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14. ABSTRACT

The proposed research project addresses the topic area of pancreatitis. The main goal will be to determine whether hyper-activation of the M3 acetylcholine receptor located on pancreatic acinar cells induces pancreatitis. We will study this through premature intra-acinar cell trypsinogen activation and measuring increased pro-inflammatory NF-kB pathway signaling as well as by targeting the muscarinic 3 receptor with an FDA approved chemical antagonist Darifenacin. Additionally, we will also study commercially available human pancreatic acinar cells to see if they respond similarly to our mouse studies.

15. SUBJECT TERMS
Pancreatic inflammation; muscarinic 3 receptor; animal model; pancreatitis therapy

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1. INTRODUCTION:

The central goal of this proposal is to establish and characterize novel models of acute and chronic pancreatitis by directly activating the muscarinic receptor 3 (M3R) on pancreatic acinar cells and test novel targeted therapies. Completion of the proposed studies is expected to establish a clinically relevant animal model of pancreatitis that is superior to the current "standard" model for developing and testing new interventions. In this proposal, we will test our central hypothesis that M3R hyperactivation induces pancreatitis through premature intra-acinar trypsinogen activation and increased proinflammatory NF-*k*B pathways and targeting M3R will be beneficial to pancreatitis therapy.

2. KEYWORDS:

Pancreatic inflammation; muscarinic 3 receptor; animal model; pancreatitis therapy.

3. ACCOMPLISHMENTS: What were the major goals of the project?

Specific aim #1: Characterize hM3R-induced acute pancreatitis (AP) and determine whether AP will progress to chronic pancreatitis (CP). In this aim, we will examine and compare dose-dependent CCK, carbachol (a muscarinic receptor agonist) and CNO (activating transgenic hM3R)-induced amylase secretion and cell damage in isolated pancreatic acinar cells. We will also determine the minimum doses of CNO needed to cause AP, characterize the severity of AP, and examine whether the hM3R-elicited AP will progress to CP and precancerous lesions.

Specific aim #2: **Identify the signaling pathways of hM3R during the initiation of pancreatitis and validate these pathways in commercially available primary human pancreatic acinar cells.** First, we will examine whether several AP-related common pathways are activated in M3R-initiated pancreatitis. These pathways including trypsinogen activation, NF-*k*B activation, ER stress and autophagy are observed in the caerulein model of pancreatitis. Second, we will use differential phosphoproteomics and Next Generation RNA-seq to discover novel pathways that are common in CCK-, carbachol- and CNO-stimulated acinar cells and also that are unique to M3R. Third, we validate these pathways in commercially available primary human pancreatic acinar cells stimulated with M3R agonist carbachol.

Specific aim #3: Test whether the M3R-specific antagonist Darifenacin (Enablex®) will ameliorate pancreatitis. Darifenacin works by blocking the M3 muscarinic acetylcholine receptor (M3R). It is an FDA-approved drug for treating symptoms of overactive bladder, such as frequent or urgent urination, and incontinence. In this study, we will test its efficacy in reducing the severity of pancreatitis in several models of pancreatitis including alcohol and high fat diet-induced pancreatitis.

What was accomplished under these goals?

Hyperstimulation of muscarinic receptor 3 caused pancreatic acinar cell damage and biphasic amylase secretion.

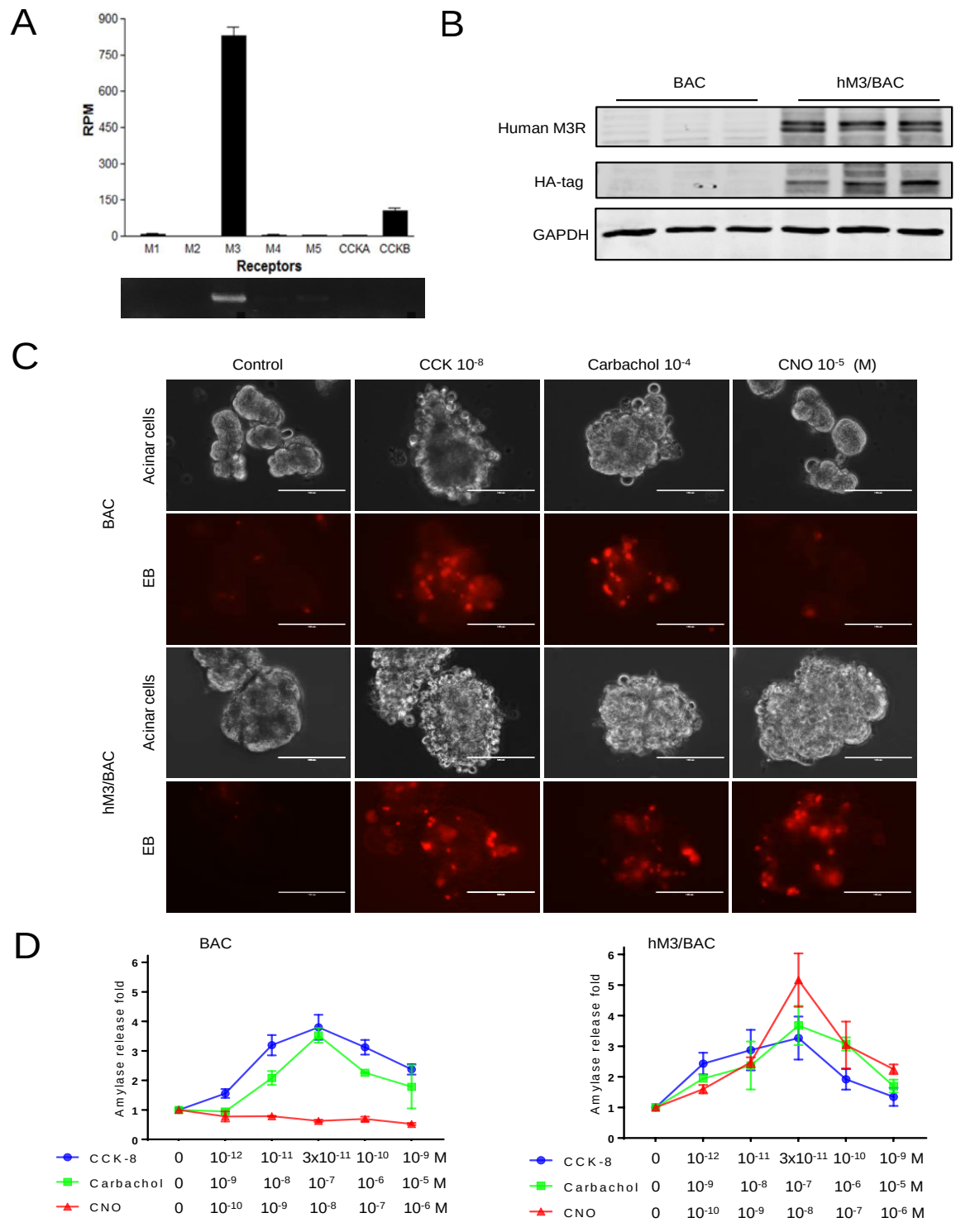
To further examine the mRNA expressions of CCKR and subtypes of muscarinic receptors in human pancreatic acinar cells, we performed unbiased RNAseq and real-time RT-PCR using total RNA prepared from isolated pancreatic acinar cells of healthy donors without exocrine pancreatic disorders (Figure 1A). With these sensitive methods, mRNA expression of CCKAR was not detected. Instead, M3R was specifically expressed in these human pancreatic acinar cells (Figure 1A). Therefore, we concluded that normal human pancreatic acinar cells specifically express M3R but not CCKAR.

Stimulation of CCKAR causes pancreatitis in rodents. However, it is unclear whether stimulation of M receptors, located on pancreatic acinar cells, was complicated in pancreatitis *in vivo*. In order to specifically activate the M3R in mouse pancreatic acinar cells, we decided to use a new genetic mouse model with conditional expression of a modified human M3 receptor by chemical-genetic approach to control the activation of receptors, the Designer receptors exclusively activated by designer drugs (DREADD). The DREADD mutant M3R (hM3R) loses responsiveness to the endogenous ligand acetylcholine, but can be activated by exogenous CNO (clozapine-N-oxide). Pancreatic specific expression of hM3R can be used to test the functionality of M3R activation without causing systematic side effects.

For conditional pancreatic acinar specific expression of hM3R, LSL-hM3R (Loxp-Stop-Loxp-hM3R) mice were crossed with BAC-Ela-CreERT (BAC) mice which express CreERT driven by the pancreatic acinar-specific elastase-I promoter. Expression of hM3R alleles was induced by administration of tamoxifen. The protein expression of hM3R was verified by Western Blot (Figure 1B). Initially, we studied the effects of CCK, carbachol and CNO stimulation in isolated pancreatic acinar cells. In pancreatic acinar cells from the control BAC mice, CCK and carbachol induced membrane blebbing and acinar membrane damage as indicated by ethidium bromide staining (Figure 1C) and biphasic amylase secretion (Figure. 1D). No response to CNO was observed in the control pancreatic acinar cells. In contrast, pancreatic acinar cells from hM3R mice responded to CCK, carbachol, as well as CNO in similar patterns (Figure 1 C-D). These data indicate that CNO and carbachol activation of M receptors caused pancreatic acinar cell damage.

Figure 1 Legend: Figure 1. Human pancreas preponderantly existed M3 receptor, which caused damage by stimulating in pancreatic acinar cells. (A). The RNA sequencing for human pancreatic acini. (B). M1-5 and CCKA-B receptors were detected by PCR in normal pancreatic acini of donor. (C). The strategy to generate BAC-Ela-CreErT; hM3Dq. TM injection induces recombination of hM3Dq alleles in elastase-expressing acinar cells of pancreas. (D). Pancreatic levels of mutant M3 receptors and HA-tag were measured by IB.

Figure 1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CCKA
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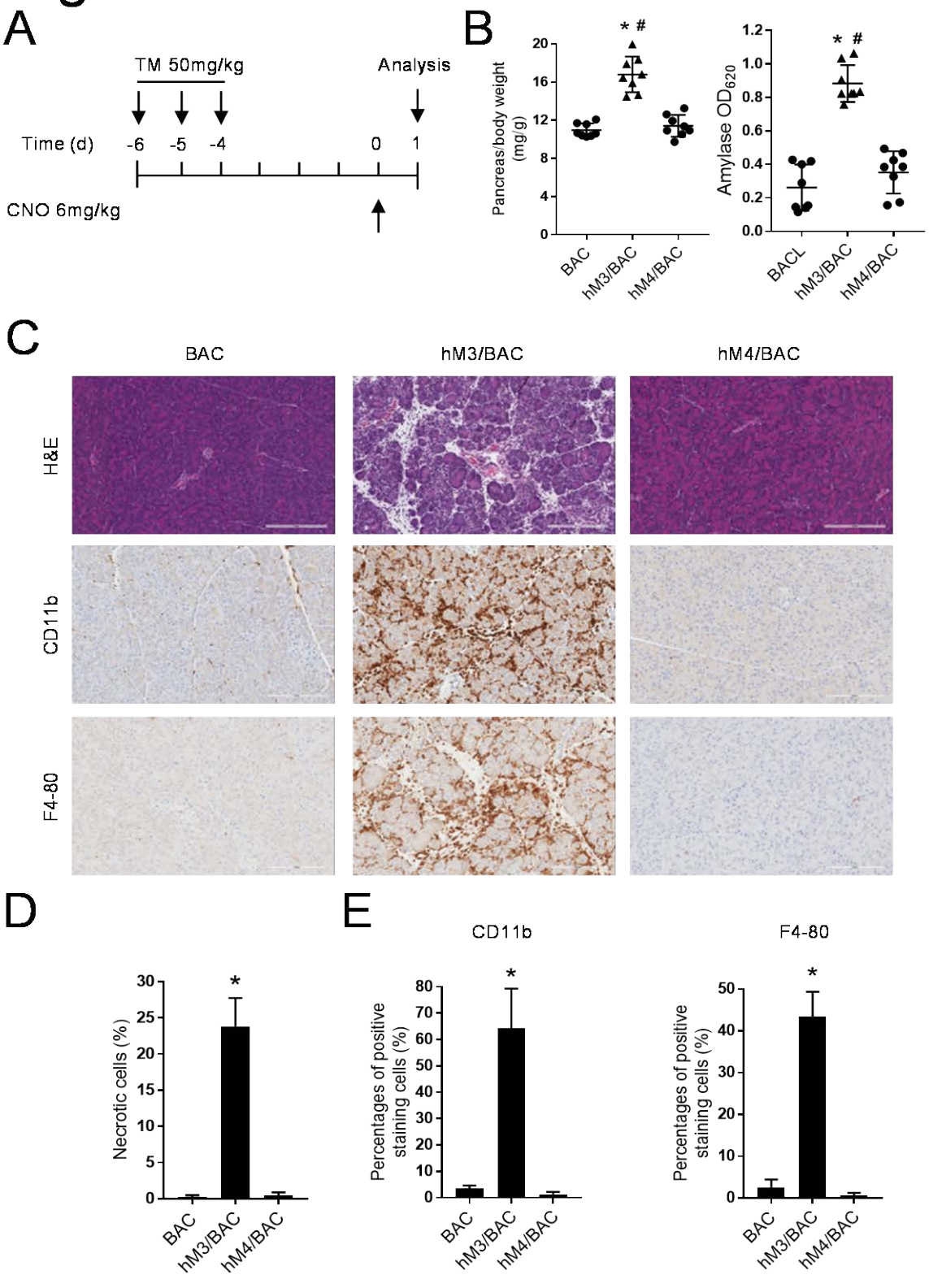
Receptors
B
BAC
hM3/BAC
Human M3R
HA-tag
GAPDH
C
Control
CCK 10-8
Carbachol 10-4
CNO 10-5 (M)
BAC
hM3/BAC
Acinar cells
EB
D
BAC
hM3/BAC
Amylase release fold
CCK-8 0 10-12 10-11 3x10-11 10-10 10-9 M
Carbachol 0 10-9 10-8 10-7 10-6 10-5 M
CNO 0 10-10 10-9 10-8 10-7 10-6 M

Activation of M3R induced the development of acute pancreatitis in hM3R mice.

To study the effects of M3R activation on pancreatic acinar cells *in vivo*, hM3R expression was induced with tamoxifen followed by CNO administration to these mice. Control BAC mice and hM4R (a DREADD mutant Gi protein-coupled muscarinic 4 receptor) mice received similar treatments. Mice were sacrificed 24 hours after the initial dose of CNO and the severity of pancreatitis was examined (Figure. 2A). Pancreatic edema and serum amylase increased in the CNO-treated hM3R mice, but not in control mice and hM4R mice (Figure. 2B). Compared with control mice, the hM3/BAC mice given CNO displayed dramatic inflammatory cell infiltration (Figure. 2C and E) and pancreatic acinar necrosis (Figure. 2C-D).

Figure 2 Legend: Figure 2. Acute pancreatitis induced by CNO in BAC-Ela-CreErT-based hM3Dq mice. (A). The strategy to gavage tamoxifen one week before for BAC, hM4/BAC and hM3/BAC mice injected with CNO (6mg/kg) were sacrificed at 24h after the CNO injection. (B). The ratio of pancreas to body weight (mg/g) and Serum activities of amylase N≥6 mice/group. (C). H&E staining and immunohistochemical analysis of pancreatic, scale bar: 200 mm, (D). Quantification of the necrosis area and the percentage of positive cells was calculated under ImageScope software. (E). The expression of inflammatory factors in pancreas were detected by q-PCR.

Figure 2


A
TM 50mg/kg
Analysis
Time (d)
-6
-5
-4
0
1
CNO 6mg/kg
B
Pancreas/body weight (mg/g)
Amylase OD620
* #
BAC
hM3/BAC
hM4/BAC
BACL
C
BAC
hM3/BAC
hM4/BAC
H&E
CD11b
F4-80
D
Necrotic cells (%)
E
CD11b
F4-80
Percentages of positive staining cells (%)

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

Specific aim #1: Characterize hM3R-induced acute pancreatitis (AP) and determine whether AP will progress to chronic pancreatitis (CP).

Regarding this aim, we have yet to determine the minimum doses of CNO needed to cause AP, characterize the severity of AP, and examine whether the hM3R-elicited AP will progress to CP and precancerous lesions. This will be our next task in completing this aim of the study.

Specific aim #2: **Identify the signaling pathways of hM3R during the initiation of pancreatitis and validate these pathways in commercially available primary human pancreatic acinar cells.**

To begin targeting this aim, we will compare CNO-induced acute pancreatitis to that of cerulein-induced pancreatitis, utilizing mice that we have already begun to breed from the LSL-hM3R mice with Pdx1-Cre mice (hM3R/Pdx1-Cre, hM3/Pdx1) to express hM3R in the pancreas. The severity of acute pancreatitis in hM3/pdx1 and hM3/BAC mice will be compared with the commonly used cerulein-induced pancreatitis model to detect pancreatic edema ad serum amylase levels as a result of hourly cerulein injections. Histological and immunohistological analysis will be performed and resulting visuals provided to support the discovered data, as well as to visually assess pancreatic damage and inflammation compared between the groups.

Additionally, there is belief that AP can progress to CP in both humans and mice because activation of M3 receptor in the pancreas may potentially cause more severe acute pancreatitis. This could have an impact on recovery time as well. We suspect that hM3R mice will be more susceptible to the development of chronic pancreatitis following recurrent acute pancreatitis. To test this hypothesis, 3 bouts of acute pancreatitis will be induced every other day by multiple cerulein injections in BAC mice or a single CNO injection in hM3/BAC and hM3/Pdx1 mice. Pancreatitis was evaluated 28 days after the first onset of acute pancreatitis. Thorough histological analysis will help determine the results of this experiment, specifically seeking markers such as fibrosis, reactive cell proliferations, chronic inflammatory cell infiltrations, and pancreatic atrophy.

Specific aim #3: Test whether the M3R-specific antagonist Darifenacin (Enablex®) will ameliorate pancreatitis.

To determine whether the severity of pancreatitis will be affected by inhibiting the M3 receptor in wild-type mice, we will use a selective muscarinic M3-receptor antagonist darifenacin. Darifenacin (Enablex®, Novartis) is an FDA-approved medication used to treat overactive bladder syndrome. It works by blocking the M3R on bladder muscles and inhibiting bladder contractions, thereby decreasing the urgency to urinate. We hope to find that inhibition of the M3 receptor is beneficial to pancreatitis, as well as helpful in gaining meaningful insight to pathomechanisms of this disease, and further explain the complex relationship between acute and chronic pancreatitis.

4. IMPACT: What was the impact on the development of the principal discipline(s) of the project?

While early in our planned study, we already have promising insight and an overall better understanding of the role of inflammation in chronic pancreatitis. Our novel mouse model will provide more accurate elucidations of pathomechanisms for human pancreatitis, due to the specificity of our targets in contrast to unreliable and non-translatable mouse models of the past. We believe that this new mouse model will unleash new potential in discovering therapies that will more reliably and more effectively treat chronic pancreatitis in humans. Our hope would be to be responsible for creating the newest and greatest standard of pancreatitis mouse models, with wide and varied applications for battling this dangerous and deadly disease.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS: Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

COVID-19 caused temporary laboratory shutdown, layoffs and furloughs to certain staff, essential mouse colony reductions across laboratories, as well as enforced schedule staggering to limit employee exposure and limit overcrowding to comply with adopted social distance policies. The result of these circumstances led to a decrease in efficiency and a higher than normal workload due to the decrease in available assistance when compared to regular operating procedures. While COVID-19 still persists, many improvements have been made and some restrictions lightened to improve overall efficiency and bring things back towards a semblance of normalcy.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

  Journal publications

Nothing to report.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers and presentations.

Nothing to report.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

All of the following lab members have performed all facets of technical work surrounding this project, some of which includes: tissue and organ harvesting immediately following euthanasia, oral gavage for tamoxifen administration, mating and tagging/genotyping pups between 14-21 days old, and euthanasia via carbon dioxide.

Dr. Baoan Ji, Principal Investigator (13 months)
Jiaxiang Chen, Visiting Research Fellow (8 months)
Jianhua Wan, Visiting Research Fellow (7 months)
Ji Shi, Visiting Research Fellow (7 months)
Ashley Haddock, Research Technologist and Laboratory Manager (13 months)

No other funding support to report.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

None.

8. **SPECIAL REPORTING REQUIREMENTS**

   COLLABORATIVE AWARDS

   QUAD CHARTS:

9. **APPENDICES:**