accomplishment of this aim will yield the results which may establish TET1 as a potential target in CCA patients.

In summary, we propose to use 2966 mice and 60 rats. Thus, the total animal number requested in this proposal is 3026.

2. Justification of the Proposed Animal Model

a. Are there non-animal alternatives available to accomplish your goals? ☐ yes ☒ no

If yes, provide a brief narrative as to why those alternatives are not being used.

b. Will you be using an established (published) animal model? ☒ *yes* ☐ *no*

If yes, provide literature citations.

1. Immunization with aspartate-β-hydroxylase-loaded dendritic cells produces antitumor effects in a rat model of intrahepatic cholangiocarcinoma. Hepatology. 2012 Jan;55(1):86-97

2. Blockage of CXCR2 suppresses tumor growth of intrahepatic cholangiocellular carcinoma.

Surgery. 2014 Apr;155(4):640-9

3. Kras(G12D) and p53 mutation cause primary intrahepatic cholangiocarcinoma. Cancer research 2012, 72:1557-67.

If no, provide the rationale for development of the model? (Note: the veterinarians must be involved in all new animal model development)

c. Have you used this animal model system before? ☒ yes ☐ no

If yes, describe any refinements you have implemented to reduce the number of animals used or to reduce the amount of pain or distress experienced by individual animals?

We have followed the veterinarian's recommendations to minimize the amount of pain or distress experienced by individual animals, this includes frequent monitoring of tumors (see monitoring information), increased monitoring of animals with potentially compromising phenotypes that may cause early death, reporting to the IACUC regarding our preliminary results.

d. Does the proposed animal model system have the potential to negatively affect the long-term health and well-being of the study animals? *(Examples include: tumor implantation, which can lead to cachexia(wasting) and/or chronic pain; cardiac failure, which may lead to respiratory distress; arthritis, which may inhibit motion and the ability to feed.)* ☒ *yes* ☐ *no*

If yes, describe all anticipated negative consequences of the animal model?

Overall, our animal models look at liver fibrosis, cirrhosis, liver cancer, the etiologies and pathogenesis of these combined with potential therapies and/or targets that may yield an eventual benefit in humans. Death is not an expected endpoint in any of our studies, but we may see clinical signs of compromise specific to each model.

More specifically

Experiment A and B:

Mice:

For orthotopic xenograft, cachexia, ascites, and/or a moribund condition may occur. We will ensure that there is endpoint criteria in place (please see 10b) for this model and mice are checked frequently to ensure that mice are euthanized before a moribund condition occurs.

Rat:

For intrahepatic tumor implantation in rats, cachexia, ascites, and/or a moribund condition may occur. We will ensure that there is endpoint criteria in place (as described at 10b) for this model and rats are checked frequently to ensure that rats are euthanized before a moribund condition occurs.

Experiment D:

Mice:

For the transgenic CCA model, there is an estimated 20% mortality rate prior to 20 weeks in $Alb^{Cre+/-}$ $p53^{lox/lox}$ $KRas^{G12D+/-}$.

CCL4 injections may cause sudden death in up to 20% of mice.

If yes, also describe how will the animals be monitored for these outcomes?

Experiment A and B:

Mice:

Orthotopic xenograft tumor model: Once the tumor cells are implanted via laparotomy, mice will be monitored a minimum of twice per day for three days post-operatively, but will also be evaluated for their clinical condition. Since this tumor is intrahepatic, we are not able to monitor the tumor visually. Instead, a minimum of every other day (including weekends) the mice will be evaluated for weight (change from baseline), body condition, signs of ascities, moribund, cachexia. Since we are implanting tumors in young, still growing mice, Body Condition Scoring will be the best measure of clinical disposition of the mouse. As per the Tumor Policy, the mouse will be euthanized if the body condition score is 1/5. The body condition score is 2/5 and the mouse has decreased activity/responsiveness. The tumor affects the rodent's gait or normal posture, ability to eat, urinate, or defecate independent of the size of the tumor. The veterinarian determines that the animal should be euthanized for humane concerns

Rat:

Intrahepatic cancer cells inoculation: Once the tumor cells are implanted via laparotomy, rats will be monitored a minimum of twice per day for three days post-operatively, but will also be evaluated for their clinical condition. Since this tumor is intrahepatic, we are not able to monitor the tumor visually. Instead, a minimum of every other day (including weekends) the rat will be evaluated for weight (change from baseline), body condition, signs of ascities, moribund, cachexia. Since we are implanting tumors in young, still growing rats, Body Condition Scoring will be the best measure of clinical disposition of the rat. As per the Tumor Policy, the rat will be euthanized if the body condition score is 1/5 • The body condition score is 2/5 and the rat has decreased activity/responsiveness • The tumor affects the rodent's gait or normal posture, ability to eat, urinate, or defecate independent of the size of the tumor • The veterinarian determines that the animal should be euthanized for humane concerns

Experiment D:

Mice:

Transgenic mouse model of: $Alb^{Cre+/-}p53^{flox/flox}$ $Kras^{G12D+/-}$ and $Alb^{Cre+/-}$ $p53^{flox/-}$ $Kras^{G12+/-}$. The median survival of the mice are 19 week and 52 weeks, respectively. There is an estimated 20% mortality rate prior to 20 week. To ensure their welfare we will monitor mice from week 16 onward to ensure welfare. Death is not and endpoint for our studies. Mice will be evaluated for distended abdomen, ascites, difficult breading, jaundice, hunch, weigh gain (>20% more than baseline from week 12 on), or altered mental status.

CCL4: We will monitor a minimum of 3 times weekly, with assessments of parameters of: weights 1-2 times per week with euthanasia if weight lost is >15% from baseline, abnormal posture/positioning including head pressing and hunched back, and an inability to ambulate, access food or other signs of being moribund. If mice are found in declining condition, they will be humanely sacrificed since death as endpoint is not the focus of our studies.

3. Description of Procedures Performed in Live Animals

a. Summary of procedures to be performed

*Provide a clear and concise sequential list of **all** procedures involving the use of live animals that will be easily understood by all members of the committee. Please use non-scientific terminology. Detailed descriptions of surgical and non-surgical but potentially painful or distressful procedures are to be included in the applicable appendices. Complete descriptions of procedures which do not involve surgery and do not present pain or distress should be described in detail below, including the use of any sedatives or anesthetics (e.g. use of sedation for restraint prior to imaging or EKG)*

Examples of procedures: tail snips, surgery, tumor induction, blood collection, metabolism procedures, behavioral studies, injections of chemical/biological agents, etc. If additional procedures will be performed as part of the present protocol, please add additional items as needed.

1. Procedure name: Breeding
   Brief description: see breeding appendix and notation below. We must generate a quadruple mutant for our studies.

2. Procedure name: Genotyping/Tail biopsy
   Brief description: To determine the genotype of mice under 21 days old, the mouse is gently restrained and a small piece (2-5mm) of the tail is snipped off perpendicular to the long axis with very sharp scissors, a scalpel or razor blade. Bleeding after a tail biopsy is stopped by a combination of pressure and styptic powder. Ear tags or ear notches will be performed for unique identification

3. Procedure name: Intrahepatic inoculation of tumor cells (mice)
   Brief description: See Appendix 1. Mice will be anesthetized, a mid-line laparotomy is performed and : 5x10^6 human H1 or 10^7 HuCCT1 CCA cells in 100 µl PBS will be inoculated into the left lateral lobe of the liver using a 30-26g needle + syringe. The body wall is closed, the skin is closed and the mouse is then recovered and monitored as described below. Please see procedural details for surgical/anesthestic descriptions.

4. Procedure name: Intrapertioneal injections of MO-1151 (or vehicle)
   Brief description: 100ul of MO-1151 at the dose of 25 mg/kg (dissolved in DMSO + Sterile Saline) or equal vehicle control will be injected every other day via i.p. to mice and rats starting on day 8 until the animal reaches it's experimental or humane endpoint.

5. Procedure name: Intrahepatic inoculation of tumor cells (rats)
   Brief description: See Appendix 1. Rats will be anesthetized, a mid-line laparotomy is performed and : 3x10^6 rat CCA cells in 100 µl PBS will be inoculated into the left lateral lobe of the liver using a 30-26g needle + syringe. The body wall is closed, the skin is closed and the rat is then recovered

and monitored as described below. Please see procedural details for surgical/anesthestic descriptions.

6. Procedure name: Chronic ethanol feeding (mice)
   Brief description: For chronic alcohol feeding, ethanol liquid diet (F1258) will be purchased from Bio Serv. The iso-caloric control diet (F1259, Bio Serv) will be used as control group. Diet will be prepared per manufacturer's directions and replaced every 48h due to the stability of the diet. The cages will be marked with "special diet" cards placed. Mice will be exposed to chronic alcohol feeding (5% alcohol liquid diet) starting at 8 weeks of age. This will continue for 4, 8 or 12 weeks and then mice are humanely euthanized and tissues harvested.

7. Procedure name: Thioacetamide in water (mice)
   Brief description: Mutant and control mice (as described above) will be exposed to TAA in drinking water. We will prepare water bottles that they have 300 mg/L TAA for mouse drinking water. Mice will be challenged with TAA in their drinking water starting at 8 weeks of age. These bottles will be changed a minimum of once per week and will have "special water" cards placed. Food is given ad lib. After 8 weeks of TAA water exposure, normal drinking water will be given and mice will be euthanized by CO2 overdose and cervical dislocation 4 weeks later and tissues will be harvested.

8. Procedure name: CCL4 injections (i.p.): (mice)
   Brief description: Mutant and control mice (as described above, 8 weeks male and female) will be subjected to CCl4 (289116, Sigma-Aldrich, diluted 1:1 in olive oil) or vehicle (olive-oil). These will be administered by intraperitoneal (i.p.) injection at a dose of 1 ml/kg of body weight twice per week for 8 weeks to induce liver cirrhosis. 4 weeks after the last injection, mice will be euthanized by CO2 overdose and cervical dislocation and tissues will be harvested.

9. Procedure name: High fat diet feeding (mice)
   Brief description: Mice will be fed with high fat diet or control diet starting from week 8 until their experimental endpoint at weeks 20, 32, 48, and 72. For high fat diet feeding, High Fat Calories (60%) diet (Soft Pellets, F3282) will be purchased from Bio Serv. The control diet (F4031, Bio Serv) will be used as control group.

10. Procedure name: 2-OG in drinking water (mice)
    Brief description: Mice will have free access to the drinking water containing 50µM 2-OG starting from week 8 until the mice are euthanized at weeks 20, 32, 48, and 72. 2-OG water is stable for 2 weeks and we will change water weekly.

11. Procedure name: Fasting
    Brief description: Mice will be fasted for 4 hours in insulin tolerance test. Mice will be fasted 16 hours for glucose tolerance test. The detail procedure will be as appendix 2_fasting described.

12. Procedure name: Insulin tolerance test (ITT)
    Brief description: For ITT test, mice will be fasted 4 hr before procedure. The blood glucose (by tail snip) will be measured before an i.p injection of insulin (0.5U/kg B.W.). A serial of very small amounts of blood samples will be used to determine blood glucose 15, 30, 60, 120 minutes post-injection of insulin. If there are signs of immediate hypoglycemia (lethargy, staggering, slow respirations), 0.2 mL of Karo Syrup will be administered by mouth and the mouse will be observed.

13. Procedure name: Glucose tolerance test (GTT)
    Brief description: For GTT test, mice will be fasted overnight. We will measure blood glucose by tail snip before giving 20% glucose injection via i.p. (2g of 20% glucose/kg B.W.). Similarly, the blood glucose levels will be determined by using a serial of very small amounts of blood samples 15, 30, 60, and 120 minutes post-injection of glucose.

14. Procedure name: Tail snips for ITT GTT

Brief description: Tail snips for ITT GTT with subsequent scab removal will be used for serial blood sampling during each of these tests. A topical anesthetic will be applied and meloxicam will be administered prior to the initial tail snip. No more than a combined total of 5mm of tail will be amputated.

15. Procedure name: Euthanasia
    Brief description: Experimental mice and rats will be euthanized with CO2 followed by cervical dislocation to confirm death. Tumors and livers will be harvested for analysis.

b. Euthanasia

Briefly list the primary and, where applicable, secondary methods of euthanasia. A secondary method is a second procedure that is used to confirm euthanasia. (Example: administration of an anesthetic as primary method followed by thoracotomy as a secondary method) Additional details regarding euthanasia will be entered in section 11.

Species	Primary Method[1]	Pharmaceutical agent (drug name, dose, route) *if applicable*	Secondary Method[2] *(Typically rodents, after CO_2 or anesthesia)*
Mouse	CO2		cervical dislocation
Rat	CO2		cervical dislocation

[1] *Examples: asphyxiation, barbiturate overdose, exsanguination under anesthesia, hypothermia (neonates only)*

[2] *This typically applies to rodents only. Examples: bilateral thoracotomy, cervical dislocation, decapitation, exsanguination*

If any of the drugs/agents included in the table above are not pharmaceutical grade, provide information regarding justification, source and formulation.

Drug/Agent name (from table above)	Justification	Preparation(*e.g. diluents, sterilization, pH balancing, storage and labeling*)
Drug: Source:	☐ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	
Drug: Source:	☐ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	

c. Collection of tissue or body fluid from live animals

Will tissue or body fluids be collected from live animals (excluding tail snips)?

☒*yes* ☐*no*

If yes, complete the table below:

Species	Tissue or Body Fluid	Method of Collection	Amount and Frequency of Collection	Agents* Administered Prior to Specimen Collection
Mouse	Tissue (Tail)	Tail biosy	1 time when the mouse is 21 days old	Biopsies limited to mice <21 days old (no anesthesia or analgesia required)
Mouse	blood	Tail snip	5 times with very small blood samples	EMLA cream, topical, 30-60 minutes before procedure

**Anesthetic, analgesic, sedative, or tranquilizer*

d. Multiple Survival Surgical Procedures

Will the animals be subjected to more than one survival surgery taking place during separate periods of anesthesia?

☐ Yes ☒ No

If Yes, list the surgical procedures sequentially and justify why it is scientifically necessary to operate on these animals more than once.

e. Other procedures. *Check all that apply*

Ensure that each item checked is described in Section 2a above. Complete and attach Appendix 2 for any procedures noted below which present the potential for inducing pain or distress.

☐ Perform behavioral studies
☐ Study the effects of trauma
☐ Use electric shock
☐ Study pain
☐ Study pain in alert animals
☐ Employ the use of forced exercise
☒ Induce cancerous tumor growth
☒ Induce organ or system failure
☐ Exposure to infectious agents(s) (complete Appendix 4)
☒ Exposure to human biological agents (complete Appendix 4)
☒ Study the effects of diet or environmental changes (describe in 7.f)
☐ Study temperature changes (describe in 7.f)
☐ Change the light cycle (describe in 7.f)
☐ Purchase pregnant female animals (complete Appendix 5 if dams will deliver live pups)

4. Substances Administered to Animals *(not anesthetics/analgesics)*

Identify all therapeutic drugs, experimental/study agents, chemicals, or other materials administered to live animals by injection, intubation, implantation, or surface application in the appropriate tables below. Do not include anesthetics and/or analgesics which are mentioned in an applicable appendix.

a. Experimental/study agents, therapeutic drugs, chemicals

Species #1: Mouse (*Please duplicate table if agents will be administered to multiple species.)*

Name and Purpose	Source	Pharmaceutical Grade? (*If no, justify below)*	1. Dose (mg/kg) and/or volume 2. Route (e.g. ip, im, po) 3. Frequency (e.g. sid, bid)	4. Timing of administration (e.g. pre-op, intra-op, post-op) 5. Expected duration of effect
Agent: MO-I-1151 Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From our collaborator Mark Olsen Ph.D.	☐ Yes ☒ No	1. Dose 25 mg/kg 2. Route i.p. 3. Frequency every other day	4. Timing Starting on day 8 given IP every other day until day 26 (Fig. 1) 5. Duration 18 days
Agent: Olive oil Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat# 8001-25-0	☐ Yes ☒ No	1. Dose 0.5 ml/kg B.W. 2. Route i.p. 3. Frequency twice per week	4. Timing starting when mice are 8 weeks old (Fig. 5) 5. Duration 8 weeks
Agent: carbon tetrachloride (CCL4) Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat# 319961	☐ Yes ☒ No	1. Dose 0.5 ml/kg B.W. 2. Route i.p. 3. Frequency twice per week	4. Timing starting when mice are 8 weeks old (Fig. 5) 5. Duration 8 weeks
Agent: Thioacetamide (TAA) Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat#163678	☐ Yes ☒ No	1. Dose 300 mg/L 2. Route drinking water 3. Frequency change every week	4. Timing starting when mice are 8 weeks old (Fig. 6) 5. Duration 8 weeks
Agent: alcohol Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat# 1.00983	☐ Yes ☒ No	1. Dose 5% alcohol 2. Route Liquid food 3. Frequency change every other day	4. Timing starting when mice are 8 weeks old (Fig. 4) 5. Duration 12 weeks
Agent: Insulin Purpose: ☒ Experimental ☐ Therapeutic	☒ RIH Pharmacy ☐Other (specify)	☒ Yes ☐ No	1. Dose 0.5U/kg B.W. 2. Route i.p. injection 3. Frequency 1 time	4. Timing when mice are on HFD or 2-OG for 10 weeks (Fig. 3) 5. Duration one time
Agent: glucose Purpose: ☒ Experimental ☐ Therapeutic	☒ RIH Pharmacy ☐Other (specify)	☒ Yes ☐ No	1. Dose 2g of 20% glucose/kg B.W 2. Route i.p. injection 3. Frequency one time	4. Timing when mice are on HFD or 2-OG for 10 weeks (Fig. 3) 5. Duration one time

Agent: a-ketoglutarate (2-OG) Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat# 75890	☐ Yes ☒ No	1. Dose 50µM 2. Route drinking water 3. Frequency change every week	4. Timing starting when mice are 8 weeks old (Fig. 3) 5. Duration 72 weeks
Agent: Saline Purpose: ☒ Experimental ☐ Therapeutic	☒ RIH Pharmacy ☐Other (specify)	☒ Yes ☐ No	1. Dose 2. Route i.p. 3. Frequency every other day	4. Timing Starting on day 8 given IP every other day until day 26 5. Duration 18 days
Agent: DMSO Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat# D2650	☐ Yes ☒ No	1. Dose 2. Route i.p. 3. Frequency every other day	4. Timing Starting on day 8 given IP every other day until day 26 5. Duration 18 days
Agent: Iso-caloric control diet Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Bio-Serv, Cat# F1259SP	☐ Yes ☒ No	1. Dose 2. Route Liquid food 3. Frequency change every other day	4. Timing starting when mice are 8 weeks old (Fig. 4) 5. Duration 12 weeks
Agent: alcoholic liquid diet Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Bio-Serv, Cat# F1258SP	☐ Yes ☒ No	1. Dose prepared with 5% alcohol 2. Route Liquid food 3. Frequency change every other day	4. Timing starting when mice are 8 weeks old (Fig. 4) 5. Duration 12 weeks

Species #2: Rat (*Please duplicate table if agents will be administered to multiple species.)*

Name and Purpose	Source	Pharmaceutical Grade? (*If no, justify below)*	1. Dose (mg/kg) and/or volume 2. Route (e.g. ip, im, po) 3. Frequency (e.g. sid, bid)	4. Timing of administration (e.g. pre-op, intra-op, post-op) 5. Expected duration of effect
Agent: MO-I-1151 Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From our collaborator Mark Olsen Ph.D.	☐ Yes ☒ No	1. Dose 25 mg/kg 2. Route i.p. 3. Frequency every other day	4. Timing Starting on day 8 given IP every other day until day 26 5. Duration 18 days
Agent: Saline Purpose: ☒ Experimental ☐ Therapeutic	☒ RIH Pharmacy ☐Other (specify)	☒ Yes ☐ No	1. Dose 2. Route i.p. 3. Frequency every other day	4. Timing Starting on day 8 given IP every other day until day 26 5. Duration 18 days

Agent: DMSO Purpose: ☒ Experimental ☐ Therapeutic	☐ RIH Pharmacy ☒Other (specify) From Sigma-Aldrich, Cat# D2650	☐ Yes ☒ No	1. Dose 2. Route i.p. 3. Frequency every other day	4. Timing Starting on day 8 given IP every other day until day 26 5. Duration 18 days

b. Justification for the use of non-pharmacological grade agents

Federal regulations require the use of pharmaceutical-grade medications wherever possible, even for acute procedures. All materials administered by parenteral routes, (e.g., intravenous, intramuscular, intraperitoneal and intracranial) must be sterile unless otherwise approved by the IACUC.

See http://grants.nih.gov/grants/olaw/faqs.htm#useandmgmt_4 (F.4.) for rationale and elaboration.

Drug/Agent name (from table above)	Justification	Preparation(*e.g. diluents, sterilization, pH balancing, storage and labeling*)
MO-I-1151	☒ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	MO-I-1151 will be prepared in DMSO and diluted with saline. It will be prepared aseptically within a hood, sterile filtered with 0.22um syringe filtered and pH tested before use. A physiologic pH is ensured of 7.2-7.6. MO-I-1151 will be prepared freshly the same day of injection and not stored.
CCL4	☒ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	CCL4 will be prepared in olive oil at 1:1 ratio. It will be prepared aseptically within a hood, sterile filtered with 0.22um syringe filtered before use. CCL4 will be prepared freshly the same day of injection and not stored.
TAA	☒ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	TAA will be prepared in sterile H2O. It will be prepared aseptically within a hood, sterile filtered with 0.22um filter and pH tested before use. A physiologic pH is ensured of 7.2-7.6. TAA will be prepared freshly the same day of replacement and not stored.
alcohol	☒ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	5% Alcohol liquid diet will be prepared by mixing 5% of ethanol with ethanol liquid diet (F1258) purchased from Bio Serv. The deionized water will be used for the preparation. The food will be changed every other day and prepared freshly the same day of change Thus, the ethanol liquid diet is not stored.

2-OG	☒ Pharmaceutical grade not available ☐ Scientific necessity (specify) ☐ Other (specify)	2-OG will be prepared in sterile H2O. It will be prepared aseptically within a hood, sterile filtered with 0.22um filter and pH tested before use. A physiologic pH is ensured of 7.2-7.6. 2-OG will be prepared freshly the same day of change and not stored. The special water will be changed every week based on the information of stability provided by Sigma-Aldrich.

c. **Biological Agents.** *If you will inject transplantable tumors, cell lines, blood products, agents prepared by recombinant methods, or other biological materials into animals you must attach documentation of testing for murine pathogen viruses. The veterinarians will provide further information regarding testing requirements.*

Agent Name	Source	Viral testing date	Dose	Route	Volume	Diluent/ Media	Frequency of injection
H1 cells	ATCC	9.19.16	5x10^6	subcuta s	50 micro	HBSS buffer	1 time
HuCCT1	ATCC	11.22.10	10^7	subcuta s	50 micro	HBSS buffer	1 time
H1	ATCC	9.19.16	5x10^6	intrahe	50 micro	HBSS buffer	1 time
HuCCT1	ATCC	11.22.10	10^7	intrahe	50 micro	HBSS buffer	1 time
BDE-Neu cell	primary	9.19.16	3x10^6	intrahe	100 micr	HBSS buffer	1 time

Note: Before initiating the proposed xenograft experiments, I will provide the documentation of rodent pathogen test to IACUC as an amendment.

5. Description of Animals

a. List all animals including strain, sex, etc.

Species	Strain	Sex	Age/ Type	# to be purchased	# to be born on-site (or fetuses for in utero studies)[1]	Total # to be used[2]	USDA pain category[3]
Mouse	Foxn1nu/Foxn1+ (Exp_A&B)	female	6 weeks	480	0	480	E
rat	Fisher-344 (Exp_A)	female	8 weeks	60	0	60	E
Mouse	Transgenic mice (Exp_C)	male and female	8 weeks	0	452	452	C
Mouse	Transgenic mice (Exp_D)	male and female	8 weeks	0	1056	1056	E
Mouse	Transgenic mice (Exp_E)	Male and female	8 weeks	4	984	988	C

Total # of animals	3036

[1] *"# to be born on-site" Includes all animals which are born on-site and fetuses used in pregnancy/in utero studies), even if they are not used for experiments or breeding*
[2] *"Total # to used" = # to be purchased + # to be born on-site*
[3] *Indicate the appropriate pain category based on the table below and the approximate number of animals in each category. If multiple procedures will be performed on an animal, the animal is placed in the category appropriate for the most painful/distressful procedure.*

Category	Definition
B	Used only for breeding purposes (no procedures)
C	Minimal, transient or no pain or distress (use of anesthesia for chemical restraint for imaging, etc.)
D	Pain or Distress Relieved by Appropriate Measures, such as use of anesthesia or analgesia
E	Unrelieved Pain or Distress

b. Justification for the number of animals requested for each species listed above.

Describe the strategy used to determine the number of animals required for the experiments described in this protocol. Statistical power analyses should be used to justify animal numbers whenever possible. Assistance with sample size estimation can be obtained from the Biostatistician (444-1493).

Check all that apply and provide specific answers to the associated questions:

☒ **Pilot study or preliminary project – Group variances unknown**

Describe the information used to estimate how many animals are needed.

Experiment C:
The experiment C is to determine if overexpression of ASPH promotes CCA initiation. We propose to challenge ASPH transgenic mice with HFD and 2-OG for 72 weeks and analyze liver tumor incidence in those animals. We propose to use 6 mice in each group as a pilot study since this is a cancer initiation study in determining if there is any tumor developed in the experimental mice. If we cannot make a conclusion based on the animal number proposed, we will re-calculate the sample size based on the results of the pilot studies and make appropriate amendment for our animal protocol.

☒ **Group Sizes determined statistically- power analysis**

Describe the statistical analysis used to estimate the number of animals needed (n). This is usually based on the expected size of the treatment effect, the variance associated with the measurement, and the desired statistical power.

Experiment A and B.
Based on our preliminary data (ref), we calculated the effect size and obtained the rough d value 0.75. We then used this to calculate minimum total sample size (two-tailed hypothesis) needed for at least 80% power for reaching a p-value less than 0.05. The minimal mouse number will be 30 in each group. To minimize the use of animals, we propose to use 15 mice in each group as pilot studies. If, unfortunately, we do not get significant difference, we will submit an amendment to request the increase of using 30 mice in each group.
(Aspartate beta-hydroxylase promotes cholangiocarcinoma progression by modulating RB1 phosphorylation. Cancer Lett. 2018 Aug 10;429:1-10. doi: 10.1016/j.canlet.2018.04.041.)

Experiment D.

Based on the preliminary data published (ref), we calculated the effect size, *Cohen's d*, and obtained the rough *d* value, 4. We then used this value to calculate minimum total sample size (two-tailed hypothesis) needed in order to get 80 % of power for reaching p-value less than 0.05. The minimal animal number will be 6 in each proposed treatment. We propose to use 9 mice in CCL4 treated groups as CCL4 has been suggested to cause 20% sudden death.
(Mutant IDH inhibits HNF-4α to block hepatocyte differentiation and promote biliary cancer. Nature. 2014 Sep 4;513(7516):110-4. doi: 10.1038/nature13441. Epub 2014 Jul 2.)

Experiment E.
Based on our preliminary data, we calculated the effect size and obtained the rough d value 1.96. We then used this to calculate minimum total sample size (two-tailed hypothesis) needed for at least 80% power for reaching a p-value less than 0.05. The minimal mouse number will be 20 in each group.

☐ **Group sizes based on quantity of harvested cells or amount of tissue required**

Describe how the amount of tissue or cells required was determined and explain how much tissue is needed based on the number of experiments you will conduct and how much tissue you expect to obtain from each animal.

Example: Need 10 g tissue. Can get 2 g tissue per animal = 5 animals required

☐ **Product testing**

If the number of animals needed is based on FDA guidelines, provide the citation from the regulations, the IND tracking number, or relevant FDA correspondence.

☐ **Other**

Please describe alternate method for sample size determination.

6. Animal Procurement

a. Source(s) of animals:

☒ Commercial Vendor (specify): Jackson lab, Envigo, and Charles River lab
☐ In-house Breeding Colony (specify PI and CMTT#):
☐ Non-Commercial (e.g. academic) Source (specify)*:
Quarantine Location: (mandatory 60 days)
☐ Brown University
☐ Charles River Laboratories
☐ Jackson Laboratories
☐ Other:

** All mice and rats entering the Lifespan animal facilities from a noncommercial source (such as an academic institution) will be quarantined for a minimum of 60 days before being released for project*

use. Please contact the CRF Director (444-5788) and CRF Import/Export Coordinator (444-4588) for quarantine procedure and required documents.

7. Animal Husbandry

a. Housing Location/Facility (Check all that apply)

- ☐ Middle House (Main Campus) animal facility
- ☒ Claverick St. facility
- ☐ Coro East barrier facility
- ☐ Coro West facility
- ☐ Other *(specify)*:

b. How many animals will be housed within the facility at any one time? *(Include separate estimates for each species)*

100 mice and 30 rats

c. Social Housing (*Check all that apply*)

Social animals should be housed in stable pairs or groups of compatible individuals unless they must be housed alone for experimental reasons or because of social incompatibility. If both group and single caging will be used, provide specifics in 7.f.

☒ Group caging ☒ Single caging

Justify the need for single caging. On occasion, there may be a need that arises to singly house male breeder mice. We will keep this for the least amount of time possible.

d. Food and Water:

☒ Standard diet ☒ Non-standard diet *(Provide details in 7.f.)*

e. Enrichment:

Is it acceptable for CAF staff to provide standard enrichment as appropriate for the species (e.g. chews, toys, and nesting materials)?

☒Yes ☐No

Justify if standard enrichment is not allowed.

f. Non-Standard Husbandry

Describe any non-standard husbandry requirements. Animals exposed to human biological agents must be housed in an ABSL-2 room.

- ☐ None. Standard husbandry for all animals.
- ☐ *Nude animal suite*
- ☒ *ABSL-2 room*
- ☐ *Special suite*
- ☐ *Metabolic cages*
- ☒ *Special food or water (specify)* high fat diet, 2-OG water, TAA water, ethanol liquid diet, control liquid diet
- ☐ *Altered day/night cycles (specify)*
- ☐ *Altered cage change cycles (specify)*

☐ Other *(specify)*

g. Identification

How will individual animals be identified? (*Check all that apply*.)

☐ No individual identification will be used
☒ Ear tags
☐ Tattoos
☒ Ear punch
☐ Temporary marker (e.g. Sharpie)
☐ Toe clipping *(neonatal mice only; requires IACUC approval)*
☐ Subcutaneous RFID microchip *(please describe in Procedures – question 3)*
☐ Other method *(please describe in Procedures – question 3)*

Summary of special food/water preparation and administration				
	Preparation	**Change frequency**	**Biohazard**	**Note**
Control diet for obesity	The control diet (F4031, Bio-Serv) will be given as it is.	weekly	No	Leftover food will be dumped in a dirty cage
High fat diet for obesity	The high fat diet (Soft Pellets, F3282) will be purchased from Bio-Serv and given as it is.	weekly	No	Leftover food will be dumped in a dirty cage
Ethanol diet (alcoholic liquid diet)	5% Alcohol liquid diet will be prepared by mixing 5% of ethanol with ethanol liquid diet (F1258) purchased from Bio Serv.	Every other day	No	The food will be prepared in PI's and leftover food will be discarded in the sink located in PI's lab.
Iso-caloric control liquid diet for alcohol	Control diet will be prepared by mixing sterilize water with control diet purchased From Bio-Serv, Cat# F1259SP	Every other day	No	The food will be prepared in PI's and leftover food will be discarded in the sink located in PI's lab.
2-OG water	Please see detail in 4b. In brief, 2% 2-OG (Cat#78590, SigmaAldrich) will be prepared in sterile H2O.	weekly	No	The leftover 2-OG water will be dumped in the sink located in PI's lab.
TAA water	Please see 4b for detail. Basically, 0.05% TAA (Cat# 163678, Sigma-Aldrich) will be prepared in sterile	weekly	Yes	Will be performed in BSL2 room. The left water will be stored in a sealed container labeled with BIOHAZARD

	H2O.			sticker and located in PI's lab

8. Animal Transport

a. Will live animals be transported outside of the animal care facility? ☒ Yes ☐ No

If *"yes," provide the procedure used to transport animals including the route and elevator(s) to be used and complete a and b below)*

Mouse cages will be covered with drapes to prevent the exposure of animal containments from humans. The cages will be transported from the animal rooms of the Central Research Facility located at 1st floor of 55 Claverick to animal dissection room #407 of the Liver Research Center located at 4th floor. After euthanization, mice ages will be covered with drapes and animal bodies will be disposed in the freezer located at the animal room washing room. Both elevators of the 55 Claverick St. building will be used for the animal transportation.

If the mice to be transported are injected with human cells, the mouse cages will be transported using a container provided by the central research facility. The container is opaque and with a lid. Thus, it will prevent exposure of human hazard to humans. After euthanizing the mice, we will put the cages in red biohazard bag, tie the bag to prevent biohazard exposure, and return it to the animal room where we do nude mouse experiments.

b. Will animals be transported outside of the animal care facility and then returned to their housing room?

☐ Yes ☒ No

If "yes," please justify why the procedures could not be performed within the animal facility and describe the containment measures that will be used to minimize the risk of introducing pathogenic agents from the lab back into the animal facility.

c. Will animals be transported outside of the animal care facility for use of equipment also used for human patients (e.g. MRI, gamma knife, radiation therapy, CT scan, etc.)?

☐ Yes ☒ No

If yes, have you received approval from the clinical area and Infection Control? ☐ Yes ☐ No

Please refer to CRF Policy Manual, section VII (J) for sanitation requirements.

9. Emergency Treatment

In an emergency, animals will be treated to relieve suffering and preserve life, or if necessary, euthanized. When possible, investigators will be contacted by CRF or veterinary staff prior to diagnostic testing, therapy, or euthanasia. In the event that contact is not possible, staff will do their best to follow the parameters listed below.

☒ No therapeutic restrictions exist.

☐ Do not use the following medications (e.g., corticosteroids, antihistamines, antibiotics):

10. Humane Endpoint Criteria for Euthanasia

a. Monitoring of animals

Individuals who will be responsible for monitoring animals must be trained to assess and recognize animal pain or distress.

i. Lab personnel who will be responsible for monitoring animals. Xuewei Bai, Chiung-Kuei Huang, Zhixiang Cheng, and Kevin Cao

ii. How often will this be done? As described in the individual procedures and appendix forms.

b. Even though euthanasia may not be planned for a particular project, the IACUC requires establishing both humane endpoints for a project and criteria to euthanize animal(s) prior to the end of the experiment in the event that the animal's condition falls outside the anticipated experimental parameters. Please define both the humane endpoints and criteria to euthanize animal(s) on this protocol for all animals from time of receipt to their final disposition.

The following signs of ill health will be used as euthanasia criteria

Clinical Observation	**Applicable to this project**
Clinical condition that does not respond to treatment (e.g. infected surgical site)	☒
Delayed wound healing, dehiscence of surgical site	☒
Difficulty in ambulation which render animal unable to access food/water	☒
Persistent and progressive dermatitis or self-trauma	☒
CNS signs such as tremors, seizures, circles that were not anticipated by the study plan	☒
Anorexia >48 hours, other lesions interfering with eating or drinking	☒
Sudden behavioral change (e.g. aggression, guarding, hiding)	☒
Weight loss of 20% or more from baseline at the start of the experiment or as compared to age/gender/strain-matched controls	☒
Markedly discolored urine, excessive urine, or no urine	☒
Severe or refractory diarrhea or decreased fecal output > 48 hours	☐
Dehydration unresponsive to oral or parental therapy	☒
Rough hair coat, hunched posture, distended abdomen, reluctance to move, or lethargy	☒
Respiratory signs such as labored breathing, wheezing, or copious nasal discharge	☒
Cumulative tumor burden exceeds the IACUC-approved tumor burden	☒
Mobility impairment due to tumor burden and/or location of tumor, regardless of tumor size	☒
Tumor ulceration, necrosis or infection	☒
Ascites due to tumor production which results in a 20% increase in body weight	☒
Hemorrhage (blood loss) from any site that is estimated to be >10% total circulating blood volume	☒
Any condition that a veterinarian (or their designee) deems severe enough to warrant euthanizing the animal and/or animals found in a moribund state	☒
Additional humane endpoints: Please see description at option 11.	☒
Additional criteria for euthanasia: As described above in intrahepatic injection rodent tumor models.	☒

11. Disposition of Animals (*Check all that apply)*

☒ **Euthanasia (methods described in question 3b above)**

☒ **Animals will/may become moribund and die before they can be humanely euthanized**. Death of animals is a planned experimental endpoint (e.g. toxicity testing), or there is a likelihood that animals may/will become moribund and die (e.g. sepsis studies, organ failure studies).

1. *Provide a scientific justification,*
2. *Estimate how many (i.e. rate or percentage) will die at the end of the experiment, and*
3. *Indicate the procedures that will be used to minimize non-euthanasia deaths.*

Experiment C:

The CCL4 induced liver fibrosis is a well established mouse liver fibrosis model. The CCL4 injections may cause 20% of sudden death. Thus, we will monitor a minimum of 3 times weekly, with assessments of parameters of: weights 1-2 times per week with euthanasia if weight lost is>15% from baseline, abnormal posture/positioning including head pressing and hunched back, and an inability to ambulate, access food or other signs of being moribund. If mice are found in declining condition, they will be humanely sacrificed since death as endpoint is not the focus of our studies.

Experiment D and E:

As KRas and p53 are the two most common mutations occurred in CCA patients, the triple transgenic mouse, Albumin$^{Cre/-}$ p53$^{lox/lox}$ KRas$^{G12D/+}$ is a highly relevant CCA cancer model. There is an estimated 20% mortality rate prior to 20 weeks; thus we will intensively monitor the mice from week 16 on to ensure their welfare. Death is not an endpoint for our studies. We will monitor mice 3-4 times per week, starting at week 16 for all mice under study, including breeders. Mice will be evaluated for signs of distended abdomen and/or ascites, difficulty breathing, jaundice(yellowing of mucus membranes), hunched appearance, significant weight gain (i.e 20% more than baseline – we will weigh once weekly from week12 on) or alter mental status (staggering, head pressing, dull or lethargic). If mice are noted to have any of these parameters, we will increase the monitoring of these mice to once per day. If mice become significantly affected and/or moribund or have signs of altered mental status (staggering, head pressing, dull/lethargic) we will euthanize these mice immediately. The attached form kindly provided by Dr. Borjeson carefully describes what we will monitor in the proposed animals.

☐ **Animals will be transferred to another protocol.**

Provide details:

12. Assurance that the Proposed Work Does not Unnecessarily Duplicate Previously Published Work on the Same Topic

In accordance with USDA regulations, PHS [9 CFR Part 2.31 (8)] and the Animal Welfare Act, I have conducted a literature search covering the period **from 01/01/1975 to 09/17/2018** using the following **databases** and **keywords.**

Keywords *(Scientific search terms related to the proposed model)*

Aim 1: Determine whether targeting ASPH with pharmaceutical approaches may inhibit CCA tumor progression in rodent CCA models.

Search Key Words: Aspartate beta-hydroxylase, overexpression of aspartate beta-hydroxylase, ASPH inhibitor MO-I-1151, cholangiocarcinoma, bile duct cancer, subcutaneous tumor, liver orthotopic tumor, ASPH, IDH1 wild-type, and small molecule inhibitor.

Aim 2: Clarify the molecular mechanisms by which ASPH modulates CCA progression by control of RB1 expression.
Search Key Words: Aspartate beta-hydroxylase, overexpression of aspartate beta-hydroxylase, cholangiocarcinoma, bile duct cancer, liver orthotopic tumor, ASPH, IDH1 wild-type, CDK2, CDK4, CDK6, RB1, and cell cycle progression.

Aim 3: Evaluate if activation of ASPH promotes CCA initiation.
Search Key Words: Aspartate beta-hydroxylase, overexpression of aspartate beta-hydroxylase, cholangiocarcinoma, bile duct cancer, high fat diet, obesity, alpha-ketoglutarate, ASPH, IDH1 wild-type, non-alcoholic fatty liver

Aim 4: Investigate how ASPH is involved in liver fibrosis associated CCA
Search Key Words: Aspartate beta-hydroxylase, knockout of aspartate beta-hydroxylase, cholangiocarcinoma, bile duct cancer, triple transgenic CCA model, ASPH, IDH1 wild-type, liver fibrosis, Carbon tetrachloride, thioacetamide, and alcoholic liver fibrosis

Aim 5: Determine how TET1 is involved in CCA progression.
Search Key Words: Methylcytosine dioxygenase TET1, knockout of TET1, cholangiocarcinoma, bile duct cancer, triple transgenic CCA model, IDH1 wild-type, liver fibrosis, Carbon tetrachloride, thioacetamide, and alcoholic liver fibrosis, IDH1 mutation.

Databases

Pubmed and Agricola

I have concluded that the activities described in this protocol are not unnecessarily duplicative of previous experiments, including my own.

Please note; OVID, Medline and PubMed search engines use the same database. You may use one of these databases plus one other database, such as Agricola. Hyperlinks to animal welfare information web sites that may be helpful in your search for alternatives to potentially painful/distressful procedures may be found on the Research Administration web site.

13. Search for Alternatives to Painful/Distressful Procedures

The Animal Welfare Act (Title 7, U.S. Code), as written and approved by Congress, emphasizes minimizing pain and distress. It states in Section 13(a)(3)(B):

"… that the principal investigator consider alternatives to any procedure likely to produce pain or distress in an experimental animal;"

The Lifespan IACUC concurs with the USDA (9CFR, Part 2, Sec. 2.31 (d)(1)(ii)) that a multiple database literature search meets the requirement of the Animal Welfare Act as the IACUC members, including the non-affiliated member, a visiting USDA Animal Care Inspector, or a member of the public can follow a printed search strategy, view the list of databases and keywords, and verify that the investigator has made a good faith effort to demonstrate whether or not alternatives exist and why he/she will or will not adopt them.

Accordingly, please document your searches for alternatives, below. Please document separate searches for each painful or distressful procedure. A minimum of two different databases are required. *Please refer to the IACUC Guidelines for Literature Searches for Alternatives (download from the forms library in IRBNet).*

The IACUC strongly encourages you to include at least one database specifically for alternatives in addition to PubMed. **For example:**

- AWIC-Animal Welfare Information Center, https://awic.nal.usda.gov/
- ALTWEB, http://altweb.jhsph.edu/resources/searchalt/
- Guide to Searching for Alternatives to the Use of Laboratory Animals,http://www.frame.org.uk/the-frame-alternatives-laboratory/
- Alternatives to Laboratory Animals, http://www.atla.org.uk/
- University of California Center for Animal Alternatives, http://www.lib.ucdavis.edu/dept/animalalternatives/

Skin and body cavity penetrations (laparotomy, thoracotomy, craniotomy, and entry into a joint space) are examples of procedures considered to be potentially painful. Prolonged restraint and procedures that result in limited mobility, malaise, etc. are examples of procedures considered to be potentially distressful. Refer to the procedure list (Item 3a, above) for a list of potentially painful/distressful procedures.

☒ **No alternatives exist. This must be documented by completing the following:**

I certify that I have reviewed the pertinent sources and have found no valid alternatives to any of the proposed procedures which may cause more than momentary pain or distress. The methods and sources used in my searches included the following databases and keywords:

☒Literature search conducted

Procedure 1: Mouse intrahepatic cholangiocarcinoma xenograft tumor

Databases searched: Pubmed and Agricola

Keywords used: intrahepatic xenograft cholangiocarcinoma, mouse, tumor, rodent, refinements, alternatives, liver orthotopic

Years searched: 1975-2018

Date search was performed: 10/29/2018

Procedure 2: Rat intrahepatic cholangiocarcinoma

Databases searched: Pubmed and Agricola

Keywords used: Rat intrahepatic cholangiocarcinoma, laparotomy, rat, rodent, tumor, rat, refinements, alternatives

Years searched: 1975-2018

Date search was performed: 10/29/2018

Procedure 3: Thioacetamide induced liver fibrosis and transgenic mouse model in cholangiocarcinoma

Databases searched: Pubmed and Agricola

Keywords used: Thioacetamide liver fibrosis model, transgenic cholangiocarcinoma mouse model, refinements, alternatives

Years searched: 1975-2018

Date search was performed: 10/29/2018

Procedure 4: carbon tetrachloride induced liver fibrosis and transgenic cholangiocarcinoma mouse model

Databases searched: Pubmed and Agricola

Keywords used: carbon tetrachloride liver fibrosis mouse model, transgenic cholangiocarcinoma mouse model, refinements, alternatives

Years searched: 1975-2018

Date search was performed: 10/29/2018

Procedure 5: Alcohol induced liver fibrosis/cirrhosis and transgenic cholangiocarcinoma mouse model

Databases searched: Pubmed and Agricola

Keywords used: Ethanol, alcohol, chronic alcohol, liver fibrosis, liver cirrhosis, transgenic cholangiocarcinoma mouse model, rodent, alternative

Years searched: 1975-2018

Date search was performed: 10/29/2018

Procedure 6: Endogenous liver tumor development in mice

Databases searched: Pubmed and Agricola

Keywords used: Albumin cre, KRas, mouse, p53 knockout, cholangiocarcinoma, liver tumor, alternative

Years searched: 1975-2018

Date search was performed: 10/29/2018

Procedure 7: Alcohol induced liver fibrosis/cirrhosis rat cholangiocarinoma model

Databases searched: Pubmed and Agricola

Keywords used: Ethanol, alcohol, chronic alcohol, liver fibrosis, liver cirrhosis, rat, cholangiocarcinoma, alternative

Years searched: 1975-2018

Date search was performed: 10/29/2018

☐ Consultation with colleagues

Provide names, affiliations, credentials, and dates of contact. Describe the colleague's area of expertise and why this colleague is qualified to provide an opinion on alternatives to the proposed painful/distressful procedures.

☐ Other information services utilized.

Elaborate, providing specific information.

☐ Alternatives exist, but are not appropriate for these studies.

Elaborate, providing specific information.

Appendix 1 – Surgical Procedures

*Duplicate this appendix for **each surgical procedure**. Procedures performed under the same period of surgical anesthesia may be combined.*

1. Identification

a. Procedure name (include sham if applicable): intrahepatic injection (Laparotomy)

b. Species: mouse

c. Number of animals: 480

d. Survival surgery:

☐ No (complete Sections 1 and 2)

☒ Yes (complete Sections 1, 2 and 3)

e. Surgical Team:

Responsibility	Lab Personnel (specify name)	Central Research Facility (CRF) staff will perform this service*
Pre-Operative Prep:	**Kevin Cao.**	☐
Anesthesiologist:	**Xuewei Bai**	☐
Surgeon:	**Chiung-Kuei Huang**	
Assistant(s):	Zhixiang Cheng	☐
	Xuewei Bai	

Note: Before initiating the proposed animal experiments, I will provide the documentation of completing AALAS rodent surgery courses for Xuewei Bai, Kevin Cao, and Zhixiang Cheng to IACUC as an amendment.

2. Procedure Details

a. Where will the surgery be performed? *(Check all that apply)*

☒ Central Animal Facility Procedure Room
☐ Central Animal Facility Large Animal Research OR (Aldrich Bldg.)
☐ Laboratory (Building and Room #):

b. Aseptic Procedures:

Sterile instruments, implants and a sterile field are required for ALL surgeries in which the animal will recover from anesthesia. Sterile materials are recommended for other procedures. Indicate methods used to ensure the sterility of these materials, excluding materials that are sterilized by the manufacturer (e.g., surgical gloves, surgical blades, suture, etc.).

Sterilization of Instruments *(Check all that apply)*	**Sterile Field**
☒ High-pressure/temperature steam (autoclave)	☒ Surgeon cap
☐ Gas sterilization (ethylene oxide)	☒ Face mask
☐ Dry heat (hot bead sterilizer)	☐ Surgeon scrub
☐ Plasma sterilization	☐ Sterile gown
☐ Chemical sterilant: type: duration of treatment:	☒ Sterile drapes
☐ Other: (*Describe*):	☒ Sterile gloves

	☐ Other: (*Describe*):

c. Pre-operative procedures

1. Identify all pre-operative procedures performed during the surgical preparation

The following will be employed for preparation of the animal(s) prior to surgery (*check all that apply*):

☐ Fasting (*rarely used in rabbits or rodents*)
Specify timing and duration:
☐ Withhold water
Specify timing and duration:
☐ IV Catheter placement
Catheter size, vessel(s) accessed
☐ Intubation
☒ Application of eye lubricant
Specify: SteriLid, TriOptic from pharmacy
☒ Warming pads to maintain body temperature
☐ Removal of hair from the surgical site
☐ shaving
☐ depilatory (e.g. Nair)
☒ Application of skin disinfectant
☒ Betadine/chlorhexidine followed by alcohol, repeated 3 times
☐ Other (describe):
☐ Other (describe):

d. Pre-operative and intraoperative anesthesia, analgesia, and other medications:

1. Identify the anesthetics, analgesics and other agents administered prior to or during surgery. Examples include antibiotics, sedatives, tranquilizers, anticholinergics, paralyzing agents, fluids, or other pharmaceuticals.

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		
Isoflurane	1-4 % in oxygen		inhalation	continuous

2. Depth of anesthesia

Identify the metrics used to confirm and monitor anesthesia during the surgical procedure.

☒ Toe pinch	☐ Heart rate (requires monitor)
☒ Eye blink	☐ Jaw tone (large animals)
☒ Respiration rate	☐ Blood pressure (large animals)
☐ Mucous membrane color	☐ Other

3. Paralytics

i. Are paralytics employed during this surgical procedure? ☐ Yes ☒ No
If "yes," will anesthesia be used when paralytics are employed? ☐ Yes ☐ No

ii. If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception? *(i.e. heart rate, ECG, etc.*)

e.Implanted Devices

1. List all implanted materials and/or devices (e.g. ligatures, telemetry units, catheters, electrodes, fracture plates, pumps, etc.)

	Implanted Device (description, size, composition, etc.)	**Anatomic Location**	**Duration (days)**
1			
2			
3			
4			

2. Specify methods used to sterilize implanted materials and/or devices. (Check all that apply).

Sterilization Method	Material and/or Device (Specify number from (e)(1) above)
☐ Received sterile from the manufacturer	
☐ High-pressure/temperature steam (autoclave)	
☐ Gas sterilization (ethylene oxide)	
☐ Dry heat (hot bead sterilizer)	
☐ Plasma sterilization	
☐ Chemical sterilant: Type: duration of treatment:	
☐ Other: (*Describe*):	

f. Surgical Procedure Description

Provide a complete narrative of the surgical procedure. *You must provide all of the relevant details without making reference to other protocols.*

Preparation procedures:

1. Anesthetize the mouse with inhalation of 1-4 vol% isoflurane in 100% oxygen for the induction of the anesthesia. A surgical plane of anesthesia is reached when there is no reaction to toe-pinch, no eye blink when touched
2. Protect the eyes from drying out by usage of eye ointment
3. As the nude mouse has no hairs, we will not shave the mouse. Place the mouse nose in the tube of the isoflurane anesthesia system, and fix the legs of the animal with stripes of silk tape
4. Mouse will have its abdomen prepared with betadine, ethanol for three times and then place sterile drapes over the mouse
5. Open the abdomen with a midline laparotomy of a length of <2cm by cutting skin with autoclaved scissors
6. Dissect the connective tissue on top of the peritoneum by using the scissor as a spreader
7. Cut the peritoneum along the linea alba to open the peritoneal cavity
8. Exposure the liver and inject the human CCA cells (prepared in 100µl HBSS) with 26g needle into left lateral lobe of the liver
9. Close deep tissue layer with simple interrupted sutures with 3.0 vicryl suture
10. Cut the ends of the sutures
11. Close skin layer with staples and sterilize the operation area with a gauze swab moistened with antiseptic solution
12. give the sustained release formula of buprenorphine (0.5-1mg/kg, one dose lasts 72h)
13. remove mouse from isoflurane and recover in home cage until awake and ambulatory. Then put back into housing room.

g. Tissue Apposition and Wound Closure.*(Check all that apply)*.

Deep Tissue:
☒ Sutures
Type: vicryl
Size: 3.0
Skin:
☐ Sutures
Type:
Size:
When will these be removed?
☐Wound clips
When will these be removed?
☒Staples
When will these be removed? post-op 7-14 days
☐Animal tissue adhesive (e.g. VetBond)

3. Post-Operative Care/Monitoring

a. Post-Operative Analgesia.*(Check all that apply)*.

☒ Analgesics will be used to provide post-operative pain relief to the animals following surgery

Identify the analgesics and anesthetics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			
SR Buprenorphine	0.5-1.0mg/kg		SQ	once	lasts 72h

☐ Post-operative pain relief will be withheld

Provide a justification for not using postoperative analgesics. Include supporting literature references to justify the exclusion of postoperative analgesia.

b. Postoperative Monitoring

1. What will be the duration of survival after surgery?

   19 days

2. What will be the frequency of monitoring from surgery to euthanasia?

   (*Note: Thetypically accepted minimum monitoring might include twice a day for 3 days then 2-3 times weekly for the duration of the experiment. Consult the veterinarians.)*

We will monitor the experimental mice receiving surgery twice a day for 3 days. Since this tumor is intrahepatic, we are not able to monitor the tumor visually. After post-op monitoring of 3 days has occurred, the mouse will be evaluated a minimum of every other day (including weekends) for weight (change from baseline), body condition, signs of ascities, moribund, cachexia. Since we are implanting tumors in young, still growing mice, Body Condition Scoring will be the best measure of clinical disposition of the mouse. As per the Tumor Policy, the mouse will be euthanized if the body condition score is 1/5 OR the body condition score is 2/5 and the mouse has decreased activity/responsiveness. or if the tumor affects the rodent's gait or normal posture, ability to eat, urinate, or defecate independent of the size of the tumor. The veterinarian determines that the animal should be euthanized for humane concerns.

3. What parameters will be monitored? *Check all that apply.*

☒Behavior and activity level
☒ Body weight
Specify frequency of weighing: every other day
☒ Overall condition
☐ Body temperature
Specify frequency
☒ Food and water intake
☒ Hydration status

☒ Other: Body Condition

4. What post-op monitoring form will you use?

☒ CRF Post-Operative Monitoring Form

☐ Other *(attach/upload)*

Who will provide post-op support?

Xuewei Bai, Chiung-Kuei Huang, Zhixiang Cheng, and Kevin Cao

c. Skin Management for Transcutaneous Implants

1. Describe management of indwelling transcutaneous implants, such as venous catheters, electrical leads, central lines, etc. Include skin care, antibiotic prophylaxis, etc.

2. How will you manage the implant (e.g. cleaning and flushing catheters)?

d. Long-Term Consequences of the Surgical Model

1. Might this surgical model lead to persistent, chronic pain or distress unrelieved by analgesics?

   ☒ Yes ☐ No

   If Yes, place these animals under pain category E under question 5a of the main ACUP and complete 2-3 below.

2. What are the potential long-term consequences or complications? *Check all that apply.*

   ☒ Long-term pain or discomfort
   ☐ Immobility
   ☐ Organ failure
   ☐ Paralysis
   ☐ Paresis (muscle weakness, partial paralysis)
   ☐ Difficulty obtaining food and/or water

   ☒ Other: ascities

3. What methods or non-pharmacological environmental comfort measures will be employed to minimize pain or distress? *Check all that apply.*

   ☒ Paper bedding
   ☒ Access to soft pellet feed
   ☒ Gel packs
   ☐ Housing Huts

   ☐ Other:

Appendix 1 – Surgical Procedures

*Duplicate this appendix for **each surgical procedure**. Procedures performed under the same period of surgical anesthesia may be combined.*

1. Identification

a. Procedure name (include sham if applicable): intrahepatic injection (Laparotomy)

b. Species: rat

c. Number of animals: 60

d. Survival surgery:

☐ No (complete Sections 1 and 2)

☒ Yes (complete Sections 1, 2 and 3)

e. Surgical Team:

Responsibility	Lab Personnel (specify name)	Central Research Facility (CRF) staff will perform this service*
Pre-Operative Prep:	**Kevin Y. Cao**	☐
Anesthesiologist:	**Xuewei Bai**	☐
Surgeon:	**Xuewei Bai**	
Assistant(s):	**Chiung-Kuei Huang**	☐
	Zhixiang Cheng	

Note: Before initiating the proposed animal experiments, I will provide the documentation of completing AALAS rodent surgery courses for Xuewei Bai, Kevin Cao, and Zhixiang Cheng to IACUC as an amendment.

2. Procedure Details

a. Where will the surgery be performed? *(Check all that apply)*

☒ Central Animal Facility Procedure Room
☐ Central Animal Facility Large Animal Research OR (Aldrich Bldg.)
☐ Laboratory (Building and Room #):

b. Aseptic Procedures:

Sterile instruments, implants and a sterile field are required for ALL surgeries in which the animal will recover from anesthesia. Sterile materials are recommended for other procedures. Indicate methods used to ensure the sterility of these materials, excluding materials that are sterilized by the manufacturer (e.g., surgical gloves, surgical blades, suture, etc.).

Sterilization of Instruments *(Check all that apply)*	**Sterile Field**
☒ High-pressure/temperature steam (autoclave)	☒ Surgeon cap
☐ Gas sterilization (ethylene oxide)	☒ Face mask
☐ Dry heat (hot bead sterilizer)	☐ Surgeon scrub
☐ Plasma sterilization	☐ Sterile gown
☐ Chemical sterilant: type: duration of treatment:	☒ Sterile drapes
☐ Other: (*Describe*):	☒ Sterile gloves
	☐ Other: (*Describe*):

c. Pre-operative procedures

1. Identify all pre-operative procedures performed during the surgical preparation

The following will be employed for preparation of the animal(s) prior to surgery (*check all that apply*):

☐ Fasting (*rarely used in rabbits or rodents*)

Specify timing and duration:

☐ Withhold water

Specify timing and duration:

☐ IV Catheter placement

Catheter size, vessel(s) accessed

☐ Intubation

☒ Application of eye lubricant

Specify: SteriLid, TriOptic from pharmacy

☒ Warming pads to maintain body temperature

☒ Removal of hair from the surgical site

☒ shaving

☐ depilatory (e.g. Nair)

☒ Application of skin disinfectant

☒ Betadine/chlorhexidine followed by alcohol, repeated 3 times

☐ Other (describe):

☐ Other (describe):

d. Pre-operative and intraoperative anesthesia, analgesia, and other medications:

1. Identify the anesthetics, analgesics and other agents administered prior to or during surgery. Examples include antibiotics, sedatives, tranquilizers, anticholinergics, paralyzing agents, fluids, or other pharmaceuticals.

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		
Isoflurane	1-4 % in oxygen		inhalation	continuous

2. Depth of anesthesia

Identify the metrics used to confirm and monitor anesthesia during the surgical procedure.

☒ Toe pinch	☐ Heart rate (requires monitor)
☒ Eye blink	☐ Jaw tone (large animals)
☒ Respiration rate	☐ Blood pressure (large animals)
☐ Mucous membrane color	☐ Other

3. Paralytics

i. Are paralytics employed during this surgical procedure? ☐ Yes ☒ No
If "yes," will anesthesia be used when paralytics are employed? ☐ Yes ☐ No

ii. If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception? *(i.e. heart rate, ECG, etc.*)

e.Implanted Devices

1. List all implanted materials and/or devices (e.g. ligatures, telemetry units, catheters, electrodes, fracture plates, pumps, etc.)

	Implanted Device (description, size, composition, etc.)	**Anatomic Location**	**Duration (days)**
1			
2			
3			
4			

2. Specify methods used to sterilize implanted materials and/or devices. (Check all that apply).

Sterilization Method	Material and/or Device (Specify number from (e)(1) above)
☐ Received sterile from the manufacturer	
☐ High-pressure/temperature steam (autoclave)	
☐ Gas sterilization (ethylene oxide)	
☐ Dry heat (hot bead sterilizer)	
☐ Plasma sterilization	
☐ Chemical sterilant: Type: duration of treatment:	
☐ Other: (*Describe*):	

f. Surgical Procedure Description

Provide a complete narrative of the surgical procedure. *You must provide all of the relevant details without making reference to other protocols.*

<u>Preparation procedures:</u>

1. Anesthetize the rat with inhalation of 1-4 vol% isoflurane in 100% oxygen for the induction of the anesthesia. A surgical plane of anesthesia is reached when there is no reaction to toe-pinch, no eye blink when touched
2. Shave the abdominal fur of the rat with an electric fur shaver and protect the eyes from drying out by usage of eye ointment
3. Place the rat nose in the tube of the isoflurane anesthesia system, and fix the legs of the animal with stripes of silk tape
4. Rat will have his abdomen prepared with betadine, ethanol for three times and then place sterile drapes over the rat
5. Open the abdomen with a midline laparotomy of a length of approximately 2 cm by cutting skin with autoclaved scissors
6. Dissect the connective tissue on top of the peritoneum by using the scissor as a spreader
7. Cut the peritoneum along the linea alba to open the peritoneal cavity
8. Exposure the liver and inject the rat CCA cells with 26g needle into left lateral lobe of the liver
9. Close deep tissue layer with simple interrupted sutures with 3.0 vicryl suture
10. Cut the ends of the sutures
11. Close skin layer with staples and sterilize the operation area with a gauze swab moistened with antiseptic solution
12. give the sustained release formula of buprenorphine (1-1.2 mg/kg, one dose lasts 72h)
13. remove rat from isoflurane and recover in home cage until awake and ambulatory. Then put back into housing room.

g. Tissue Apposition and Wound Closure.*(Check all that apply)*.

Deep Tissue:
☒ Sutures
Type: vicryl
Size: 3.0
Skin:
☐ Sutures
Type:
Size:
When will these be removed?
☐Wound clips
When will these be removed?
☒Staples
When will these be removed? post-op 7-14 days
☐Animal tissue adhesive (e.g. VetBond)

3. Post-Operative Care/Monitoring

a. Post-Operative Analgesia.*(Check all that apply)*.

☒ Analgesics will be used to provide post-operative pain relief to the animals following surgery

Identify the analgesics and anesthetics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			
SR Buprenorphine	1.0 - 1.2 mg/kg		SQ	once	lasts 72h

☐ Post-operative pain relief will be withheld

Provide a justification for not using postoperative analgesics. Include supporting literature references to justify the exclusion of postoperative analgesia.

b. Postoperative Monitoring

1. What will be the duration of survival after surgery?

   19 days

2. What will be the frequency of monitoring from surgery to euthanasia?

   (*Note: Thetypically accepted minimum monitoring might include twice a day for 3 days then 2-3 times weekly for the duration of the experiment. Consult the veterinarians.)*

The frequency of monitoring will be as previously described (reference). Basically, we will monitor the experimental rats receiving surgery twice a day for 3 days. Since this tumor is intrahepatic, we are not able to monitor the tumor visually. After post-op monitoring of 3 days has occured, the rat will be evaluated a minimum of every other day (including weekends) for weight (change from baseline), body condition, signs of ascities, moribund, cachexia. Since we are implanting tumors in young, still growing rats, Body Condition Scoring will be the best measure of clinical disposition of the rat. As per the Tumor Policy, the rat will be euthanized if the body condition score is 1/5 • The body condition score is 2/5 and the rat has decreased activity/responsiveness • The tumor affects the rodent's gait or normal posture, ability to eat, urinate, or defecate independent of the size of the tumor • The veterinarian determines that the animal should be euthanized for humane concerns

Reference: A novel "patient-like" model of cholangiocarcinoma progression based on bile duct inoculation of tumorigenic rat cholangiocyte cell lines. Hepatology. 2008 Apr;47(4):1178-90.

3. What parameters will be monitored? *Check all that apply.*

☒ Behavior and activity level
☒ Body weight
Specify frequency of weighing: every other day
☒ Overall condition
☐ Body temperature
Specify frequency
☒ Food and water intake
☒ Hydration status

☐ Other:

4. What post-op monitoring form will you use?

☒ CRF Post-Operative Monitoring Form
☐ Other *(attach/upload)*

Who will provide post-op support?

Chiung-Kuei Huang and Yoshifumi Iwagami

c. Skin Management for Transcutaneous Implants

1. Describe management of indwelling transcutaneous implants, such as venous catheters, electrical leads, central lines, etc. Include skin care, antibiotic prophylaxis, etc.

2. How will you manage the implant (e.g. cleaning and flushing catheters)?

d. Long-Term Consequences of the Surgical Model

1. Might this surgical model lead to persistent, chronic pain or distress unrelieved by analgesics?

   ☒ Yes ☐ No

   If Yes, place these animals under pain category E under question 5a of the main ACUP and complete 2-3 below.

2. What are the potential long-term consequences or complications? *Check all that apply.*

   ☒ Long-term pain or discomfort
   ☐ Immobility
   ☐ Organ failure
   ☐ Paralysis
   ☐ Paresis (muscle weakness, partial paralysis)
   ☐ Difficulty obtaining food and/or water

   ☒ Other: ascities

3. What methods or non-pharmacological environmental comfort measures will be employed to minimize pain or distress? *Check all that apply.*

   ☒ Paper bedding
   ☒ Access to soft pellet feed
   ☒ Gel packs
   ☐ Housing Huts

   ☐ Other:

Appendix 2 – Non-Surgical Procedures that have the Potential to cause Pain and/or Distress

Pain management is expected for all procedures that may cause more than momentary pain or distress. All procedures where anesthetics and/or analgesics are used to relieve pain are considered to be potentially painful. *Injection of Complete Freund's Adjuvant (CFA), infliction of trauma and burns, bacterial infection, induction of disease states such as severe arthritis, and some behavioral tests are examples of procedures that are considered to have the potential to produce pain. Prolonged restraint, food or water deprivation, procedures which result in limited mobility, malaise, etc. are examples of procedures considered to be potentially distressful.*

Duplicate this appendix for each potentially painful/distressful procedure. ***You must also complete the Search for Alternatives in the Main body of the ACUP.***

1. Identification

a. Procedure name (include sham if applicable): carbon tetrachloride (CCL4) injection

b. Species: mouse

c. Number of animals: 24

d. Survival:

☐ No

☒ Yes

2. Procedure Details

a. **Where will the procedure be performed**? (*Check all that apply*)

☐ Central Animal Facility Procedure Room (select location)

- ☐ Middle House (Main Campus) animal facility
- ☒ Claverick St. facility
- ☐ Coro East barrier facility
- ☐ Coro West facility

☐ Laboratory (Building and Room #):

b. **Procedure Description**.
Provide a complete narrative of the procedure. Provide all of the relevant details for this protocol without making reference to other protocols.

CCl4 or vehicle (olive-oil) will be administered by intraperitoneal (IP) injection at a dose of 1 ml/kg of body weight twice per week for 8 weeks to induce liver cirrhosis. The mice will be challenged with CCl4 starting from 8-weeks old. CCl4-induced acute liver toxicity that may lead to sudden death of the experimental mice (approx. 20%). We will monitor animals as described below. We will collect liver samples at week 20.

c. **Pain and/or Distress**

1. How is pain/distress assessed and monitored during the procedure? Include a description of the signs of pain/distress that will be monitored.

The CCL4 injections themselves do not cause pain or distress, however, acute liver toxicity that does lead to sudden death is a possibility. We will monitor mice as described under 3.b.2

2. Analgesia or anesthesia will be utilized to minimize pain or distress during the procedure.
Yes ☐ No ☒ *If Yes, Complete the table below.*

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		

3. Will sedatives, analgesics, or anesthetics be withheld for scientific reasons? Yes ☐ No ☒

If yes, explain and include supporting literature references.

d. **Paralytics**

1. Are paralytics employed during potentially painful/distressful procedures? Yes ☐ No ☒

a) If yes, will anesthesia be used when paralytics are employed? Yes ☐ No ☒

b) If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception?

3. **Post-Procedure Care/Monitoring**

a. Post-Procedure Analgesia. *(Check all that apply)*.

☐ Analgesics will be used to provide pain relief to the animals following the procedure

Identify the analgesics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			

☐ Post-procedure pain relief will be withheld

Provide a justification for not using post-procedure analgesics. Include supporting literature references to justify the exclusion of post-procedure analgesia.

☐ Post-procedure pain relief is unnecessary – pain or discomfort is minimal and/or transient

☒ Environmental comfort measures will be employed to minimize pain or distress

Check all that apply.

☒ Paper bedding
☒ Access to soft pellet feed
☒ Gel packs
☒ Housing Huts
☐ Other:

b. Post-procedure Monitoring

1. What will be the duration of survival after the procedure? After 8 weeks of CCl4 treatment, we will sacrifice the experimental mice.
2. What will be the frequency of monitoring from procedure to euthanasia?

   (*Note: The typically accepted minimum monitoring might include frequent monitoring for the short-term following the procedure and then tapering to standard weekly observations thereafter. Consult the veterinarians for further guidance.)*

   The injections may cause sudden death. Thus, we will monitor a minimum of 3 times weekly, with assessments of parameters of: weights 1-2 times per week with euthanasia if weight lost is >15% from baseline, abnormal posture/positioning including head pressing and hunched back, and an inability to ambulate, access food or other signs of being moribund. If mice are found in declining condition, they will be humanely sacrificed since death as endpoint is not the focus of our studies.

3. Do you expect that there will be any long-term consequences from this procedure (e.g. long-term pain or discomfort, immobility, difficulty obtaining food or water)? ☒ Yes ☐ No
   If Yes, explain

As this is a liver fibrosis animal model, we expect those experimental mice will have discomfort as liver fibrotic patients have.

4. What parameters will be monitored? *Check all that apply.*

   ☒ Behavior and activity level
   ☒ Body weight
   Specify frequency of weighing weekly
   ☒ Overall condition
   ☐ Body temperature
   Specify frequency
   ☒ Food and water intake
   ☒ Hydration status
   ☐ Other:

5. Who will provide post-procedural support? Chiung-Kuei Huang, Zhixiang Cheng, and Kevin Cao

6. **You are required to document post-procedural monitoring.** Indicate which form(s) you will use.

   ☒ CRF Sample Post-Operative/Post-Procedure Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ Lifespan/RIH Tumor Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ A procedure/project specific form will be used to document monitoring. **A**ttach/upload the monitoring form to be used for this procedure.

Appendix 2 – Non-Surgical Procedures that have the Potential to cause Pain and/or Distress

Pain management is expected for all procedures that may cause more than momentary pain or distress. All procedures where anesthetics and/or analgesics are used to relieve pain are considered to be potentially painful. *Injection of Complete Freund's Adjuvant (CFA), infliction of trauma and burns, bacterial infection, induction of disease states such as severe arthritis, and some behavioral tests are examples of procedures that are considered to have the potential to produce pain. Prolonged restraint, food or water deprivation, procedures which result in limited mobility, malaise, etc. are examples of procedures considered to be potentially distressful.*

Duplicate this appendix for each potentially painful/distressful procedure. ***You must also complete the Search for Alternatives in the Main body of the ACUP.***

1. Identification

a. Procedure name (include sham if applicable): Chronic alcohol feeding

b. Species: Mouse

c. Number of animals: 72

d. Survival:

☐ No

☒ Yes

2. Procedure Details

a. **Where will the procedure be performed**? (*Check all that apply*)

☐ Central Animal Facility Procedure Room (select location)

- ☐ Middle House (Main Campus) animal facility
- ☒ Claverick St. facility
- ☐ Coro East barrier facility
- ☐ Coro West facility

☐ Laboratory (Building and Room #):

b. **Procedure Description**.
Provide a complete narrative of the procedure. Provide all of the relevant details for this protocol without making reference to other protocols.

1. The regular diet will be replaced with 5% ethanol liquid diet or iso-caloric control diet.

2. The experimental mice will be fed with 5% ethanol liquid diet or iso-caloric control diet for 4, 8, or 12 weeks.

3. The experimental mice will be euthansized after alchol feeding schedule.

c. **Pain and/or Distress**

1. How is pain/distress assessed and monitored during the procedure? Include a description of the signs of pain/distress that will be monitored.

Animals receiving chronic alcohol feeding that causes no pain or distress, or only momentary or silght pain or distress, may not require the use of pain-relieving drugs.

2. Analgesia or anesthesia will be utilized to minimize pain or distress during the procedure.
Yes ☐ No ☒ *If Yes, Complete the table below.*

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		

3. Will sedatives, analgesics, or anesthetics be withheld for scientific reasons? Yes ☐ No ☒

If yes, explain and include supporting literature references.

d. **Paralytics**

1. Are paralytics employed during potentially painful/distressful procedures? Yes ☐ No ☒

a) If yes, will anesthesia be used when paralytics are employed? Yes ☐ No ☒

b) If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception?

3. **Post-Procedure Care/Monitoring**

a. Post-Procedure Analgesia. *(Check all that apply)*.

☐ Analgesics will be used to provide pain relief to the animals following the procedure

Identify the analgesics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			

☐ Post-procedure pain relief will be withheld

Provide a justification for not using post-procedure analgesics. Include supporting literature references to justify the exclusion of post-procedure analgesia.

☐ Post-procedure pain relief is unnecessary – pain or discomfort is minimal and/or transient

☒ Environmental comfort measures will be employed to minimize pain or distress

Check all that apply.

☒ Paper bedding

☒ Access to soft pellet feed

☒ Gel packs

☒ Housing Huts

☐ Other:

b. Post-procedure Monitoring

1. What will be the duration of survival after the procedure? The chronic alcohol feeding will take 12 weeks. We will sacrifice the experimental mice right after 12 weeks of alcohol feeding.
2. What will be the frequency of monitoring from procedure to euthanasia?

   (*Note: The typically accepted minimum monitoring might include frequent monitoring for the short-term following the procedure and then tapering to standard weekly observations thereafter. Consult the veterinarians for further guidance.)*

   We will monitor the experimental animals a minimum of 1-2 times per week. We change the diet out every 48 hours so mice are, in general, evaluated more frequently. We do not expect to see any overt clinical signs with the liver cirrhosis as our model. Mice typically do well on the diet and tend not to decrease in body mass >15%. Note that we do not perform an alcohol-withdrawal component with this model, thus those clinical signs are not expected. Any mouse showing signs of morbidity, including a decline in body weight >15% from baseline, inability to access food or water, or signs of being moribund will be humanely euthanized.

3. Do you expect that there will be any long-term consequences from this procedure (e.g. long-term pain or discomfort, immobility, difficulty obtaining food or water)? ☐ Yes ☒ No
   If Yes, explain

4. What parameters will be monitored? *Check all that apply.*

   ☒ Behavior and activity level

   ☒ Body weight

   Specify frequency of weighing weekly

   ☒ Overall condition

   ☐ Body temperature

   Specify frequency

   ☒ Food and water intake

   ☒ Hydration status

   ☐ Other:

5. Who will provide post-procedural support? Chiung-Kuei Huang, Zhixiang Cheng, and Kevin Cao

6. **You are required to document post-procedural monitoring.** Indicate which form(s) you will use.

   ☒ CRF Sample Post-Operative/Post-Procedure Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ Lifespan/RIH Tumor Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ A procedure/project specific form will be used to document monitoring. **A**ttach/upload the monitoring form to be used for this procedure.

Appendix 2 – Non-Surgical Procedures that have the Potential to cause Pain and/or Distress

Pain management is expected for all procedures that may cause more than momentary pain or distress. All procedures where anesthetics and/or analgesics are used to relieve pain are considered to be potentially painful. *Injection of Complete Freund's Adjuvant (CFA), infliction of trauma and burns, bacterial infection, induction of disease states such as severe arthritis, and some behavioral tests are examples of procedures that are considered to have the potential to produce pain. Prolonged restraint, food or water deprivation, procedures which result in limited mobility, malaise, etc. are examples of procedures considered to be potentially distressful.*

Duplicate this appendix for each potentially painful/distressful procedure. ***You must also complete the Search for Alternatives in the Main body of the ACUP.***

1. Identification

a. Procedure name (include sham if applicable): Fasting

b. Species: mouse

c. Number of animals: 192

d. Survival:

☐ No

☒ Yes

2. Procedure Details

a. **Where will the procedure be performed**? (*Check all that apply*)

☒ Central Animal Facility Procedure Room (select location)

- ☐ Middle House (Main Campus) animal facility
- ☒ Claverick St. facility
- ☐ Coro East barrier facility
- ☐ Coro West facility

☐ Laboratory (Building and Room #):

b. **Procedure Description**.
Provide a complete narrative of the procedure. Provide all of the relevant details for this protocol without making reference to other protocols.

1. Weigh mice and record mouse body weight.

2. To do glucose tolerance test, food will be removed for 16 hours. Mice will be moved to a new, clean cage without food or feces. Mice will have full access to water during the entire fasting period.

3. Weigh mice to ensure that the experimental mice are back to regular body weights before another fasting. If they have not returned to their previous pre-fasting baseline body weight, the second test (ITT) will be postponed until they have.

4. To do insulin tolerance test, food will be removed 4 hours before the test. Mice will be moved to a new, clean cage without food or feces. Mice will have water access during tne entire fasting period.

All cages undergoing fasting will be marked to alert animal care staff.

c. **Pain and/or Distress**

1. How is pain/distress assessed and monitored during the procedure? Include a description of the signs of pain/distress that will be monitored.

We will provide food after glucose tolerance test and insulin tolerance test.

2. Analgesia or anesthesia will be utilized to minimize pain or distress during the procedure.
Yes ☐ No ☒ *If Yes, Complete the table below.*

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		

3. Will sedatives, analgesics, or anesthetics be withheld for scientific reasons? Yes ☐ No ☒

If yes, explain and include supporting literature references.

d. **Paralytics**

1. Are paralytics employed during potentially painful/distressful procedures? Yes ☐ No ☒

a) If yes, will anesthesia be used when paralytics are employed? Yes ☐ No ☐
b) If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception?

3. **Post-Procedure Care/Monitoring**

a. Post-Procedure Analgesia. *(Check all that apply)*.

☐ Analgesics will be used to provide pain relief to the animals following the procedure

Identify the analgesics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			

☐ Post-procedure pain relief will be withheld

Provide a justification for not using post-procedure analgesics. Include supporting literature references to justify the exclusion of post-procedure analgesia.

☒ Post-procedure pain relief is unnecessary – pain or discomfort is minimal and/or transient

☐ Environmental comfort measures will be employed to minimize pain or distress

Check all that apply.

☐ Paper bedding
☐ Access to soft pellet feed
☐ Gel packs
☐ Housing Huts
☐ Other:

b. Post-procedure Monitoring

1. What will be the duration of survival after the procedure? two weeks after the ITT procedure, we will sacrifice mice
2. What will be the frequency of monitoring from procedure to euthanasia?

   (*Note: The typically accepted minimum monitoring might include frequent monitoring for the short-term following the procedure and then tapering to standard weekly observations thereafter. Consult the veterinarians for further guidance.)*

   The experimental mice will be checked once at the end of day receiving procedures. After that, they will be checked weekly.

3. Do you expect that there will be any long-term consequences from this procedure (e.g. long-term pain or discomfort, immobility, difficulty obtaining food or water)? ☐ Yes ☒ No
   If Yes, explain

4. What parameters will be monitored? *Check all that apply.*

   ☒ Behavior and activity level
   ☒ Body weight
   Specify frequency of weighing Once before each fasting period.
   ☒ Overall condition
   ☐ Body temperature
   Specify frequency
   ☐ Food and water intake
   ☒ Hydration status
   ☐ Other:

5. Who will provide post-procedural support? Chiung-Kuei Huang, Kevin Cao, Zhixiang Cheng

6. **You are required to document post-procedural monitoring.** Indicate which form(s) you will use.

   ☒ CRF Sample Post-Operative/Post-Procedure Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ Lifespan/RIH Tumor Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ A procedure/project specific form will be used to document monitoring. **A**ttach/upload the monitoring form to be used for this procedure.

Appendix 2 – Non-Surgical Procedures that have the Potential to cause Pain and/or Distress

Pain management is expected for all procedures that may cause more than momentary pain or distress. All procedures where anesthetics and/or analgesics are used to relieve pain are considered to be potentially painful. *Injection of Complete Freund's Adjuvant (CFA), infliction of trauma and burns, bacterial infection, induction of disease states such as severe arthritis, and some behavioral tests are examples of procedures that are considered to have the potential to produce pain. Prolonged restraint, food or water deprivation, procedures which result in limited mobility, malaise, etc. are examples of procedures considered to be potentially distressful.*

Duplicate this appendix for each potentially painful/distressful procedure. ***You must also complete the Search for Alternatives in the Main body of the ACUP.***

1. Identification

a. Procedure name (include sham if applicable): Insulin tolerance and glucose tolerance test in aspartate beta-hydroxylase trasgenic mouse

b. Species: mouse

c. Number of animals: 192

d. Survival:

☐ No

☒ Yes

2. Procedure Details

a. **Where will the procedure be performed**? (*Check all that apply*)

☐ Central Animal Facility Procedure Room (select location)

- ☐ Middle House (Main Campus) animal facility
- ☐ Claverick St. facility
- ☐ Coro East barrier facility
- ☐ Coro West facility

☒ Laboratory (Building and Room #): 55 Claverick St., Rm# 407

b. **Procedure Description**.
Provide a complete narrative of the procedure. Provide all of the relevant details for this protocol without making reference to other protocols.

A) Glucose tolerance test (GTT):

A1. Mice will be weighed before fasting. Fast mice overnight for approximately 16 hours by transferring mice to clean cages with no food or faeces in hopper or bottom of cage. We will ensure that they have access to drinking water at all times.

A2. Prepare an experiment record sheet, syringe and sticks for glucose measurement and glucose solution.

A3. Weight the mouse.

A4. Calculate and record the volume of 20% glucose solution required (2g of glucose/kg body mass) for IP injection as follows: volume of IP glucose injection (µl) = 10 x body weight (g).

A5. Application of topical anesthetic cream:

A6. Apply a small amount of topical anesthetic cream, EMLA cream, to the tail of the mouse, spreading over the tail evenly

A7. Gently massage it in for ~10 seconds to enhance the effect of the anesthetic cream ensuring that the proposed incision site is fully covered

A8. Allow an appropriate length of time (60 minutes) for the local anaesthetic to take effect (Duration 3-5 hours).

A9. Inject one dose of Meloxicam (2mg/kg, SC) before cutting mouse tail

A10. Using manual restraint, wipe the tail with Alcohol Prep Pad (for antiseptic purpose) and cut 1-2mm of the tip of the tail using a fresh or sterilized with 75% ethanol scalpel blade

A11. Discard first small drop of blood. A small drop of blood (<5µl) is placed on the test strip of the blood glucose meter. This is the baseline glucose level (t = 0) and is recorded in the experiment record sheet.

A12. Remove the mouse from the cage.

A13. Inject the mouse intraperitoneally with the appropriate amount of glucose solution, as previously determined (point 3) and note the time-point of injection.

A14. The blood glucose levels are measured at 15, 30, 60 and 120 minutes (t = 15, t = 30, t = 60 and t = 120) after glucose injection, by placing a small drop of blood on a new test strip and recording the measurements. Start the bleeding again by removing the clot from the first incision, massage the tail if blood flow is inadequate. Results are recorded in the record sheet. The experimental mice will be monitored for the entire 2 hours of experiment.

A15. Ensure that further blood loss from the incision is minimal by briefly applying pressure to the incision after each measurement.

A16. At the end of the experimental session, place the mouse in a clean cage with water and food available ad libitum.

A17. Monitor the animals carefully to observe any abnormal behavior(s) for 15 minutes.

A18. If there is still bleeding, we will use styptic powder to stop bleeding in the mouse.

A19. Return mice to the animal room after the final time point.

A20. We will check mice once before end of the day to make sure there is no more bleeding in the experimental mice.

A21. Mice will stay in animal room for another week before insulin tolerance test

B) Insulin tolerance test (ITT):

B1. Weigh the mice to ensure that they are back to their pre-fasting baseline weights before doing ITT test.

B2. Fast mice for 4 h only by taking away food early in the morning. The mice will still have water access during fasting.

B3. Apply a small amount of topical anesthetic cream, EMLA cream, to the tail of the mouse, spreading over the tail evenly

B4. Gently massage it in for ~10 seconds to enhance the effect of the anesthetic cream ensuring that the proposed incision site is fully covered

B5. Allow an appropriate length of time (60 minutes) for the local anaesthetic to take effect (Duration 3-5 hours).

B6. Inject one dose of Meloxicam (2mg/kg, SC) before cutting 2mm mouse tail

B7. Using manual restraint, wipe the tail with Alcohol Prep Pad (for antiseptic purpose) and cut 1-2mm of the tip of the tail using a fresh or sterilized with 75% ethanol scalpel blade

B8. Discard first small drop of blood.

B9. Remove approximately 5µl of blood (one drop) from the tail via a tail tip cut and transfer directly onto a glucose indicator strip.

B10. Measure blood glucose immediately in a glucometer.

B11. Give the mouse an intraperitoneal injection of insulin (0.5 U/kg) with a 27 G needle.

B12. Continue to take blood samples from the initial tail cut before the insulin injection and at 15, 30, 45, 60 and 120 min. Start the bleeding again by removing the clot from the first incision, massage the tail if blood flow is inadequate. Results are recorded in the record sheet.

B13. Between each of these time points, return the mouse to its cage and monitor it every minute. The experimental mice will be monitored for the entire 2 hours of experiment.

B14. Ensure that further blood loss from the incision is minimal by briefly applying pressure to the incision after each measurement. At the end of the experiment add food to the cage and make sure that a plentiful supply of water is available to the animals.

B15. Monitor the animals carefully to observe any abnormal behavior(s) for 15 minutes.

B16. If there is still bleeding, we will use syptic powder to stop bleeding in the mouse.

B17. Return mice to the animal room. Monitor the animals carefully to observe any abnormal behavior(s).

B18. We will check mice once before end of the day to make sure there is no more bleeding in the experimental mice.

**Note: No more than a total (from GTT and ITT combined) of 5 mm of the tail will be amputated.

**Note: Tail snips and subsequent scab removal is a standard and well established method to collect very small serial blood samples in mice. General anesthesia cannot be used because it affects heart rate and blood flow and induces hyperglycemia in mice, and so tests of glucose metabolism should not be performed in anesthetized mice and should instead be performed in conscious mice.

(See Ayala JE, Samuel VT, Morton GJ, et al. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Disease Models & Mechanisms*. 2010;3(9-10):525-534. doi:10.1242/dmm.006239.)

c. **Pain and/or Distress**

1. How is pain/distress assessed and monitored during the procedure? Include a description of the signs of pain/distress that will be monitored.

   The experimental animals will have a momentary painful/distressful procedure in the tail when initial tail snips are done. Thus, we will use a small amount of topical anesthetic cream for anestheia purpose and administer a single dose of meloxicam.

2. Analgesia or anesthesia will be utilized to minimize pain or distress during the procedure.
   Yes ☒ No ☐ *If Yes, Complete the table below.*

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		
EMLA cream			topical	Once, 30-60 minutes before procedure

3. Will sedatives, analgesics, or anesthetics be withheld for scientific reasons? Yes ☐ No ☒

   If yes, explain and include supporting literature references.

d. **Paralytics**

1. Are paralytics employed during potentially painful/distressful procedures? Yes ☐ No ☒

a) If yes, will anesthesia be used when paralytics are employed? Yes ☐ No ☐

b) If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception?

3. **Post-Procedure Care/Monitoring**

a. Post-Procedure Analgesia. *(Check all that apply)*.

☒ Analgesics will be used to provide pain relief to the animals following the procedure

Identify the analgesics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			
Meloxicam	2mg/kg		sc	once	Once before the initial tail snip to provide 24 hours of pain relief

☐ Post-procedure pain relief will be withheld

Provide a justification for not using post-procedure analgesics. Include supporting literature references to justify the exclusion of post-procedure analgesia.

☐ Post-procedure pain relief is unnecessary – pain or discomfort is minimal and/or transient

☐ Environmental comfort measures will be employed to minimize pain or distress

Check all that apply.

☐ Paper bedding
☐ Access to soft pellet feed
☐ Gel packs
☐ Housing Huts
☐ Other:

b. Post-procedure Monitoring

1. What will be the duration of survival after the procedure? two weeks after procedure, we will sacrifice mice
2. What will be the frequency of monitoring from procedure to euthanasia?

   (*Note: The typically accepted minimum monitoring might include frequent monitoring for the short-term following the procedure and then tapering to standard weekly observations thereafter. Consult the veterinarians for further guidance.)*

   The experimental mice will be checked once more at the end of day to ensure there is no further bleeding. After that, they will be checked weekly.

3. Do you expect that there will be any long-term consequences from this procedure (e.g. long-term pain or discomfort, immobility, difficulty obtaining food or water)? ☐ Yes ☒ No
   If Yes, explain

4. What parameters will be monitored? *Check all that apply.*

   ☒ Behavior and activity level
   ☒ Body weight
   Specify frequency of weighing Once before each fasting event.
   ☒ Overall condition
   ☐ Body temperature
   Specify frequency
   ☐ Food and water intake
   ☒ Hydration status
   ☐ Other:

5. Who will provide post-procedural support? Chiung-Kuei Huang, Kevin Cao, Zhixiang Cheng

6. **You are required to document post-procedural monitoring.** Indicate which form(s) you will use.

☒ CRF Sample Post-Operative/Post-Procedure Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

☐ Lifespan/RIH Tumor Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

☐ A procedure/project specific form will be used to document monitoring. **A**ttach/upload the monitoring form to be used for this procedure.

Appendix 2 – Non-Surgical Procedures that have the Potential to cause Pain and/or Distress

Pain management is expected for all procedures that may cause more than momentary pain or distress. All procedures where anesthetics and/or analgesics are used to relieve pain are considered to be potentially painful. *Injection of Complete Freund's Adjuvant (CFA), infliction of trauma and burns, bacterial infection, induction of disease states such as severe arthritis, and some behavioral tests are examples of procedures that are considered to have the potential to produce pain. Prolonged restraint, food or water deprivation, procedures which result in limited mobility, malaise, etc. are examples of procedures considered to be potentially distressful.*

Duplicate this appendix for each potentially painful/distressful procedure. ***You must also complete the Search for Alternatives in the Main body of the ACUP.***

1. Identification

a. Procedure name (include sham if applicable): Thioacetamide (TAA) feeding using drinking water

b. Species: mouse

c. Number of animals: 12

d. Survival:

☐ No

☒ Yes

2. Procedure Details

a. **Where will the procedure be performed**? (*Check all that apply*)

☐ Central Animal Facility Procedure Room (select location)

- ☐ Middle House (Main Campus) animal facility
- ☒ Claverick St. facility
- ☐ Coro East barrier facility
- ☐ Coro West facility

☐ Laboratory (Building and Room #):

b. **Procedure Description**.
Provide a complete narrative of the procedure. Provide all of the relevant details for this protocol without making reference to other protocols.

1. prepare 300mg thioacetamide in 1 liter water
2. Replace regular water with 300mg/L thioacetamide in drinking water weekly
3. Continue the feeding schedule for 8 weeks
4. the experimental mice will be euthansized with CO2 at the end of feeding schedule

c. **Pain and/or Distress**

1. How is pain/distress assessed and monitored during the procedure? Include a description of the signs of pain/distress that will be monitored.

Animals receiving thioacetamide that causes no pain or distress, or only momentary or silght pain or distress, may not require the use of pain-relieving drugs.

2. Analgesia or anesthesia will be utilized to minimize pain or distress during the procedure.
Yes ☐ No ☒ *If Yes, Complete the table below.*

Agent	Dose/volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. once, continuous, other)
	mg/kg	mL		

3. Will sedatives, analgesics, or anesthetics be withheld for scientific reasons? Yes ☐ No ☒

If yes, explain and include supporting literature references.

d. **Paralytics**

1. Are paralytics employed during potentially painful/distressful procedures? Yes ☐ No ☒

a) If yes, will anesthesia be used when paralytics are employed? Yes ☐ No ☒

b) If animals are under anesthesia and the influence of paralytics, how will animals be monitored for pain perception?

3. **Post-Procedure Care/Monitoring**

a. Post-Procedure Analgesia. *(Check all that apply)*.

☐ Analgesics will be used to provide pain relief to the animals following the procedure

Identify the analgesics that will be given.

Agent	Dose /Volume		Route (e.g. iv, ip, sc)	Frequency of Administration (e.g. times/day)	Duration of Treatment (e.g. days)
	mg/kg	mL			

☐ Post-procedure pain relief will be withheld

Provide a justification for not using post-procedure analgesics. Include supporting literature references to justify the exclusion of post-procedure analgesia.

☐ Post-procedure pain relief is unnecessary – pain or discomfort is minimal and/or transient

☒ Environmental comfort measures will be employed to minimize pain or distress

Check all that apply.

☒ Paper bedding

☒ Access to soft pellet feed

☒ Gel packs

☒ Housing Huts

☐ Other:

b. Post-procedure Monitoring

1. What will be the duration of survival after the procedure? After 8 weeks of TAA treatment, we will sacrifice the experimental mice.
2. What will be the frequency of monitoring from procedure to euthanasia?

   (*Note: The typically accepted minimum monitoring might include frequent monitoring for the short-term following the procedure and then tapering to standard weekly observations thereafter. Consult the veterinarians for further guidance.)*

   We will monitor the experimental animals a minimum of 1-2 times per week. We change the special water every few days so mice are, in general, evaluated more frequently. We do not expect to see any overt clinical signs with this model. Mice clinically are not overtly affected due to the slow progression of this model, and tend not to decrease in body mass >15%. Any mouse showing signs of morbidity, including a decline in body weight >15% from baseline, inability to access food or water, or signs of being moribund will be humanely euthanized.

3. Do you expect that there will be any long-term consequences from this procedure (e.g. long-term pain or discomfort, immobility, difficulty obtaining food or water)? ☒ Yes ☐ No
   If Yes, explain

As this is a liver fibrosis animal model, we expect those experimental mice will have discomfort as liver fibrotic patients have.

4. What parameters will be monitored? *Check all that apply.*

   ☒ Behavior and activity level

   ☒ Body weight

   Specify frequency of weighing weekly

   ☒ Overall condition

   ☐ Body temperature

   Specify frequency

   ☒ Food and water intake

   ☒ Hydration status

   ☐ Other:

5. Who will provide post-procedural support? Chiung-Kuei Huang, Zhixiang Cheng, Xuewei Bai, Joud

Mulla, and Kevin Cao

6. **You are required to document post-procedural monitoring.** Indicate which form(s) you will use.

   ☒ CRF Sample Post-Operative/Post-Procedure Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ Lifespan/RIH Tumor Monitoring Form. *(The sample form can be found in the CRF P&P Manual Appendix, and also in the IRBNet Forms Library.)*

   ☐ A procedure/project specific form will be used to document monitoring. **A**ttach/upload the monitoring form to be used for this procedure.

Appendix 4 – Use of Hazardous Substances in Live Animals

Use this Appendix to list all carcinogens, teratogens, mutagens, toxic chemicals, volatile substances, infectious agents, viral vectors, etc. that will be given to animals in the proposed study. ***Duplicate this appendix for each individual hazardous agent to be used in live animals.***

Biohazard and Laboratory Safety Committee (BLSC), Recombinant DNA Committee (RDC) or Radioactive Safety Committee approval may be required prior to implementation.

1. Description

a. Agent: carbon tetrachloride (CCl4)

b. Species that will be treated with the agent: mouse

c. Hazard Type:

☒ Chemical

☐ Biological

☐ Recombinant DNA

☐ Radioactive (see also item 3, below)

d. Will animals experience pain as a result of the treatment? ☒ Yes ☐ No

e. Status of BLSC, RDC or Radioactive Safety Committee review:

☐ Pending

☒ Complete. Approved.

Include CMTT# 5059-17, and approval date 12/4/17

Append a copy of the approval letter to this application as a PDF

2. Hazard Containment

a. Location where agent will be administered (Bldg. & Room #): 55 Claverick St. Rm#8

b. How long will the animal survive after treatment? Ater 8 weeks of CCl4 treatment, we will sacrifice the experimental mice. However, this is a category E procedure and the injections may cause sudden death of approximately 10% of animals based on the literature standard due to hepatic toxicity.

c. Will these animals be housed in the animal facility after treatment? ☐ Yes ☒ No

d. Will the animals be involved in any other procedures after treatment with this agent? ☐ Yes ☒ No

If so, what procedure(s)?

(NOTE: The IACUC and RDC have developed a Standard Operating Procedure (S.O.P.) for the care and handling of animals on non-replicating viral vector protocols. This S.O.P. is available from CRF or Research Administration.)

3. For the Use of Radioactive Agents Only:

Authorization #:

Name of authorized user:

Details on the management of radioisotopes (ordering, containment, storage, transport, handling, disposal, etc.):

Appendix 4 – Use of Hazardous Substances in Live Animals

Use this Appendix to list all carcinogens, teratogens, mutagens, toxic chemicals, volatile substances, infectious agents, viral vectors, etc. that will be given to animals in the proposed study. ***Duplicate this appendix for each individual hazardous agent to be used in live animals.***

Biohazard and Laboratory Safety Committee (BLSC), Recombinant DNA Committee (RDC) or Radioactive Safety Committee approval may be required prior to implementation.

1. Description

a. Agent: Human H1 and HuCCT1 CCA cells manipulated with pLKO.1-shRNA-luciferase, pLKO.1-shRNA-ASPH, plenti-Crispr/Cas9-control, plenti-Crispr/Cas9-ASPH, pCDH-EV, and pCDH-ASPH via a lentiviral system Rat BDE CCA cell lines manipulated with pLKO.1-shRNA-luciferase, pLKO.1-shRNA-rat-ASPH, pCDH-EV, and pCDH-rat-ASPH via a lentiviral system.

b. Species that will be treated with the agent: mouse for human H1 and HuCCT1 CCA cells; rat for BDE CCA cells

c. Hazard Type:

- ☐ Chemical
- ☒ Biological
- ☐ Recombinant DNA
- ☐ Radioactive (see also item 3, below)

d. Will animals experience pain as a result of the treatment? ☒ Yes ☐ No

e. Status of BLSC, RDC or Radioactive Safety Committee review:

- ☐ Pending
- ☒ Complete. Approved.

Include CMTT# 5055-18, and approval date 10/9/18

Append a copy of the approval letter to this application as a PDF

2. Hazard Containment

a. Location where agent will be administered (Bldg. & Room #): Central Animal Facility Procedure Room

b. How long will the animal survive after treatment? 19 days

c. Will these animals be housed in the animal facility after treatment? ☒ Yes ☐ No

d. Will the animals be involved in any other procedures after treatment with this agent? ☒ Yes ☐ No

If so, what procedure(s)? Intraperitoneal injection of MO-I-1151 25mg/kg or vehicle (DMSO+saline) control every other day.

(NOTE: The IACUC and RDC have developed a Standard Operating Procedure (S.O.P.) for the care and handling of animals on non-replicating viral vector protocols. This S.O.P. is available from CRF or Research Administration.)

3. For the Use of Radioactive Agents Only:

Authorization #:

Name of authorized user:

Details on the management of radioisotopes (ordering, containment, storage, transport, handling, disposal, etc.):

Appendix 4 – Use of Hazardous Substances in Live Animals

Use this Appendix to list all carcinogens, teratogens, mutagens, toxic chemicals, volatile substances, infectious agents, viral vectors, etc. that will be given to animals in the proposed study. ***Duplicate this appendix for each individual hazardous agent to be used in live animals.***

Biohazard and Laboratory Safety Committee (BLSC), Recombinant DNA Committee (RDC) or Radioactive Safety Committee approval may be required prior to implementation.

1. Description

a. Agent: Plasmids: pLKO.1-shRNA-luciferase, pLKO.1-shRNA-ASPH, pLenti-Crispr/Cas9-control, pLenti-Crispr/Cas9-ASPH, pLKO.1-shRNA-rat-ASPH, pCDH-empty vector (EV), pCDH-ASPH, pCDH-rat-ASPH. Human CCA cell lines will be manipulated with pLKO.1-shRNA-luciferase, pLKO.1-shRNA-ASPH, plenti-Crispr/Cas9-control, plenti-Crispr/Cas9-ASPH, pCDH-EV, and pCDH-ASPH via a lentiviral system. Rat CCA cells will be manipulated with pLKO.1-shRNA-luciferase, pLKO.1-shRNA-rat-ASPH, pCDH-EV, and pCDH-rat-ASPH. The cells will be implanted into the experimental mice or rats. The lentivirus will not be used to directly infect live animals.

b. Species that will be treated with the agent: mouse and rats

c. Hazard Type:

☐ Chemical
☒ Biological
☒ Recombinant DNA
☐ Radioactive (see also item 3, below)

d. Will animals experience pain as a result of the treatment? ☒ Yes ☐ No

e. Status of BLSC, RDC or Radioactive Safety Committee review:

☐ Pending
☒ Complete. Approved.
Include CMTT# 0246-15, and approval date 09/07/18

Append a copy of the approval letter to this application as a PDF

2. Hazard Containment

a. Location where agent will be administered (Bldg. & Room #): Central Animal Facility Procedure Room

b. How long will the animal survive after treatment? 19 days

c. Will these animals be housed in the animal facility after treatment? ☒ Yes ☐ No

d. Will the animals be involved in any other procedures after treatment with this agent? ☒ Yes ☐ No
If so, what procedure(s)? Intraperitoneal injection of MO-I-1151 25mg/kg or vehicle (DMSO+saline) control every other day.

(NOTE: The IACUC and RDC have developed a Standard Operating Procedure (S.O.P.) for the care and handling of animals on non-replicating viral vector protocols. This S.O.P. is available from CRF or Research Administration.)

3. For the Use of Radioactive Agents Only:

Authorization #:

Name of authorized user:

Details on the management of radioisotopes (ordering, containment, storage, transport, handling, disposal, etc.):

Appendix 4 – Use of Hazardous Substances in Live Animals

Use this Appendix to list all carcinogens, teratogens, mutagens, toxic chemicals, volatile substances, infectious agents, viral vectors, etc. that will be given to animals in the proposed study. ***Duplicate this appendix for each individual hazardous agent to be used in live animals.***

Biohazard and Laboratory Safety Committee (BLSC), Recombinant DNA Committee (RDC) or Radioactive Safety Committee approval may be required prior to implementation.

1. Description

a. Agent: thioacetamide (TAA)

b. Species that will be treated with the agent: mouse

c. Hazard Type:

☒ Chemical

☐ Biological

☐ Recombinant DNA

☐ Radioactive (see also item 3, below)

d. Will animals experience pain as a result of the treatment? ☐ Yes ☒ No

e. Status of BLSC, RDC or Radioactive Safety Committee review:

☐ Pending

☒ Complete. Approved.

Include CMTT# 5059-17, and approval date 12/4/17

Append a copy of the approval letter to this application as a PDF

2. Hazard Containment

a. Location where agent will be administered (Bldg. & Room #): 55 Claverick St. Rm#8

b. How long will the animal survive after treatment? Normall, the TAA liver fibrosis model can survive until 1 year old. However, after 8 weeks of TAA treatment, we will sacrifice the experimental mice.

c. Will these animals be housed in the animal facility after treatment? ☐ Yes ☒ No

d. Will the animals be involved in any other procedures after treatment with this agent? ☐ Yes ☒ No

If so, what procedure(s)?

(NOTE: The IACUC and RDC have developed a Standard Operating Procedure (S.O.P.) for the care and handling of animals on non-replicating viral vector protocols. This S.O.P. is available from CRF or Research Administration.)

3. For the Use of Radioactive Agents Only:

Authorization #:

Name of authorized user:

Details on the management of radioisotopes (ordering, containment, storage, transport, handling, disposal, etc.):

Appendix 5 – Breeding of Animals

YOU ARE REQUIRED TO KEEP ACCURATE RECORDS OF THE NUMBER OF ANIMALS PRODUCED AND THEIR ULTIMATE DISPOSITION. *Breeding reports are due monthly. All animals born, whether of desired genotype or not, are considered to be "used" on the breeding report.*

1. Person Responsible for Submitting Monthly Breeding Reports

Name: Chiung-Kuei Huang

Email: chiung-kuei_huang@brown.edu

2. Justification for Breeding

☒ Animals are not commercially available

☐ *In utero* studies

☐ Reproduction studies

☐ Other

3. Interbreeding and/ or Crossbreeding

Will different strains of transgenic/knockout animals be interbred or crossbred? ☒ Yes ☐ No

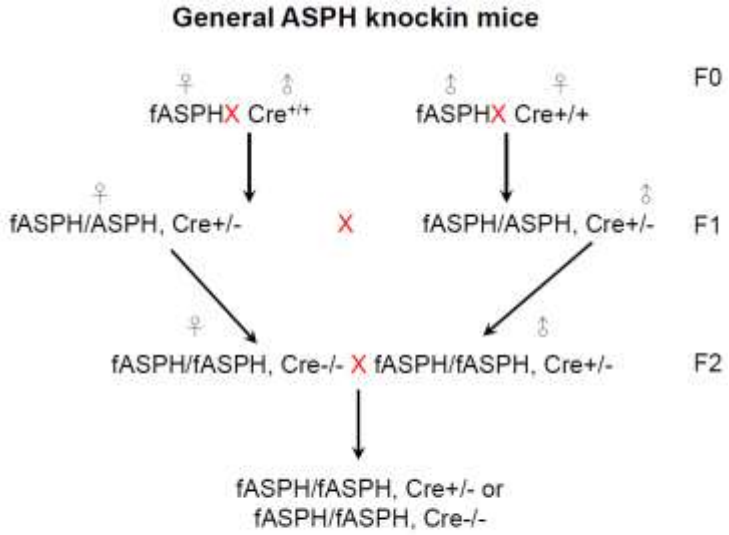
Note: If YES, then further review by the Recombinant DNA Committee may be required.

4. Breeding Scheme

Strain 1	Strain 2	Desired offspring genotype	# Anticipated to be born-desired genotype	Additional offspring(*e.g. undesired genotype*)	# Anticipated to be born - additional offspring
fASPH/fASPH	**Ella Cre+/+**	**Male and female fASPH/ASPH, Ella Cre+/-**	**10**		**10**
fASPH/ASPH, Ella Cre+/-	**fASPH/ASPH, Ella Cre+/-**	**fASPH/fASPH, Ella Cre+/-**	**5**	**fASPH/fASPH, Ella Cre +/+; fASPH/ASPH, Ella Cre+/+, fASPH/ASPH, Ella Cre+/-, fASPH/ASPH, Ella Cre+/+, fASPH/ASPH, Ella Cre-/-; fASPH/fASPH, Ella Cre-/-**	**75**
fASPH/ASPH, Ella Cre+/-	**fASPH/ASPH, Ella Cre+/-**	**fASPH/fASPH, Ella Cre-/-**	**5**	**fASPH/fASPH, Ella Cre +/+; fASPH/fASPH, Ella Cre+/-**	**155**

				fASPH/ASPH, Ella Cre+/+, fASPH/ASPH, Ella Cre+/-, fASPH/ASPH, Ella Cre+/+, fASPH/ASPH, Ella Cre-/-	
fASPH/fASPH, Ella Cre+/-	**fASPH/fASPH, Cre-/-**	**fASPH/fASPH Ella Cre+/-**	**96**		**0**
fASPH/fASPH, Ella Cre+/-	**fASPH/fASPH, Cre-/-**	**fASPH/fASPH Ella Cre-/-**	**96**		**0**

For especially complicated breeding schemes, describe additional details here if needed.



fASPH - loxP flanked ASPH
X – mating
F1: Founder 1
F2: Founder 2

5. Disposition of surplus animals?

- ☒ Donate to CRF
- ☒ Euthanize
- ☐ Transfer to another protocol
- ☐ Other:

6. Genotyping

Indicate methods used to genotype offspring (*check all that apply*)

☒**Tail biopsy***(see Mouse Tail Biopsy Policy in CRF Policy Manual).*

- ☐ Biopsies limited to mice ≤21 days old (no anesthesia or analgesia required)

☐ Biopsies taken from mice > 21 days old (requires anesthesia/analgesia and a *Search for Alternatives to this painful and/or Distressful Procedures).*

Specify Anesthesia/analgesia to be used:

☐**Toe Clip***(seeRodent Toe Clipping Policy in CRF Policy Manual).*

☐ Biopsies limited to mice ≤7 days old (no anesthesia or analgesia required)

☐ Biopsies taken from mice >7 days old (requires anesthesia/analgesia and a *Search for Alternatives to this painful and/or Distressful Procedures).*

Specify Anesthesia/analgesia to be used:

☐**Other Tissue**

Specify tissue to be biopsied and/or used for genotype determination:

Specify Anesthesia/analgesia to be used:

7. Pregnant Females

Will this protocol involve the purchase and/or use of pregnant females?☐ Yes ☒ No

If Yes:

a. Willfetuses be manipulated in utero?☐ Yes ☐ No

b.Will the dams be allowed to give birth to live pups?☐ Yes ☐ No

Genetically Modified Animal Breeding Programs

All mice and rats entering the Lifespan animal facilities from a noncommercial source (such as an academic institution) will be quarantined for a minimum of 60 days before being released for project use. *Lifespan and the IACUC highly recommends that novel or unique rodent stocks or strains that are not commercially available have a fail-save system in place, such as cryopreservation of gametes or housing at an additional off-site contract location (Charles River, Jackson Laboratories) in the case of a catastrophic event.*

1. DNA/Transgene or Gene to be Altered.

a. Specify DNA/Transgene or Gene to be Altered: Aspartate beta-hydroxylase

b. Specify extent of gene disruption

☒ Global disruption

☐ Tissue/organ specific

Specify tissue/organ affected:

c. Method of monitoring presence of transgene

☒Genotyping

☐Other

Specify other method of monitoring transgene:

2. Anticipated Consequences of Gene Disruption

List any anticipated functional (phenotypic) consequences of genetic manipulation that may adversely affect the animals (e.g. alter mobility, cause pain or distress, affect eating and drinking, etc.). Note: The PI is responsible for notifying the IACUCof any significant phenotypic consequences that were not anticipated

and described *a priori.*

Not known yet

3. Special Care and/or Monitoring

Describe any special care or monitoring that may be required

We will need to feed the experimental animals with high fat diet by chow diet and α-ketoglutarate by drinking water.

The ear punch procedure will be used to monitor and identify each animal when the tail biopsy is performed.

Appendix 5 – Breeding of Animals

YOU ARE REQUIRED TO KEEP ACCURATE RECORDS OF THE NUMBER OF ANIMALS PRODUCED AND THEIR ULTIMATE DISPOSITION. *Breeding reports are due monthly. All animals born, whether of desired genotype or not, are considered to be "used" on the breeding report.*

1. Person Responsible for Submitting Monthly Breeding Reports

Name: Chiung-Kuei Huang

Email: chiung-kuei_huang@brown.edu

2. Justification for Breeding

☒ Animals are not commercially available

☐ *In utero* studies

☐ Reproduction studies

☐ Other

3. Interbreeding and/ or Crossbreeding

Will different strains of transgenic/knockout animals be interbred or crossbred? ☒ Yes ☐ No

Note: If YES, then further review by the Recombinant DNA Committee may be required.

4. Breeding Scheme

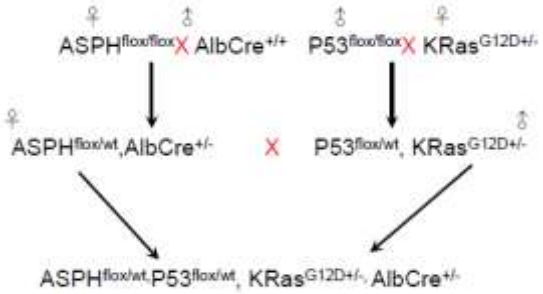
Strain 1	**Strain 2**	**Desired offspring genotype**	**# Anticipated to be born- desired genotype**	**Additional offspring(*e.g. undesired genotype*)**	**# Anticipated to be born - additional offspring**
$ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$	$ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$	$ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$; $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ or $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/+}$	**132**	$ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$ $ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/+}$ $ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D-/-,}$ $AlbCre^{+/-}$ $ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D+/+,}$ $AlbCre^{+/-}$ $ASPH^{flox/wt,}P53^{wt/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ $ASPH^{flox/wt,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ $ASPH^{flox/flox,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ $ASPH^{flox/flox,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$ $ASPH^{flox/flox,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/+}$ $ASPH^{flox/flox,}P53^{flox/wt}$, $KRas^{G12D-/-,}$ $AlbCre^{+/-}$	924

				$ASPH^{flox/flox,}P53^{flox/wt}$, $KRas^{G12D+/+,}$ $AlbCre^{+/-}$ $ASPH^{flox/flox,}P53^{wt/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ $ASPH^{flox/flox,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$	

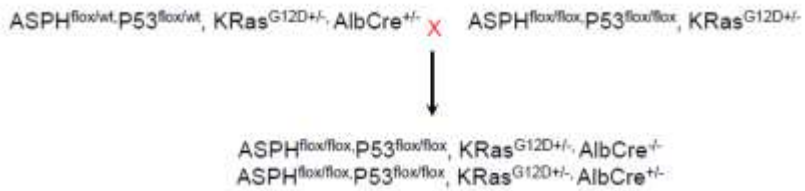
For especially complicated breeding schemes, describe additional details here if needed.

We will purchase $KRas^{G12D+/-}$, $P53^{flox/flox}$ and Albumin $Cre^{+/+}$ mice from Jackson lab. To generate $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$ and $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ mice, we have to breed them. The mating strategy will be as listed in figure 1. As gender is not an established risk factor for cholangiocarcinoma, we will use both genders for our proposed study.

Figure 1 is the mating strategy for mice to be generated.



To get $ASPH^{flox/wt}$,$AlbCre^{+/-,}$ we will generate 10 mice (1 litter)
To get $P53^{flox/wt}$, $KRas^{G12D+/-}$, we will generate 10 mice (1 litter)
To get $ASPH^{flox/wt,}P53^{flox/wt}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$, we will generate 16 mice. It is because the chance to get the mouse will be ½ ($ASPH^{flox/wt)}$ x ½ ($P53^{flox/wt}$) x ½ ($KRas^{G12D+/-}$) x ½ ($AlbCre^{+/-}$) = 1/16.



To get $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$, the chance will be ½ x ½ x ½ x ½ =1/16.
To get $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$, the chance will be ½ x ½ x ½ x ½ =1/16.

Transgenic mouse number:
To generate $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$, we will need to generate the minimal mouse number, 1056, for each experimental group. It is because the chance of getting them is only 1/16 and we plan to have 66 mice in each group (Thus, 66x16=1056). Similarly, we have to generate 1056 mice

in order to obtain 66 $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ mice. As both genotypes of mice will be generated under the identical mating strategy and since we are using both the AlbCre heterozygotes and AlbCre homozygoes, we theoretically only need to breed 1056 mice to obtain the 66 $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{-/-}$ and 66 $ASPH^{flox/flox,}P53^{flox/flox}$, $KRas^{G12D+/-,}$ $AlbCre^{+/-}$ mice, therefore reducing the numbers generated for each proposed group.

We will request to breed 1056 mice for the experiment D.

5. Disposition of surplus animals?

- ☒ Donate to CRF
- ☒ Euthanize
- ☐ Transfer to another protocol
- ☐ Other:

6. Genotyping

Indicate methods used to genotype offspring (*check all that apply*)

☒ **Tail biopsy***(see Mouse Tail Biopsy Policy in CRF Policy Manual).*

- ☒ Biopsies limited to mice ≤21 days old (no anesthesia or analgesia required)
- ☐ Biopsies taken from mice > 21 days old (requires anesthesia/analgesia and a *Search for Alternatives to this painful and/or Distressful Procedures).*

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  Specify Anesthesia/analgesia to be used:

☐ **Other Tissue**

Specify tissue to be biopsied and/or used for genotype determination:

Specify Anesthesia/analgesia to be used:

7. Pregnant Females

Will this protocol involve the purchase and/or use of pregnant females? ☐ Yes ☒ No

If Yes:

a. Willfetuses be manipulated in utero? ☐ Yes ☐ No

b.Will the dams be allowed to give birth to live pups? ☐ Yes ☐ No

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1. DNA/Transgene or Gene to be Altered.

a. Specify DNA/Transgene or Gene to be Altered: Aspartate beta-hydroxylase

b. Specify extent of gene disruption

☒ Global disruption

☒ Tissue/organ specific

Specify tissue/organ affected: liver

c. Method of monitoring presence of transgene

☒ Genotyping

☐ Other

Specify other method of monitoring transgene:

2. Anticipated Consequences of Gene Disruption

List any anticipated functional (phenotypic) consequences of genetic manipulation that may adversely affect the animals (e.g. alter mobility, cause pain or distress, affect eating and drinking, etc.). Note: The PI is responsible for notifying the IACUCof any significant phenotypic consequences that were not anticipated and described *a priori*.

This is a cholangiocaricnoma mouse model. There is an estimated 20% mortality rate prior to 20 weeks in $Alb^{Cre+/-}$ $p53^{lox/lox}$ $KRas^{G12D+/-}$.

3. Special Care and/or Monitoring

Describe any special care or monitoring that may be required

The ear punch procedure will be used to monitor and identify each animal when the tail biopsy is performed.

We will challenge the experimental mice with carbon tetrachloride via i.p. injection (twice weekly for 8 weeks), thioacetamide (300mg/L) via drinking water for 8 weeks, and 5% chronic alcohol feeding for 12 weeks.

Transgenic mouse model of: $Alb^{Cre+/-}$ $p53^{flox/flox}$ $Kras^{G12D+/-}$ and $Alb^{Cre+/-}$ $p53^{flox/-}$ $Kras^{G12+/-}$. The median survival of the mice are 19 week and 52 weeks, respectively. There is an estimated 20% mortality rate prior to 20 week. To ensure their welfare we will monitor mice from week 16 onward to ensure welfare. Death is not and endpoint for our studies. Mice will be evaluated for distended abdomen, ascites, difficult breading, jaundice, hunch, weigh gain (>20% more than baseline from week 12 on), or altered mental status.

Appendix 5 – Breeding of Animals

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3. Interbreeding and/ or Crossbreeding

Will different strains of transgenic/knockout animals be interbred or crossbred? ☒ Yes ☐ No

Note: If YES, then further review by the Recombinant DNA Committee may be required.

4. Breeding Scheme

For especially complicated breeding schemes, describe additional details here if needed.

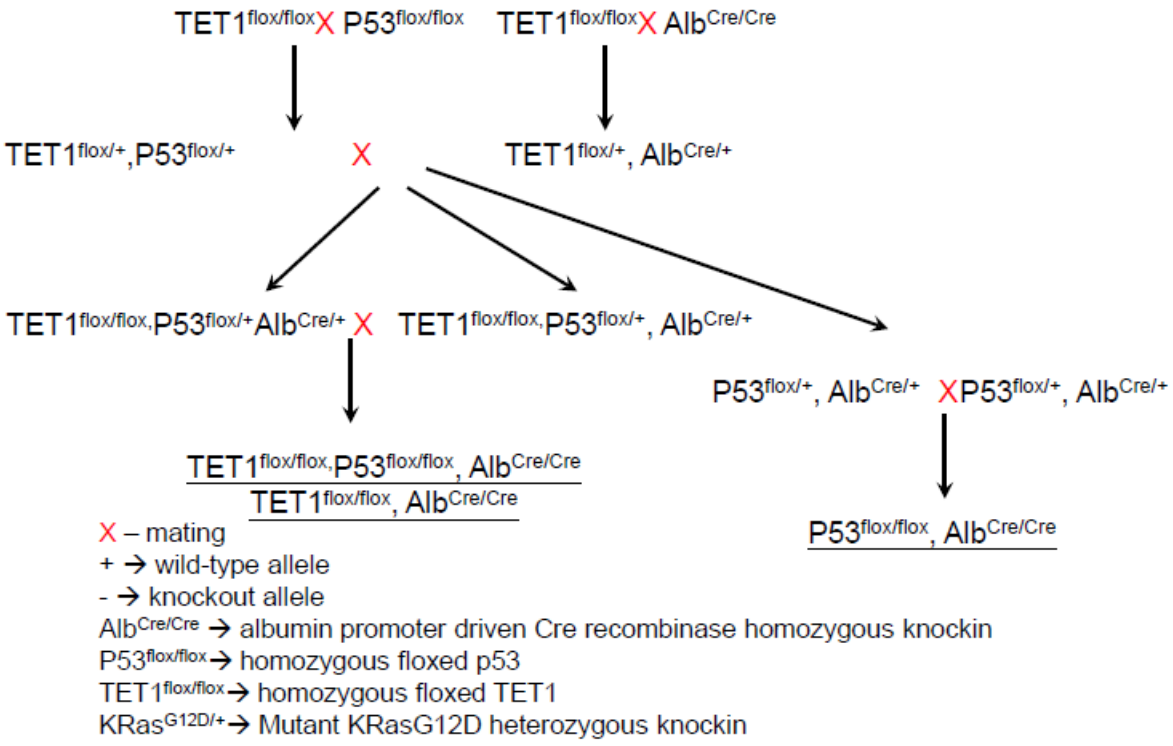
Strain 1	**Strain 2**	**Desired offspring genotype**	**# Anticipated to be born- desired genotype**	**Additional offspring(*e.g. undesired genotype*)**	**# Anticipated to be born - additional offspring**
$TET1^{flox/flox,}$	$P53^{flox/flox}$	Male and female $TET1^{flox/+}$ $P53^{flox/+}$	**10**		**0**
$TET1^{flox/flox,}$	$Alb^{Cre/Cre}$	Male and female $TET1^{flox/+}$ $Alb^{Cre/+}$	**10**		**0**
$TET1^{flox/+}$ $P53^{flox/+}$	$TET1^{flox/+}$ $Alb^{Cre/+}$	$TET1^{flox/flox}$ $P53^{flox/+}$ $Alb^{Cre/+}$ $P53^{flox/+}Alb^{Cre/+}$	4		**28**
$TET1^{flox/flox}$ $P53^{flox/+}$ $Alb^{Cre/+}$	$TET1^{flox/flox}$ $P53^{flox/+}$ $Alb^{Cre/+}$	$TET1^{flox/flox}$ $P53^{flox/flox}$ $Alb^{Cre/Cre}$	2		**30**

$P53^{flox/+}Alb^{Cre/+}$	$P53^{flox/+}Alb^{Cre/+}$	$P53^{flox/flox}$ $Alb^{Cre/Cre}$	**2**		**30**
$TET1^{flox/flox}$	$KRas^{G12D/+}$	$TET1^{flox/+}$ $KRas^{G12D/+}$	**4**		**4**
$TET1^{flox/+}$ $KRas^{G12D/+}$	$TET1^{flox/+}P53^{flox/+}$	$TET1^{flox/flox}P53^{flox/+}$ $KRas^{G12D/+}$ $P53^{flox/+}$ $KRas^{G12D/+}$	**4**		**28**
$P53^{flox/+}$ $KRas^{G12D/+}$	$P53^{flox/flox}$	$P53^{flox/flox}$, $KRas^{G12D+/-}$	**4**		**12**
$TET1^{flox/flox}$ $P53^{flox/flox}$ $Alb^{Cre/Cre}$	$TET1^{flox/flox}P53^{flox/+}$ $KRas^{G12D/+}$	$TET1^{flox/flox}P53^{flox/flox}$ $KRas^{G12D/+}$ $Alb^{Cre/+}$ $TET1^{flox/flox}P53^{flox/+}$ $KRas^{G12D/+}$ $Alb^{Cre/+}$	**40**		**40**
$P53^{flox/flox}$ $KRas^{G12D/+}$	$P53^{flox/flox}$ $Alb^{Cre/Cre}$	$P53^{flox/flox}KRas^{G12D/+}Alb^{Cre/+}$	**20**		**20**
$P53^{flox/flox}$ $KRas^{G12D/+}$	$Alb^{Cre/Cre}$	$P53^{flox/+}KRas^{G12D/+}$ $Alb^{Cre/+}$	**20**		**20**
$TET1^{+/-}$	$P53^{flox/flox}$	$TET1^{+/-}P53^{flox/+}$	**4**		**4**
$TET1^{+/-}$	$Alb^{Cre/Cre}$	$TET1^{+/-}Alb^{Cre/+}$	**4**		**4**
$TET1^{+/-}$ $P53^{flox/+}$	$TET1^{+/-}$ $Alb^{Cre/+}$	$TET1^{+/-}$ $p53^{flox/+}Alb^{Cre/+}$	**2**		**14**
$TET1^{+/-}$ $p53^{flox/+}Alb^{Cre/+}$	$TET1^{+/-}$ $p53^{flox/+}Alb^{Cre/+}$	$TET1^{+/-}$ $p53^{flox/flox}Alb^{Cre/Cre}$ $TET1^{+/-}Alb^{Cre/Cre}$	**8**		**248**
$TET1^{+/-}$	$KRas^{G12D/+}$	$TET1^{+/-}KRas^{G12D/+}$	**2**		**6**
$TET1^{+/-}$ $KRas^{G12D/+}$	$P53^{flox/flox}$	$TET1^{+/-}$ $p53^{flox/+}KRas^{G12D/+}$	**2**		**6**
$TET1^{+/-}$ $p53^{flox/+}KRas^{G12D/+}$	$P53^{flox/flox}$	$TET1^{+/-}$ $p53^{flox/flox}KRas^{G12D/+}$	**4**		**28**
$TET1^{+/-}$ $p53^{flox/flox}KRas^{G12D/+}$	$TET1^{+/-}$ $p53^{flox/flox}Alb^{Cre/Cre}$	$TET1^{-/-}$ $p53^{flox/flox}Alb^{Cre/+}$ $TET1^{+/+}p53^{flox/flox}Alb^{Cre/+}$	**40**		**120**

$TET1^{+/-}$ $p53^{flox/flox}KRas^{G12D/+}$	$TET1^{+/-}$ $Alb^{Cre/Cre}$	$TET1^{-/-}$ $p53^{flox/+}Alb^{Cre/+}$ $TET1^{+/+}p53^{flox/+}Alb^{Cre/+}$	**40**		**120**

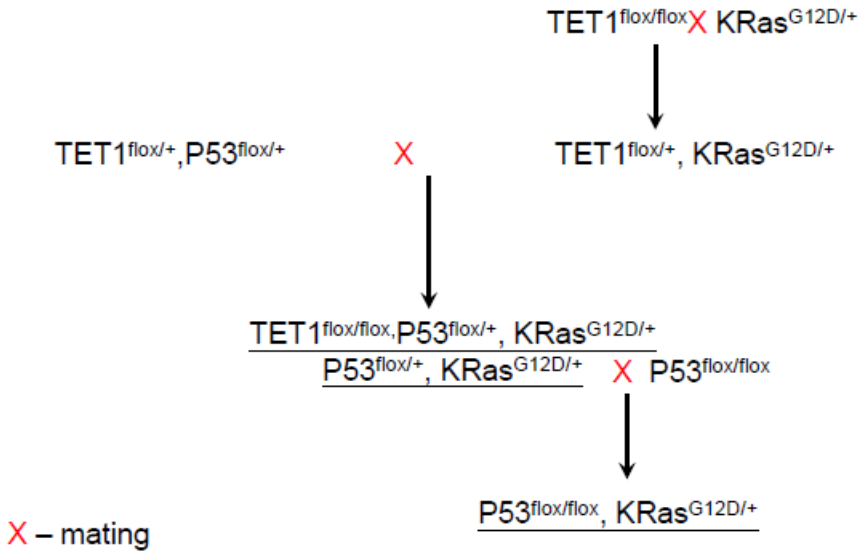
Total mice to be bred will be 984.

TET1 knockout with P53 knockout and $KRas^{G12D}$ knockin in the liver

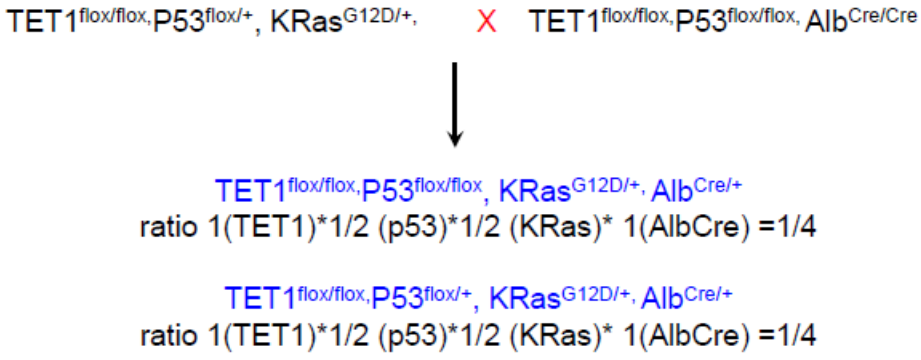


X – mating
+ → wild-type allele
- → knockout allele
$Alb^{Cre/Cre}$ → albumin promoter driven Cre recombinase homozygous knockin
$P53^{flox/flox}$ → homozygous floxed p53
$TET1^{flox/flox}$ → homozygous floxed TET1
$KRas^{G12D/+}$ → Mutant KRasG12D heterozygous knockin

TET1 knockout with P53 knockout and $KRas^{G12D}$ knockin in the liver (homozygous $KRas^{G12D/G12D}$ embryonic lethal)

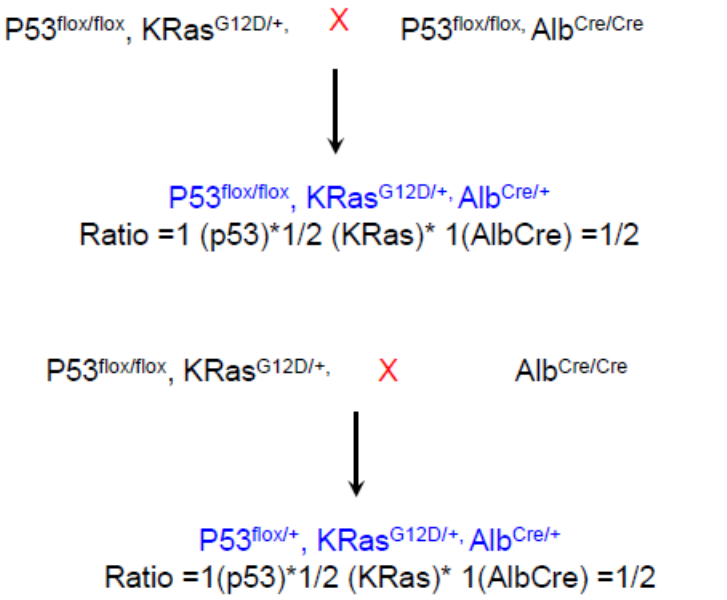


TET1 knockout with P53 knockout and $KRas^{G12D}$ knockin in the liver



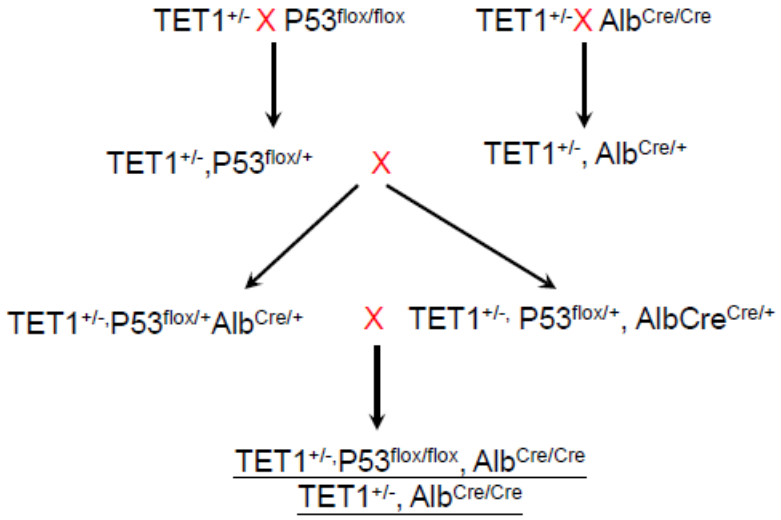
X – mating Blue color indicates the experimental animal.

TET1 knockout with P53 knockout and KRas^{G12D} knockin in the liver

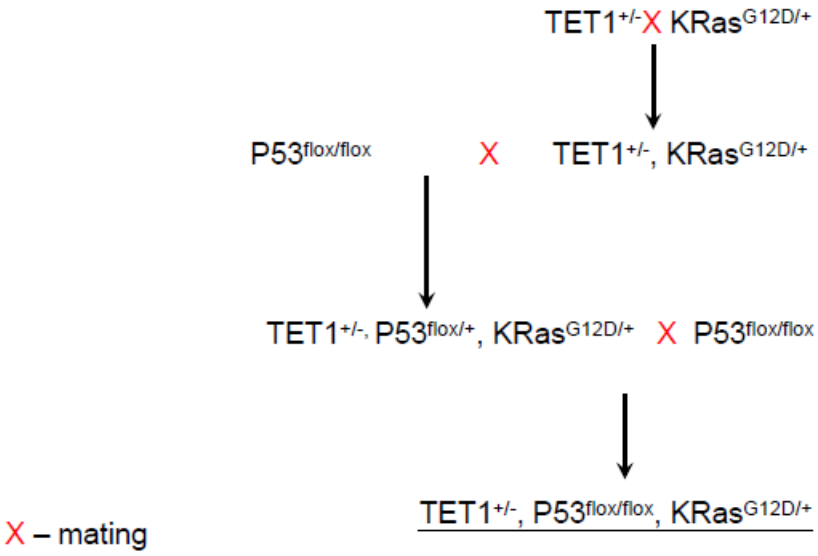


X – mating Blue color indicates the experimental animal.

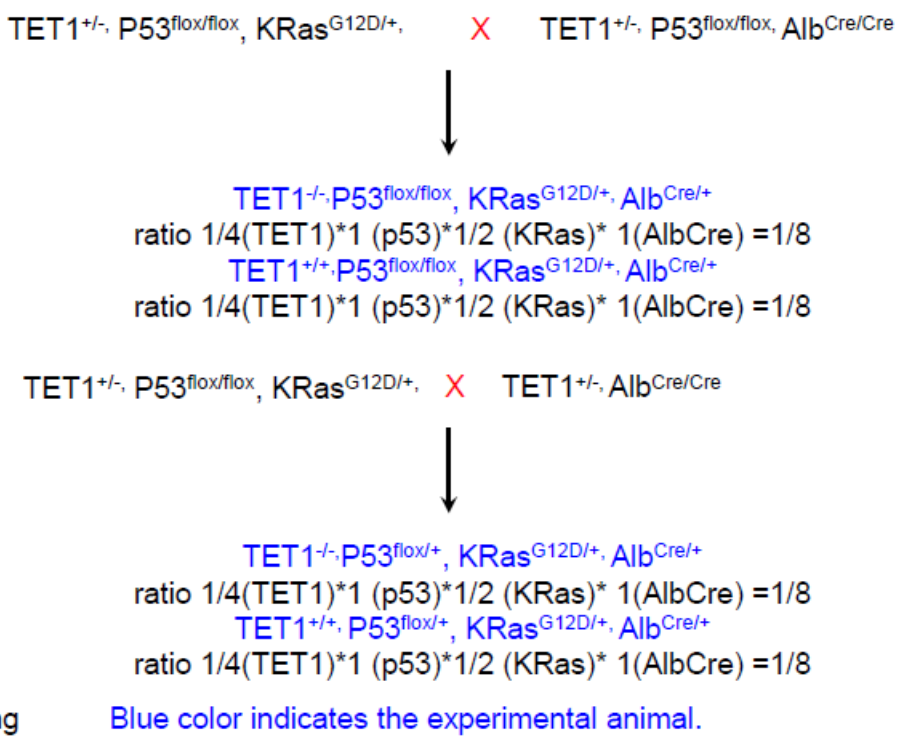
TET1 knockout in whole body with P53 knockout and KRas^{G12D} knockin in the liver (TET1-/- has reduced fertility)



TET1 knockout in whole body with P53 knockout and KRas^{G12D} knockin in the liver (TET1-/- has reduced fertility)



TET1 knockout with P53 knockout and KRas^{G12D} knockin in the liver (TET1-/- has reduced fertility)



X – mating Blue color indicates the experimental animal.

5. Disposition of surplus animals?

- ☒ Donate to CRF
- ☒ Euthanize
- ☐ Transfer to another protocol
- ☐ Other:

6. Genotyping

Indicate methods used to genotype offspring (*check all that apply*)

☒**Tail biopsy***(see Mouse Tail Biopsy Policy in CRF Policy Manual).*

- ☒ Biopsies limited to mice ≤21 days old (no anesthesia or analgesia required)
- ☐ Biopsies taken from mice > 21 days old (requires anesthesia/analgesia and a *Search for Alternatives to this painful and/or Distressful Procedures).*

  Specify Anesthesia/analgesia to be used:

☐**Toe Clip***(seeRodent Toe Clipping Policy in CRF Policy Manual).*

- ☐ Biopsies limited to mice ≤7 days old (no anesthesia or analgesia required)
- ☐ Biopsies taken from mice >7 days old (requires anesthesia/analgesia and a *Search for Alternatives to this painful and/or Distressful Procedures).*

  Specify Anesthesia/analgesia to be used:

☐**Other Tissue**

Specify tissue to be biopsied and/or used for genotype determination:

Specify Anesthesia/analgesia to be used:

7. Pregnant Females

Will this protocol involve the purchase and/or use of pregnant females?☐ Yes ☒No

If Yes:

a. Will fetuses be manipulated in utero?☐ Yes ☐ No

b. Will the dams be allowed to give birth to live pups?☐ Yes ☐ No

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All mice and rats entering the Lifespan animal facilities from a noncommercial source (such as an academic institution) will be quarantined for a minimum of 60 days before being released for project use. *Lifespan and the IACUC highly recommends that novel or unique rodent stocks or strains that are not commercially available have a fail-save system in place, such as cryopreservation of gametes or housing at an additional off-site contract location (Charles River, Jackson Laboratories) in the case of a catastrophic event.*

1. DNA/Transgene or Gene to be Altered.

a. Specify DNA/Transgene or Gene to be Altered: TET1

b. Specify extent of gene disruption

☒ Global disruption

☒ Tissue/organ specific

Specify tissue/organ affected: liver

c. Method of monitoring presence of transgene

☒Genotyping

☐Other

Specify other method of monitoring transgene:

2. Anticipated Consequences of Gene Disruption

List any anticipated functional (phenotypic) consequences of genetic manipulation that may adversely affect the animals (e.g. alter mobility, cause pain or distress, affect eating and drinking, etc.). Note: The PI is responsible for notifying the IACUCof any significant phenotypic consequences that were not anticipated and described *a priori.*

This is a cholangiocaricnoma mouse model. There is an estimated 20% mortality rate prior to 20 weeks in $Alb^{Cre+/-}$ $p53^{lox/lox}$ $KRas^{G12D+/-}$.

3. Special Care and/or Monitoring

Describe any special care or monitoring that may be required

The ear punch procedure will be used to monitor and identify each animal when the tail biopsy is performed.

Transgenic mouse model of: $Alb^{Cre+/-}$ $p53^{flox/flox}$ $Kras^{G12D+/-}$ and $Alb^{Cre+/-}$ $p53^{flox/-}$ $Kras^{G12+/-}$. The median survival of the mice are 19 week and 52 weeks, respectively. There is an estimated 20% mortality rate prior to 20 week. To ensure their welfare we will monitor mice from week 16 onward to ensure welfare. Death is not and endpoint for our studies. Mice will be evaluated for distended abdomen, ascites, difficult breading, jaundice, hunch, weigh gain (>20% more than baseline from week 12 on), or altered mental status. We will submit a report back after the first 5-10 KO mice are produced and the percent mortality, percent liver effected and length of survival are known.

Appendix 6 – De Novo (Three Year) Renewal

1. Progress

Briefly describe the progress made in achieving the specific aims of this project over the past three years and describe any substantive changes that have been made since the initial review.

- *Which studies in the original protocol have been completed during the past three years? Provide narrative description.*
- *What is yet to be completed? Provide narrative description.*
- *Which parts of the protocol will not be pursued and why?*
- *Include references to any publications that have resulted from this research.*

Our specific Aim 1 is to evaluate if targeting aspartate beta-hydroxylase (ASPH) with molecluar strategies as well as pharmaceutical approaches may inhibt cholangiocarcinoma (CCA) tumor progression in subcutaneous xenograft murine model.

We have made amendment on the proposed animal experiments by including ASPH overexpression CCA cells in xenograft tumor models. In our studies, we found that knockdown of ASPH or ASPH small molecule inhibitor both suppressed CCA malignant progression in subcutaneous and xenograft models. The results have been published in PLOS One and Cancer Letters. We are still working on determing if overexpression of ASPH promotes CCA progression. We are still investigating if overexpression of ASPH promtoes CCA progression.

Our specific Aim 2 is to determine whether targeting ASPH with molecluar strategies as well as pharmaceutical approaches may inhibt CCA tumor progression in the rat intrahepatic model.

We have amended our aim 2 by changing the aim as "determine the moleuclar mechanism by which ASPH modulates CCA progression.

In our aim 2, we found that knockdown of ASPH inhibited CCA progressoin in a rat CCA model. However, targeting ASPH with MO-I-1151 did not suppress cancer metastasis in a rat CCA model.

As we proposed to detemine if ASPH promotes CCA progression through RB1 phosphorylation in our amendment, we are still doing these experiments.

Our Specific Aim 3 is to evaluate if targeting ASPH with molecular strategies as well as pharmaceutical approaches may inhibit CCA tumor progression in a transgenic rodent model, which may be a more apt model to mimic the progression of endogenous cholangiocarcinoma.

We did not complete the aim yet. The mice are still under high fat diet and alpha-ketoglutarate challenges..

Our specific Aim 4 is to evaluate if ASPH is involved in liver fibrosis associated cholangiocarcinoma progression.

In our aim 4, we proposed to overexpress ASPH in a triple transgenic CCA murine model. As the mouse breeding strategy takes several generations of breeding, it takes couple years to complete. We are on the right track of the mouse breeding.

In our aim 5, we aimed at clarifying the role of TET1 in CCA progression.

We just got the protocol approved on 9/10/2018. We did not have any progress for the aim 5.

Published reference:

Anti-Tumor Effects of Second Generation β-Hydroxylase Inhibitors on Cholangiocarcinoma Development and Progression.

Huang CK, Iwagami Y, Aihara A, Chung W, de la Monte S, Thomas JM, Olsen M, Carlson R, Yu T,

Dong X, Wands J.

PLoS One. 2016 Mar 8;11(3):e0150336. doi: 10.1371/journal.pone.0150336. eCollection 2016.

Aspartate beta-hydroxylase promotes cholangiocarcinoma progression by modulating RB1 phosphorylation.

Huang CK, Iwagami Y, Zou J, Casulli S, Lu S, Nagaoka K, Ji C, Ogawa K, Cao KY, Gao JS, Carlson RI, Wands JR.

Cancer Lett. 2018 Aug 10;429:1-10. doi: 10.1016/j.canlet.2018.04.041. Epub 2018 May 5

2. Record of Animal Use

Information needed to complete this section is included on the progress report form which was sent to you by mail.

Species	Total Number of Animals Approved (# initially approved + # added via amendment)	Total Number of Animals Used, All 3 Years	Number Remaining within the facility to be transferred to this de novo application once approved
mouse	3618	578	200
rat	195	52	0

Will additional animals beyond those that remain, will be required to complete this project?

☐ Yes ☒ No

If yes, explain.

3. Problems/Adverse Events

During the last three years, have any unanticipated adverse events, morbidity or mortality occurred?

☐ Yes ☒ No

If yes, describe the cause(s) if known, and how the problems were resolved.

Huang mice monitoring form

Cage #: _____ **Protocol number: 0233-15**

Species: Mice **Strain:_____**
PI: Huang **Phone number: _____** **Cell phone:_____**

Monitoring parameters:

- **Performed 3-4 times per week starting at 16 weeks of age**
- **Weigh mice 1 time per week, starting at 12 weeks of age**
- **Evaluate for: distended abdomen and/or ascites, difficulty breathing, jaundice (yellowing of mucus membranes), hunched appearance, significant weight gain (>20% than baseline) altered mental status (staggering, head pressing, dull or lethargic)**
- **If mice are noted to have any of these parameters, we will increase the monitoring of these mice to once per day**
- **If mice become significantly affected and/or moribund or have signs of altered mental status (staggering, head pressing, dull/lethargic)altered mental status (dull, lethargic, head pressing), weight gain >20% from baseline (12 weeks) we will euthanize**

Date	Weight	Bright/alert?	Distended abd or ascites?	Jaundice?	Altered mental status?	Other observations (i.e. specific animal number)
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	
		Y/N	Y / N	Y / N	Y / N	